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Optimization of ammonium acquisition and metabolism by potassium in rice (*Oryza sativa* L. cv. IR-72)

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ABSTRACT

We present the first characterization of K^+ optimization of N uptake and metabolism in an NH_4^+-tolerant species, tropical lowland rice (cv. IR-72). ^{13}N radiotracing showed that increased K^+ supply reduces futile NH_4^+ cycling at the plasma membrane, diminishing the excessive rates of both unidirectional influx and efflux. Pharmacological testing showed that low-affinity NH_4^+ influx may be mediated by both K^+ and non-selective cation channels. Suppression of NH_4^+ influx by K^+ occurred within minutes of increasing K^+ supply. Increased K^+ reduced free $[NH_4^+]$ in roots and shoots by 50–75%. Plant biomass was maximized on 10 mM NH_4^+ and 5 mM K^+, with growth 160% higher than 10 mM NO_3^--grown plants, and 220% higher than plants grown at 10 mM NH_4^+ and 0.1 mM K^+. Unlike in NH_4^+-sensitive barley, growth optimization was not attributed to a reduced energy cost of futile NH_4^+ cycling at the plasma membrane. Activities of the key enzymes glutamine synthetase and phosphoenolpyruvate carboxylase (PEPC) were strongly stimulated by elevated K^+, mirroring plant growth and protein content. Improved plant performance through optimization of K^+ and NH_4^+ is likely to be of substantial agronomic significance in the world's foremost crop species.

Key-words: cereals; channels; glutamine synthetase; influx, efflux; ion transport; nitrogen; phosphoenolpyruvate carboxylase.

INTRODUCTION

Nitrogen (N) is the nutrient most limiting to plant growth and yield, and is commonly taken up from the soil in one of two inorganic forms: ammonium (NH_4^+) or nitrate (NO_3^-) (Barker & Mills 1980). Both forms can be found in terrestrial ecosystems over wide concentration ranges (Miller & Cramer 2004), but in some soils one form may dominate, as in the rice paddies of tropical Asia, where NH_4^+ is the predominant N source (Yu 1985). At low (micromolar) concentrations, NH_4^+ is an adequate N source for many plant species, but most cannot tolerate higher (millimolar) concentrations, exhibiting toxicity symptoms including declines in growth and yield (Britto & Kronzucker 2002). Exceptions to this include late successional conifers such as white spruce (Kronzucker, Siddiqi & Glass 1997; Kronzucker *et al.* 2003), species of *Vaccinium* (Greidanus *et al.* 1972), tea (*Camellia sinensis*; Ruan *et al.* 2007), and tropical lowland rice (Magalhães & Huber 1989), the focus of the present study. Presently, no single mechanism can fully account for NH_4^+ toxicity (or tolerance; Britto & Kronzucker 2002; Roosta & Schjoerring 2008), but leading explanations include the depletion of C supply due to the requirement for NH_4^+ assimilation in roots (Schortemeyer, Stamp & Feil 1997; Finnemann & Schjoerring 1999; Cruz *et al.* 2006), the energy loss associated with futile transmembrane NH_4^+ cycling (Britto *et al.* 2001; Kronzucker *et al.* 2001), the effects of reduced pH in the root zone (Chaillou *et al.* 1991), and the NH_4^+-induced deficiency of mineral cations (Barker, Maynard & Lachman 1967; Van Beusichem, Kirkby & Baas 1988; Kafkafi 1990).

The last of these mechanisms helps explain why increased external K^+ concentration ($[K^+]_{ext}$) can protect sensitive plant species from NH_4^+ toxicity (Cao, Glass & Crawford 1993; Spalding *et al.* 1999; Santa-Maria, Danna & Czibener 2000; Kronzucker, Szczerba & Britto 2003; Szczerba, Britto & Kronzucker 2006). In addition, as we have previously shown that barley, an NH_4^+-sensitive species, the excessive flux of NH_4^+ into and out of root cells can be substantially reduced by the elevation of $[K^+]_{ext}$, both immediately and in the steady state (Szczerba *et al.* 2008a). This rapid, potent and permanent inhibition of NH_4^+ transport was interpreted to be an important factor in the alleviation of toxicity symptoms and restoration of normal growth that are characteristic of increased K^+ supply in the presence of high NH_4^+ (Britto & Kronzucker 2002). It was further suggested that relief from toxicity was due in part to a decline in respiratory demand for futile ammonium cycling across the plasma membrane, a potentially important source of NH_4^+ stress in barley. In another study (Britto *et al.* 2001), energy-demanding futile cycling of NH_4^+ was observed in barley but not, however, in tropical lowland rice, a species which is rare among cereals in being not only NH_4^+-tolerant, but NH_4^+-preferring. Nevertheless, as we have more recently shown, even rice displays growth some inhibition on NH_4^+ (relative to NO_3^-), when the K^+ supply is very low, but recovers with elevated K^+, at least partly due to the restoration of adequate K^+ transport and accumulation (Szczerba *et al.* 2008b).

