RESEARCH PAPER

Alleviation of rapid, futile ammonium cycling at the plasma membrane by potassium reveals K⁺-sensitive and -insensitive components of NH₄⁺ transport

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Abstract

Futile plasma membrane cycling of ammonium (NH₄⁺) is characteristic of low-affinity NH₄⁺ transport, and has been proposed to be a critical factor in NH₄⁺ toxicity. Using unidirectional flux analysis with the positron-emitting tracer ¹³N in intact seedlings of barley (Hordeum vulgare L.), it is shown that rapid, futile NH₄⁺ cycling is alleviated by elevated K⁺ supply, and that low-affinity NH₄⁺ 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), NH₄⁺ influx (at an external [NH₄⁺] of 10 mM) of 92 μmol g⁻¹ h⁻¹ was observed, with an efflux:influx ratio of 0.75, indicative of rapid, futile NH₄⁺ cycling. Elevating K⁺ supply into the low-affinity K⁺ transport range (1.5–40 mM) reduced both influx and efflux of NH₄⁺ by as much as 75%, and substantially reduced the efflux:influx ratio. The reduction of NH₄⁺ fluxes was achieved rapidly upon exposure to elevated K⁺, within 1 min for influx and within 5 min for efflux. The channel inhibitor La³⁺ decreased high-capacity NH₄⁺ influx only at low K⁺ concentrations, suggesting that the K⁺-sensitive component of NH₄⁺ influx may be mediated by non-selective cation channels. Using respiratory measurements and current models of ion flux energetics, the energy cost of concomitant NH₄⁺ 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 NH₄⁺ flux mechanisms in plants.

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

Introduction

Ammonium (NH₄⁺) 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, NH₄⁺ 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, NH₄⁺ uptake is mediated by a high-capacity, energetically passive, low-affinity transport system (LATS). However, low-affinity NH₄⁺ influx is accompanied by an efflux of NH₄⁺ 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 NH₄⁺ under these conditions has been shown to be energetically costly in NH₄⁺-sensitive plant species (Kronzucker et al., 2001), and it has been postulated that a primary cause of NH₄⁺ toxicity in plants is the energy lost due to the active removal of NH₄⁺ that has entered root cells at an uncontrolled rate (Britto et al., 2001; Kronzucker et al., 2001).

NH₄⁺ 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, 1988a; Gerendas et al., 1997; Santa-Maria et al., 2000; Szczerba et al., 2006a). The mechanism underlying this has not been unravelled, but it may be through direct competition between NH₄⁺ and other cations for entry through common uptake pathways. In particular, potassium (K⁺) channels are considered prime candidates for low-affinity NH₄⁺ transport, as NH₄⁺ 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...
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 NH₄⁺ nutrition is that an increase in external K⁺ concentration ([K⁺]ₑ) protects sensitive plant species from NH₄⁺ toxicity (Cao et al., 1993; Spalding et al., 1999; Santa-María et al., 2000; Kronzucker et al., 2003b; Szczerba et al., 2006a). 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., 2003b; Szczerba et al., 2006a). In studies using intact barley seedlings, Kronzucker et al. (2003b) and Szczerba et al. (2006a) showed that, at low external K⁺ concentrations, K⁺ fluxes into the roots were much lower in seedlings grown with 10 mM NH₄⁺ than those grown with 10 mM nitrate (NO₃⁻), but at high external K⁺, these fluxes were dependent on N source. Additionally, the K⁺ flux from root to shoot, which was the flux most suppressed by NH₄⁺ at low external [K⁺], was nearly identical in plants grown with NO₃⁻ or NH₄⁺ at the higher K⁺ concentration (Kronzucker et al., 2003b). These effects, in particular the suppression of K⁺ influx at the plasma membrane under low-K⁺, high-NH₄⁺ conditions, are likely to be due to the inhibitory action of NH₄⁺ upon high-affinity KUP/HAK/KT transporters (Spalding et al., 1999).

While inhibition of K⁺ uptake and modification of K⁺ efflux by NH₄⁺ has been demonstrated, the reciprocal effect has only been sparsely investigated (Scherer et al., 1984; Vale et al., 1988b; Wang et al., 1996; Nielsen and Schojerring, 1998). Nielsen and Schojerring (1998) found that 100 mM K⁺ reduced the influx of NH₄⁺ by 50% in leaf apoplasm of Brassica napus L. Other studies have demonstrated moderate suppression of NH₄⁺ isothemys by K⁺, but not to the same extent as the suppression of K⁺ influx by NH₄⁺ (Scherer et al., 1984; Vale et al., 1988b; Wang et al., 1996). While many of these studies were conducted at low external concentrations of both NH₄⁺ and K⁺, none considered growth conditions mediated by LATS for either ion.

To investigate how NH₄⁺ fluxes are influenced by external [K⁺], and how this interaction may underlie potassium’s alleviation of ammonium toxicity, NH₄⁺ fluxes were examined in intact barley seedlings using the short-lived positron-emitting radiotracer ¹³N. It was hypothesized that increasing external [K⁺] would: (i) decrease unidirectional NH₄⁺ fluxes across the plasma membrane; (ii) reduce the high NH₄⁺ efflux:influx ratio that is symptomatic (and perhaps a causative agent) of NH₄⁺ toxicity; and (iii) lessen the energy burden associated with toxic NH₄⁺ fluxes. All three hypotheses were borne out in the study. It is proposed that low-affinity NH₄⁺ 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 K₂SO₄); 0.1, 1.5, 5, and 40 mM, and NH₄⁺ [as (NH₄)₂SO₄], 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, F65T12), with an irradiation of 200 μmol photons m⁻² s⁻¹ at plant height, for 16 h day⁻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 ¹³N (τ₁/₂=9.97 min; as ¹⁵NH₄⁺) provided by the CAMH cyclotron facility (University of Toronto, Ontario, Canada). Labelled seedlings were attached to efflux funnels and eluted of radioactive tracer with successive 20 ml aliquots of non-radioactive desorption solution, identical to the growth solution. Each 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 X = ln φ₀ + k⁻¹t (in which φ₀ is tracer efflux at elution time t, φ₀ 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, φ₀, was determined from φ₀ divided by the specific activity of the cytosol (SA₅₀) at the end of the labelling period: SA₅₀ is estimated by using external specific activity (SAₐ), labelling time t, and the rate constant k, which describes tracer exchange with the cytosol, which are related in the exponential rise function SA₅₀=SA₀(1−e⁻ᵀ_SA₅₀) (Kronzucker et al., 1995). Net flux, φ₀, was found using total plant ¹⁵N retention after desorption (Kronzucker et al., 1995). Influx, φ₀, was calculated from the sum of φ₀ and φ₀. Cytosolic [NH₄⁺] ([NH₄⁺]₅₀) was determined using the flux turnover equation, [NH₄⁺]₅₀=Ωφ₀/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).
homogenized under liquid N2 using a mortar and pestle, followed by storage at –80 °C. Cytosolic exchange half-times are listed in parentheses (SEM of 4–9 replicates (SEM was, on average, 15% of the mean).)

To measure tissue NH4+ content, barley seedlings were harvested and desorbed for 5 min in 10 mM CaSO4 to remove extracellular NH4+. Roots and shoots were then separated and weighed, then transferred to polyethylene plastic vials and frozen in liquid N2 for storage at –80 °C. Approximately 0.5 g of root or shoot tissue was homogenized under liquid N2 using a mortar and pestle, followed by the addition of 6 ml of formic acid (10 mM) for the purpose of extracting NH4+ (Husted et al., 2000). Subsamples (1 ml) of the homogenate were centrifuged at 2.5×104 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 5×105 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 NH4+ 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-mercaptopethanol in 1 d before use. Prior to the addition of 2-mercaptopethanol, 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 Oxycal control system (Hansatech Instruments, Norfolk, UK). Seedlings were placed in a cuvette with 2.5 ml of air-saturated growth solution. The decline in O2 concentration was monitored for 15 min, but only the initial linear decline was used to calculate O2 depletion rates. The energy costs of ion transport were calculated based upon the following equation:

\[
\frac{1}{U_{\text{theor}}} = \frac{(H/P)\times(P/O_{2})}{(H/I) \times M_{f}}
\]

where \(1/U_{\text{theor}}\) is the cost of active ion transport (mol O2 mol−1 ion), \(H/I\) is the proton/ion stoichiometry, \(M_{f}\) 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 NH4+ and K+ transport are assumed to involve an electrogenic unipart that requires charge balancing via the outward pumping of one proton per NH4+ 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 O2 consumption to ATP production is ~5 ATP/O2. Therefore, in the application of this model to the primary unidirectional influx of NH4+ or K+, across the root plasma membrane, \(H/I=1\), \(H/P=1\), and \(P/O_{2}=5\), resulting in \(1/U_{\text{theor}}=0.2\) (mol O2 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., 2006b).