Because of its remarkable resistance to NH_4^+ toxicity, and paramount agronomic importance, we have further pursued

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our investigation into this key relationship between K^+ and NH_4^+ in further detail, with special focus on the transport and metabolism of NH_4^+, in rice. More specifically, we have used growth data to examine NH_4^+ stress and its alleviation by K^+, the short-lived radioisotope ^{13}N to trace NH_4^+ fluxes and accumulation, and analyses of the activities of glutamine synthetase (GS) and phosphoenolpyruvate carboxylase (PEPC), two key enzymes involved in the assimilation of NH_4^+ into organic N.

MATERIALS AND METHODS

Plant culture

Rice seeds (*Oryza sativa* L. cv. 'IR-72') were surface-sterilized for 10 min in 1% sodium hypochlorite, and germinated in water for 2 d prior to placement in 12 L vessels (0.02 mM $[K^+]_{ext}$) or 4 L and 12 L vessels (all other K^+ conditions) containing aerated, modified Johnson's solution (2 mM $MgSO_4$; 1 mM $CaCl_2$; 0.3 mM NaH_2PO_4; 0.1 mM Fe-EDTA; 20 μM H_3BO_3; 9 μM $MnCl_2$; 1.5 μM $CuSO_4$; 1.5 μM $ZnSO_4$; 0.5 μM Na_2MoO_4), pH 6–6.5, for an additional 19 d. The growth solutions were modified to provide five concentrations of potassium (as K_2SO_4), at 0.02, 0.1, 1.5, 5 and 40 mM, and nitrogen (10 mM or 0.1 mM) as $(NH_4)_2SO_4$, or (10 mM) as $Ca(NO_3^-)_2$. Solutions were exchanged frequently to ensure that plants remained at nutritional steady state, and to ensure that solution pH was maintained between 6 and 6.5. Solutions were exchanged on days 8, 12, 15, 17, 19 and 20 (plants were grown for 21 d in total, including the first two spent in water for germination). Plants were cultured in climate-controlled walk-in growth chambers under fluorescent lights, providing a tropical environment for the seedlings, with a day/night temperature cycle of 30°C/20°C, an irradiation of 425 μmol photons m^{-2} s^{-1} at plant height for 12 h d^{-1} (Sylvania Cool White, 96T12/CW/VHO), and a relative humidity of 70%.

On day 20 (1 d prior to experimentation), seedlings were bundled together in groups of 3–8 at the stem base using a plastic collar, 0.5 cm in height. For ^{13}N experiments, seedlings were transferred on day 20 to an experimental radiotracer facility that had similar irradiance and temperature as the growth chamber.

Compartmental analysis

Compartmental analysis by tracer efflux was used to estimate unidirectional NH_4^+ fluxes and pool sizes (Lee & Clarkson 1986; Siddiqi, Glass & Ruth 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 and 60 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, Toronto, ON, 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. 2a) was used to resolve the kinetics of the slowest exchanging (intracellular) phase in these experiments (Kronzucker *et al.* 1995; Britto & Kronzucker 2003). Chemical efflux, Φ_{co}, was determined from $\Phi_{co(i)}^*$, divided by the specific activity of the intracellular $^{13}NH_4^+$- releasing pool (SA_{int}) at the end of the labelling period; SA_{int} was estimated by using external specific activity (SA_o), labelling time t, and the rate constant k, which are related in the exponential rise function $SA_{int} = SA_o(1 - e^{-kt})$ (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}. Freely exchangeable root NH_4^+ ($NH_4^+{}_{exch}$) was determined using the flux turnover equation, $NH_4^+{}_{exch} = \Phi_{oc}/k$ (Britto & Kronzucker 2001).

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 or 5 min (the two labelling times were used to investigate the rapidity of NH_4^+ influx response to changing $[K^+]_{ext}$). 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.

Pharmacological agents

Using the general procedure described above, direct influx measurements were conducted in the presence of one of the following channel inhibitors: caesium (Cs^+), lanthanum (La^{3+}), tetraethylammonium (TEA^+) or zinc (Zn^{2+}). Prior to

radiotracing, seedlings were placed for 10 min in growth solution for equilibration with 10 mM of inhibitor. Labelling with ^{13}N, 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, rice seedlings were harvested and desorbed for 5 min in 10 mM $CaSO_4$ to remove extracellular NH_4^+. Roots and shoots were separately weighed and transferred to polyethylene plastic vials with 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 10 mM formic acid to extract NH_4^+ (Husted *et al.* 2000). Subsamples (1 mL) of the homogenate were centrifuged at 2.53 *g* and 2 °C for 10 min. The supernatant was transferred to 2 mL polypropylene tubes with 0.45 μm nylon filters (Costar, Corning Inc., Lowell, MA, USA) and centrifuged at 53 000 *g* (2 °C) for 5 min. The resulting supernatant was analysed by the *o*-phthalaldehyde (OPA) method to determine total tissue NH_4^+ content, as described in detail elsewhere for use with spectrophotometry (Goyal, Rains & Huffaker 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 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

Root respiration was determined in excised roots from 21-day-old rice seedlings using a Hansatech oxygen electrode and Oxygraph control system (Hansatech Instruments, Norfolk, UK). Roots were cut into approximately 3-mm-long sections under solution using a razor blade, and aged for a minimum of 3 h in the appropriate aerated growth solution. About 0.3 g of root material was placed into 3 mL of growth solution, and the cuvette was sealed. The decline in O_2 concentration was monitored for 15 min, with the initial, linear, decline used to calculate O_2 depletion rates.

Phosphoenolpyruvate carboxylase activity

Approximately 0.5 g of root was ground by mortar and pestle under liquid N_2. 5 mL of buffer containing 50 mM TRIS-HCL (pH 7.5), 10 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM EDTA and 14 mM 2-mercaptoethanol was added to ground roots, which were then homogenized by mortar and pestle (1 mM PMSF and 10 μg mL^{-1} leupeptin were added to minimize proteolysis).

The homogenate was centrifuged at 14 000 *g* for 30 min. PEPC activity was determined by coupling its activity to malate dehydrogenase-catalysed NADH oxidation in a 3.0 mL final volume of standard buffer containing 100 mM TRIS-HCL (pH 8.0) (Bioshop, Burlington, Ontario, Canada), 5 mM $MgCl_2$ (Sigma, St Louis, MO, USA), 2.5 mM PEP (Roche, Indianapolis, IN, USA), 0.2 mM NADH (Roche), 10 mM $NaHCO_3$ (Sigma) and 15 μg mL^{-1} MDH (Boehringer Mannheim; Roche) to initiate PEPC activity. NADH oxidation was determined spectrophotometrically at 340 nm (Roosta & Schjoerring 2008).

Glutamine synthetase activity

Root GS activity was measured using the 'transferase' assay (Lea & Blackwell 1993). Approximately 0.5 g of root was ground (as above) in a mortar and pestle, then homogenized in 5 mL GS extraction buffer containing 50 mM Tris-HCl, 1 mM EDTA (VWR, Mississauga, Ontario, Canada), 2 mM dithiothreitol (Sigma), 10 mM $MgSO_4$ (Sigma), 5 mM glutamate (Sigma), 10% v/v ethanediol [Ethylene glycol (synonym)] (Sigma) and 0.1% insoluble polyvinylpyrrolidone (PVP) (Sigma); buffer pH was set to 7.8 using 1 M NaOH. The homogenated extract was centrifuged at 17 000 *g* for 45 min at 4 °C. GS activity was measured in a buffer consisting of 100 mM Tris-HCl at pH 7.8, 5 mM NH_2OH (Sigma), 50 mM $MgSO_4$ (Sigma), 50 mM glutamate (Sigma) and 20 mM ATP (Sigma). 0.375 mL of assay buffer was pre-incubated at 30°C, followed by addition of 0.3 mL supernatant. The reaction was allowed to proceed for 30 min, and terminated by the addition of 1 mL $FeCl_3$ reagent (Sigma) [2.5% w/v $FeCl_3$, 5% w/v trichloroacetic acid (Sigma) in 1.5 M HCl]. Controls were performed under identical conditions, except that ATP was absent. The resulting precipitate was centrifuged at 10 000 *g* for 5 min, and the absorbance of the supernatant was measured at 540 nm, and compared with a standard curve of glutamyl hydroxymate (Sigma).