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 NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ influx or the efflux:influx ratio. Thus, the constant residual flux observed throughout the LATS range is identified as the K⁺-insensitive component of NH₄⁺ influx.

Table 1. Tissue NH₄⁺ content of roots and shoots of barley seedlings, grown with 10 mM [NH₄⁺]ext, as determined by the indophenol and OPA methods

<table>
<thead>
<tr>
<th>Assay method</th>
<th>[K⁺]ext (mM)</th>
<th>Root content [μmol g⁻¹ (root FW)]</th>
<th>Shoot content [μmol g⁻¹ (root FW)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indophenol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Berthelot)</td>
<td>0.1</td>
<td>69.60±2.66 a</td>
<td>43.82±0.72 a</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>22.60±0.49 b</td>
<td>9.47±0.41 b</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>14.31±0.46 c</td>
<td>9.39±0.39 b</td>
</tr>
<tr>
<td>OPA</td>
<td>0.1</td>
<td>71.42±1.00 a</td>
<td>39.80±1.76 a</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>22.93±1.46 b</td>
<td>10.61±0.19 b</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>15.07±0.32 c</td>
<td>10.72±0.22 b</td>
</tr>
</tbody>
</table>

Elevated K⁺ rapidly decreases unidirectional NH₄⁺ influx

Figure 3 shows the influx of NH₄⁺ into intact barley seedlings, as determined by short-term (5 min) accumulation of ³¹NH₄⁺. In agreement with compartmental analysis, NH₄⁺ 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, NH₄⁺ 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 NH₄⁺, but also its efflux (Fig. 4). After introducing an elevated (5 mM) concentration of K⁺ midway through an elution protocol, NH₄⁺ efflux declined notably within a few minutes. Within 15 min following the shift in [K⁺]ext, the half-time of cytosolic NH₄⁺ 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 NH₄⁺ efflux (Fig. 4, inset).

La³⁺ application mimics K⁺ inhibition of NH₄⁺ influx

The channel inhibitors TEA, Cs⁺, and La³⁺, which have been shown to reduce channel-mediated fluxes of K⁺ and NH₄⁺ (Wegner et al., 1994; Nielsen and Schjoerring, 1998), were used to help identify the mechanisms underlying the K⁺-sensitive and -insensitive components of
NH₄⁺ transport (Fig. 5). Interestingly, Cs⁺ and TEA both stimulated NH₄⁺ influx, under high (40 mM) and low (0.1 mM) [K⁺]ₑₓ, with TEA in particular increasing NH₄⁺ influx by nearly 40%. In contrast, La³⁺ application reduced the influx of NH₄⁺ by 60% under low [K⁺]ₑₓ (0.1 mM). At elevated [K⁺]ₑₓ, no reduction in NH₄⁺ influx was observed. The effects of La³⁺ upon K⁺ influx were also tested, using ⁴²K as a tracer (Fig. 5, inset).

Fig. 4. Effect of K⁺ alteration on ¹³NH₄ efflux from the roots of intact barley seedlings grown with 10 mM [NH₄⁺]ₑₓ and 0.1 mM [K⁺]ₑₓ. The arrow indicates time of shift in [K⁺]ₑₓ 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 ¹³NH₄ efflux from the roots of intact barley seedlings grown with 10 mM [NH₄⁺]ₑₓ and 5 mM [K⁺]ₑₓ. The arrow indicates time of shift in [K⁺]ₑₓ 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 NH₄⁺ 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⁺]ₑₓ (P < 0.05). Inset: direct K⁺ influx measurements using short-term labelling, in the presence La³⁺ (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 NH₄⁺ and K⁺ fluxes can be energetically costly**