Protein concentration determination

Frozen plant material was stored at –80 °C in Eppendorf tubes for protein content determination using the method of Jones, Hare & Compton (1988). Approximately 0.5 g of frozen tissue was ground in a mortar and pestle under liquid N_2, homogenized in 5 mL of 0.1 M NaOH, vortexed in a centrifuge tube for 3 s, and allowed to sit at room temperature for 30 min. Samples were remixed for 3 s by vortex, then centrifuged for 5 min at high speed (>5000 *g*), and the supernatant was decanted and remixed for 3 s by vortex. One hundred microlitres of aliquots was removed and mixed in a test tube with 5 mL of diluted (1:4) Bradford reagent with added (3 mg mL^{-1}) PVP. After 15 min, contents of each tube were transferred to quartz spectrophotometric cuvettes. Absorbance of samples was read at 595 nm

against a blank with dye reagent and 0.1 M NaOH. Protein concentration was read against a RuBP standard curve made in 0.1 M NaOH (Jones *et al.* 1988).

RESULTS

Growth patterns under combinations of N and K supply

Fresh weights of 21-day-old rice seedlings were strongly affected by K^+ supply under all N regimes (Fig. 1a). When $[K^+]_{ext}$ was low (0.02 mM), plants showed the highest fresh weight on 10 mM NO_3^-, and the lowest on 10 mM NH_4^+, at which other symptoms of NH_4^+ toxicity were observed (e.g. leaf chlorosis and necrosis). Raising $[K^+]_{ext}$ from 0.02 mM to 0.1 mM significantly increased fresh weight, particularly under 10 mM NH_4^+, at which a fresh weight increase of 304% was accompanied by a relief from visible NH_4^+ toxicity symptoms. Growth at 0.1 mM $[K^+]_{ext}$ did not significantly differ between 10 mM NH_4^+ and 10 mM NO_3^- treatments, but was slightly lower under the low-N condition (0.1 mM NH_4^+). Further increasing $[K^+]_{ext}$ to 1.5 mM significantly increased fresh weight only under 10 mM NH_4^+, which supported the highest growth among N regimes at this $[K^+]_{ext}$. Optimal growth for all N conditions was seen at 5 mM $[K^+]_{ext}$, with substantially higher fresh weight at 10 mM NH_4^+ relative to the other N treatments. At the highest $[K^+]_{ext}$ of 40 mM, however, fresh weight was significantly reduced for all N treatments.

In the 10 mM NH_4^+ condition, the whole-plant fresh weight trend was reflected in both root and shoot growth, and root:shoot ratios increased with $[K^+]_{ext}$ (not shown). The fresh weight trend was also paralleled by differences in dry weights among N and K combinations (not shown).

Tracer flux and exchangeable tissue-NH_4^+ measurements in high-NH_4^+-grown plants

Figure 2a shows representative plots of time-dependent $^{13}NH_4^+$ efflux from roots of intact rice seedlings grown at 10 mM NH_4^+ and under the five $[K^+]_{ext}$ conditions. Efflux and tissue-retention data in these experiments provided values for steady-state unidirectional fluxes across the plasma membrane (Fig. 2b), and for rapidly exchangeable tissue NH_4^+ (Fig. 5a). Unidirectional influx and efflux of NH_4^+ varied dramatically with $[K^+]_{ext}$ (Fig. 2b). NH_4^+ influx was maximal at the lowest $[K^+]_{ext}$ (0.02 mM), and when $[K^+]_{ext}$ was raised to 0.1 mM, influx was reduced from 85 to 65 μmol g^{-1} (root fresh weight) h^{-1}. Further increasing $[K^+]_{ext}$ continued to reduce NH_4^+ influx, reaching about 30% of the maximum at 40 mM. Elevated K^+ similarly affected the efflux of NH_4^+, with peak values also observed at 0.02 mM $[K^+]_{ext}$. Increased $[K^+]_{ext}$ reduced NH_4^+ efflux, reaching 32% of the maximum at 40 mM. The ratio of NH_4^+ efflux to influx was also decreased by elevating $[K^+]_{ext}$, from 0.87 (at 0.02 mM $[K^+]_{ext}$) to as little as 0.63 (at 5 mM $[K^+]_{ext}$).

NH_4^+ influx values found using efflux analysis were confirmed by direct, short-term (5 min) influx measurements (Fig. 4a; see Szczerba *et al.* 2006). Again, NH_4^+ fluxes were maximal at 0.02 mM $[K^+]_{ext}$ (reaching the same peak value of 85 μmol g^{-1} h^{-1}). When $[K^+]_{ext}$ was increased to 0.1 mM, NH_4^+ influx was reduced by 39% to 61 μmol g^{-1} h^{-1} and further reduced by 38% to 38 μmol g^{-1} h^{-1} at 1.5 mM $[K^+]_{ext}$, with little change as $[K^+]_{ext}$ was raised further.

Direct, short-term influx experiments were also used to show that the suppressive effect of elevated $[K^+]_{ext}$ on NH_4^+ influx occurs very rapidly, reducing the flux at 0.1 mM $[K^+]_{ext}$, within 1 min after application of 40 mM $[K^+]_{ext}$, to values close to what were found at the 40 mM $[K^+]_{ext}$ steady state (Fig. 4b).

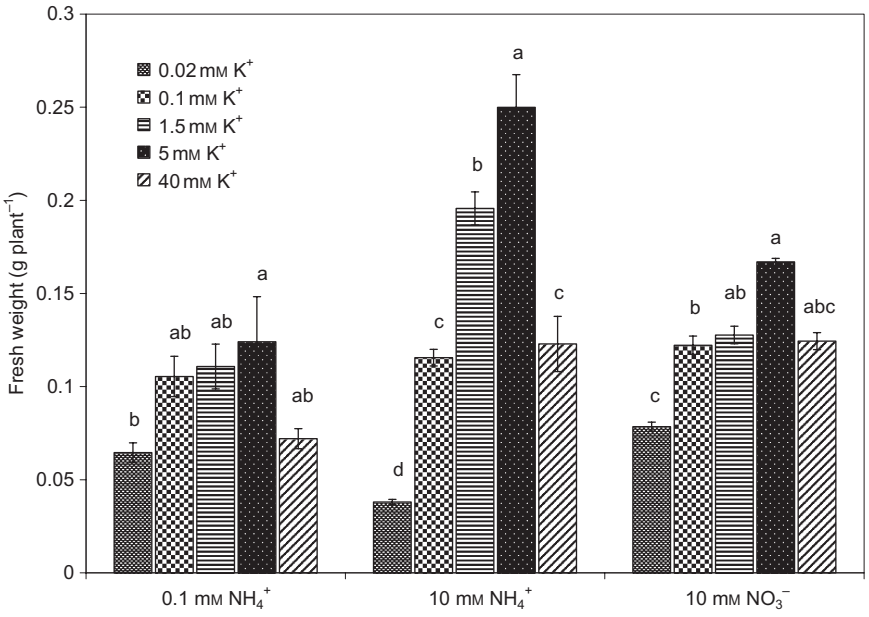

Figure 1. Total fresh weights of 21-day-old rice (cv. IR-72) seedlings grown with 10 mM NH_4^+, 10 mM NO_3^- or 0.1 mM NH_4^+, and at five concentrations of K^+. Letters indicate significantly different means within N treatments (two-factor analysis of variance with Bonferroni post-test, $P < 0.05$). Error bars indicate ± SEM.