The energetics of NH₄⁺ transport were analysed using previously reported plasma membrane electrical potentials from the same plant system (Szczerba et al., 2006a) and cytosolic concentrations of NH₄⁺ 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⁺, NH₄⁺, and a univalent anion (upper estimate), or to a simple solution of (NH₄)₂SO₄ (lower estimate) (Nobel, 1991; Lide, 2007). Although the cytosolic activities of NH₄⁺ were significantly different at low and high [K⁺]ₑxtern, the electrochemical potential gradient for NH₄⁺ was inwardly directed in both cases. Thus, NH₄⁺ 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 NH₄⁺ toxicity, root respiration experiments were conducted (Fig. 7). Specific respiratory costs of the active components of K⁺ and NH₄⁺ 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., 2006a). In all conditions tested, the respiratory costs not associated with NH₄⁺ and K⁺ fluxes were similar. The two conditions with the lowest energy requirement for NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ fluxes and measured plasma membrane electrical potentials (ΔΨ, from Szczerba et al., 2006a). Also included are measured cytosolic NH₄⁺ concentrations and ranges of NH₄⁺ activities calculated using two estimates for the ionic strength (I) of the cytosol: (i) based on K⁺, NH₄⁺, and a monovalent anion—upper value; or (ii) I based on a simple solution of (NH₄)₂SO₄—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 NH₄⁺ 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 NH₄⁺ 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; Siddiqui et al., 1991; Britto et al., 2001; Kronzucker et al., 2003b; Szczerba et al., 2006a). This technique initially led to the discovery of rapid, futile cellular NH₄⁺ cycling in a number of plant systems (Britto et al., 2001, 2002; Kronzucker et al., 2003a; Britto and Kronzucker, 2006), and to the close association of this phenomenon with NH₄⁺ toxicity (Kronzucker et al., 2001). In the present study, the high unidirectional fluxes of NH₄⁺ 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 NH₄⁺ toxicity in plants can be relieved by increasing potassium availability (Cao et al., 1993; Spalding et al., 1999; Santa-Maria et al., 2000; Kronzucker et al., 2003b; Szczerba et al., 2006a), it was hypothesized that changes in external [K⁺] would alter the magnitude of NH₄⁺ fluxes and the degree of futile cycling in NH₄⁺-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 NH₄⁺, 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 NH₄⁺ 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 NH₄⁺ of ~70%, showing that the amelioration of NH₄⁺ toxicity by increasing [K⁺]ext was paralleled by a reduction in NH₄⁺ tissue content (Fig. 6, Table 1). The [NH₄⁺]cyt values for the toxic condition, while high, are in agreement with values reported in a separate study on NH₄⁺ toxicity in barley seedlings (Britto et al., 2001). Moreover, the tissue NH₄⁺ values are well in excess of what is required to account for the [NH₄⁺]cyt estimates, demonstrating that a substantial NH₄⁺ 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 NH₄⁺ at the plasma membrane, subtraction of cytosolic content from whole-root content of NH₄⁺ 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 NH₄⁺ 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 NH₄⁺. The rapidity of this response suggests that K⁺ regulates NH₄⁺ fluxes possibly by acting allosterically on an ammonium transporter, or by competing directly with NH₄⁺ 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 NH₄⁺ influx seen in the rightmost column in Fig. 3. The lack of an immediate stimulatory effect on NH₄⁺ 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 NH₄⁺-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 NH₄⁺ transporters also occurs over a longer time scale.

K⁺-sensitive NH₄⁺ influx pathway

It is not yet fully resolved how NH₄⁺ enters the plant cell, particularly in the low-affinity range. The present study provides new insight into possible candidates and characteristics of low-affinity NH₄⁺ transport. The dramatic reduction of NH₄⁺ 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 NH₄⁺ influx operate simultaneously, one sensitive and the other insensitive to [K⁺]ext. Because NH₄⁺ 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 NH₄⁺ 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⁺, Ca²⁺, K⁺, and NH₄⁺ (Demidchik et al., 2002b). 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, NH₄⁺ influx at 0.1 mM [K⁺]ext was strongly inhibited by the relatively broad-spectrum channel blocker La³⁺, but no La³⁺ 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 La³⁺-sensitive NSCC pathway, making it unavailable for NH₄⁺ transport. Evidence that K⁺ is indeed also transported through the La³⁺-sensitive pathway is seen in the inhibitory effect of
La³⁺ 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 NH₄⁺; see Spalding et al., 1999, and Introduction). The lack of inhibition of NH₄⁺ influx by Cs⁺ and TEA is also consistent with permeation through NSCCs. Indeed, it is tempting to attribute K⁺-sensitive, La³⁺-sensitive NH₄⁺ 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 NH₄⁺ and K⁺ as competing substrates; relative insensitivity to TEA and Cs⁺; and strong inhibition by the lanthanide Gd³⁺ as well as by La³⁺ itself. The observation that, in the present study, TEA and Cs⁺ actually stimulated NH₄⁺ 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 Ca²⁺ (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 Gd³⁺ was effective in inhibiting the TEA-stimulated fluxes, a result very similar to the La³⁺ suppression of TEA-stimulated NH₄⁺ influx observed in the present study.