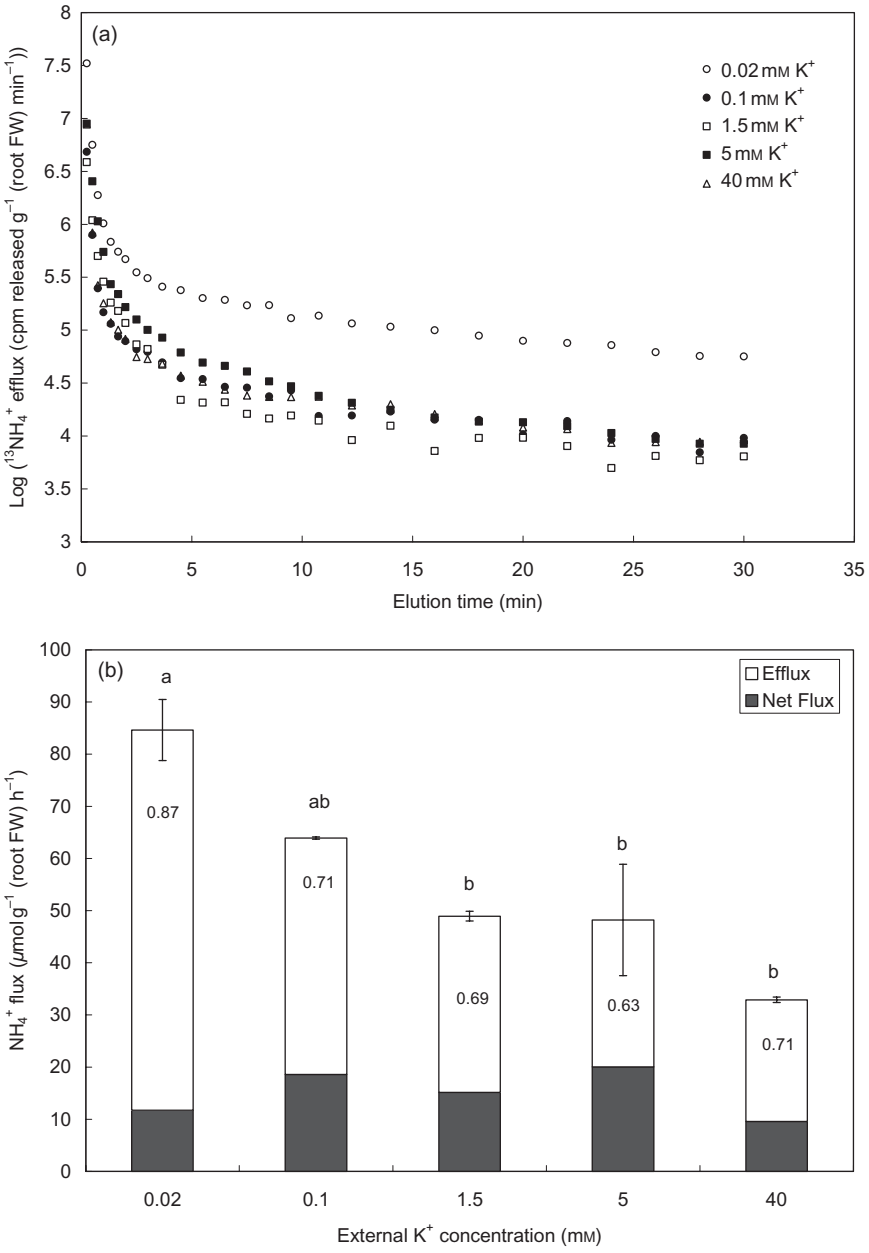

Figure 2. (a) Representative semi-logarithmic plots of steady-state $^{13}NH_4^+$ efflux from roots of intact rice seedling grown and eluted with 10 mM NH_4^+ and five concentrations of K^+. (b) Steady-state NH_4^+ fluxes in intact rice seedlings grown with 10 mM NH_4^+ and five concentrations of K^+, as determined by compartmental analysis. Total height of bars indicates influx (efflux + net flux). Numbers within each bar indicate the ratio of efflux to influx. Letters indicate significantly different influx means (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM of influx.

The channel inhibitors, La^{3+}, TEA, Cs^+ and Zn^{2+}, shown to reduce putatively channel-mediated fluxes of both K^+ and NH_4^+ (Wegner, De Boer & Raschke 1994; Nielsen & Schjoerring 1998), were used to help identify the mechanisms underlying the K^+-sensitive and -insensitive components of NH_4^+ transport (Fig. 4c). La^{3+}, Cs^+ and Zn^{2+} reduced NH_4^+ influx at low $[K^+]_{ext}$ (0.02 mM) by about 25%, while TEA reduced NH_4^+ influx by about 50%. At higher (5 mM) $[K^+]_{ext}$, La^{3+}, TEA and Zn^{2+} reduced NH_4^+ influx by 31%, while the K^+ channel inhibitor Cs^+ had no effect.

Rapidly exchangeable NH_4^+ ($NH_4^+{}_{exch}$) in roots of plants grown at 10 mM NH_4^+ was quantified using tracer efflux (Fig. 5a), and was found to decline significantly with increased $[K^+]_{ext}$. $NH_4^+{}_{exch}$ was highest (28 μmol g^{-1}) at low $[K^+]_{ext}$ (0.02 mM), and declined to 22 μmol g^{-1} when $[K^+]_{ext}$ was increased to 0.1 mM. Increasing $[K^+]_{ext}$ to 1.5 mM further reduced $NH_4^+{}_{exch}$ to 18 mM, but no significant effects on $NH_4^+{}_{exch}$ were observed by continued increases in $[K^+]_{ext}$.

$NH_4^+{}_{exch}$ was found to be in good agreement with tissue NH_4^+ concentrations (Table 1), suggesting that the majority

Table 1. Tissue NH_4^+ content of rice seedlings, grown with 10 mM NH_4^+ and five concentrations of K^+

	Tissue NH_4^+ content (μmol NH_4^+ g^{-1} (fresh weight))	
$[K^+]_{ext}$ (mM)	Root	Shoot
0.02	37.57 ± 0.66	75.70 ± 1.40
0.1	28.08 ± 1.43	48.79 ± 1.11
1.5	17.35 ± 0.83	18.64 ± 0.15
5	15.36 ± 0.37	15.81 ± 1.00
40	12.44 ± 0.29	13.03 ± 0.15

of tissue NH_4^+ is rapidly exchangeable. As with the tracer method, tissue analysis showed an inverse relationship between $[K^+]_{ext}$ and root NH_4^+ content. Shoot tissue NH_4^+ was also determined, and it also showed this inverse relationship.

In vitro activities of GS and PEPC

The activity of the main NH_4^+ assimilatory enzyme, GS, was measured in roots of plants grown at 10 mM NH_4^+ and the five $[K^+]_{ext}$ conditions (Fig. 6a). GS activities were extremely low at the lowest (0.02 mM) $[K^+]_{ext}$, but increased dramatically with increasing $[K^+]_{ext}$, up to a maximum in plants grown at 5 mM $[K^+]_{ext}$. At the highest $[K^+]_{ext}$ tested (40 mM), however, GS activity was significantly reduced.

A similar pattern was seen when activity of the anapleurotic carbon-fixing enzyme PEPC was also measured in roots (Fig. 6b). Again, activities were lowest at the lowest $[K^+]_{ext}$ (0.02 mM), with significant increases as $[K^+]_{ext}$ was increased stepwise to 0.1, 1.5 and 5 mM. At 40 mM $[K^+]_{ext}$, a significant decrease was observed as with GS.

Protein content

Root, shoot and total protein content were measured in plants grown at 10 mM NH_4^+ (Fig. 5b). Under all $[K^+]_{ext}$ conditions, protein content was higher in shoots than in roots. Following the trend seen with GS and PEPC, the lowest protein content was measured at low $[K^+]_{ext}$ (0.02 mM) in both shoot and root. Significant increases in protein were observed as $[K^+]_{ext}$ was raised to 0.1 and 1.5 mM, with a significant decline at the highest $[K^+]_{ext}$ of 40 mM.

Root oxygen consumption

Respiration was measured using excised roots (Fig. 3). In all cases, root oxygen consumption was approximately 30 μmol O_2 g^{-1} (root fresh weight) h^{-1}, except at 5 mM $[K^+]_{ext}$, where O_2 consumption was significantly, but not dramatically, lower (Fig. 3).

DISCUSSION

Optimization of plant performance at 5 mM $[K^+]_{ext}$

Growth measurements at the lowest $[K^+]_{ext}$ confirm that rice, normally NH_4^+-tolerant, can display NH_4^+ stress under this special condition (Fig. 1). Nevertheless, compared with 10 mM NO_3^- (and 0.1 mM NH_4^+), maximal growth was observed with NH_4^+ (10 mM) as a sole N source, when $[K^+]_{ext}$ was raised to 1.5 and 5 mM K^+ (Fig. 1). This indicates that, when K^+ is adequate, rice indeed prefers ammonium over nitrate. Previous kinetic comparisons have shown that rice can ably acquire both N sources (Kronzucker *et al.* 2000), and can also benefit from their co-provision (Kronzucker *et al.* 1999). However, the K^+ optimization of growth on NH_4^+ is much more significant than the improvements seen

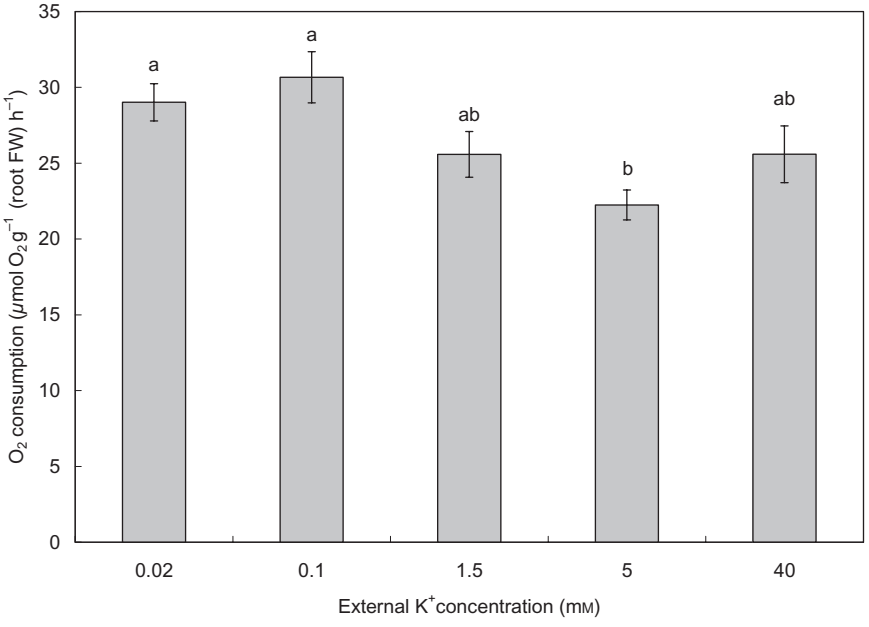

Figure 3. Rates of oxygen uptake in roots of aged, excised rice seedlings grown with 10 mM NH_4^+ and five concentrations of K^+. Letters indicate significantly different means (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM.