Many studies, in a variety of organisms including bacteria, yeast, animals, and plants, have suggested that NH₄⁺ enters the cell through K⁺-specific channels (Wang et al., 1996; Nielsen and Schjoerring, 1998; Hess et al., 2006), and the K⁺ suppression of NH₄⁺ influx (Fig. 2) supports the idea that K⁺ channels are responsible for the K⁺-sensitive component of low-affinity NH₄⁺ uptake, instead of, or in addition to, NSCCs. At higher [K⁺]ₑₓ, these channels would be occupied by K⁺, limiting NH₄⁺ influx to NH₄⁺-specific pathways. Consistent with this observation was the finding that La³⁺ (known to block K⁺ channels as well as NSCCs; Wegner et al., 1994) blocked NH₄⁺ influx at low [K⁺]ₑₓ, in addition to reducing K⁺ influx at both high and low [K⁺]ₑₓ (Fig. 5). However, the stimulation of NH₄⁺ influx by the K⁺-channel blockers Cs⁺ and TEA does not support NH₄⁺ permeation through K⁺ channels. Thus, the proposal that NSCCs are responsible for the K⁺-sensitive component of root NH₄⁺ 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 NH₄⁺ influx with La³⁺ and Cs⁺ treatments, respectively.

K⁺-insensitive NH₄⁺ influx pathway

In addition to K⁺-sensitive NH₄⁺ conductance, a substantial portion of low-affinity NH₄⁺ entry into barley root cells is mediated by a K⁺-insensitive mechanism (Fig. 2). This mechanism is resistant to increases in [K⁺]ₑₓ 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 NH₄⁺ 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 NH₄⁺ transport is down-regulated by high NH₄⁺, both genetically and functionally (Rawat et al., 1999), which eliminates its likelihood as a candidate for NH₄⁺ influx under the NH₄⁺ supply (10 mM) used in the present study. Another possibility is that NH₄⁺ 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 CO₂, glycerol, urea, NH₄⁺, and NH₃ (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 NH₄⁺ influx, casting doubt on the role of aquaporins in low-affinity NH₄⁺ transport. The elimination of these two candidates suggests that NH₄⁺ enters root cells under high-K⁺, high-NH₄⁺ conditions via NH₄⁺-specific channels, the molecular identity of which remains to be determined.

Energetics of NH₄⁺ and K⁺ unidirectional fluxes

It is instructive to examine, from an energetics perspective, the unidirectional NH₄⁺ fluxes observed here. The thermodynamic analysis shows that, under all experimental conditions, NH₄⁺ exchange across the plasma membrane takes the form of a ‘leak–pump’ scenario, i.e. with passive NH₄⁺ influx coupled to active NH₄⁺ efflux (Fig. 6). In this respect, bidirectional NH₄⁺ transport follows a pattern that has been observed in the low-affinity exchange of other major cations, such as K⁺ (Szczeparka et al., 2006a) 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 NH₄⁺ and K⁺ transport were 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 NO₃⁻ anion (Schuurwater 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 NH₄⁺ and K⁺. The plants that displayed rapid, futile cycling of NH₄⁺ 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 NH4+ fluxes in the low-affinity transport range, and offers insight into the mechanism by which K+ is able to alleviate NH4+ toxicity. Elevated K+ eliminates a major fraction of low-affinity NH4+ influx, and substantially reduces the amount of futile cycling of this toxic ion. Intriguingly, this effect contrasts sharply with the effect of NH4+ on K+ transport, where high-affinity influx is diminished by NH4+, but low-affinity influx remains unaffected (Spalding et al., 1999; Kronzucker et al., 2003b; Szczerba et al., 2006a). It is proposed that low-affinity NH4+ transport may be mediated by the dual operation of non-selective, K+-sensitive cation channels on the one hand, and K+-insensitive, NH4+-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 NH4+ 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 NH4+ fluxes under both high- and low-K+ conditions show virtually the same degree of La3+ resistance (Fig. 5); and (ii) NH4+ 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 La3+- and K+-insensitive component of low-affinity NH4+ influx is operative under all K+ conditions. This study demonstrates how pivotal a role K+ plays in the regulation of NH4+ toxicity, reducing the energy burden of toxic NH4+ fluxes, and substantially improving growth under a high-NH4+ nutritional regime.

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