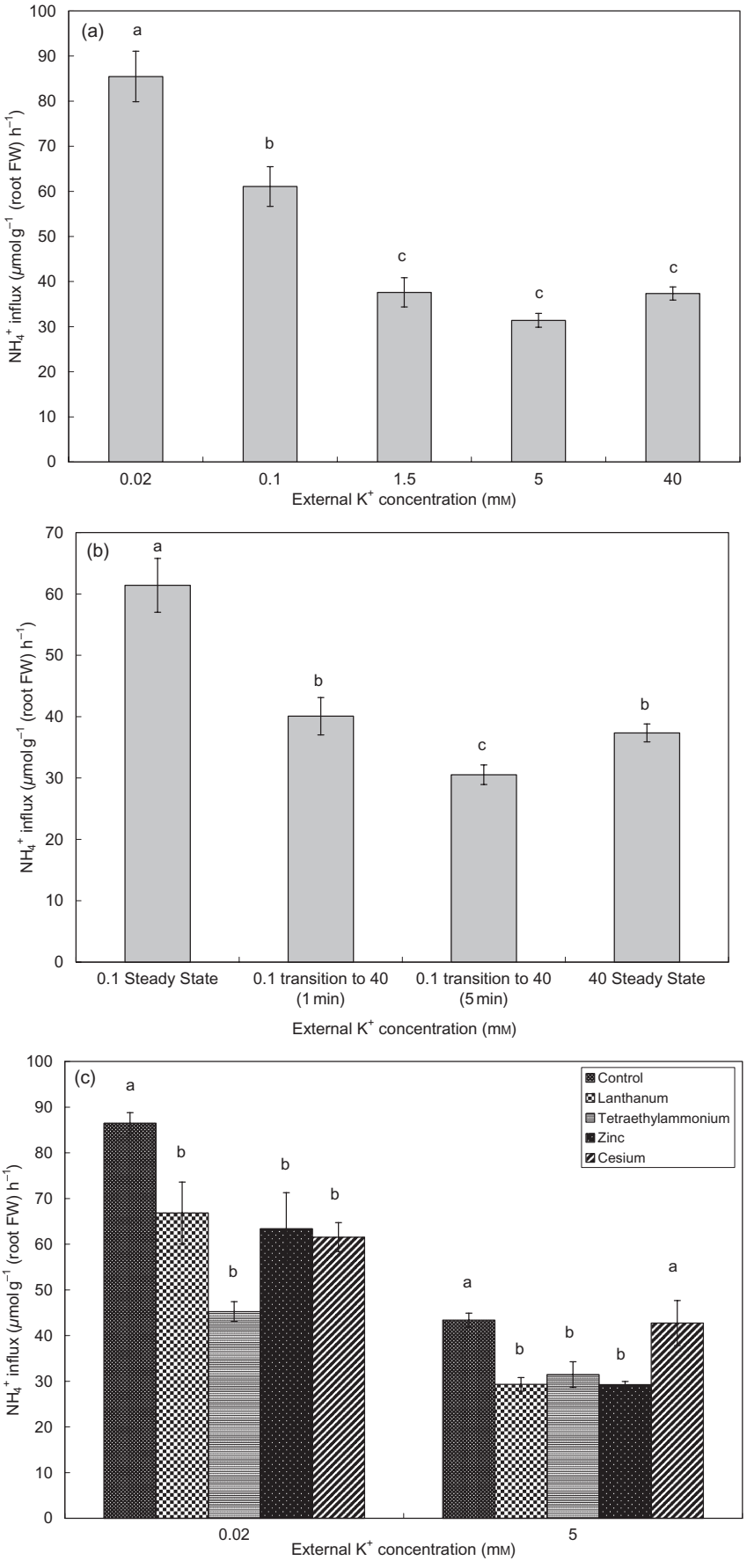

Figure 4. (a) Direct NH_4^+ influx, determined by short-term (5 min) ^{13}N labelling of intact rice seedlings, grown with 10 mM NH_4^+ and five concentrations of K^+. Letters indicate significantly different means (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM. (b) Immediacy of K^+ effect on $^{13}NH_4^+$ influx in intact rice seedlings grown and measured at 10 mM NH_4^+ and one of four K^+ conditions: 0.1 mM steady-state throughout, or 0.1 mM steady-state but labelled at 40 mM for 1 or 5 min. The 40 mM K^+ steady-state condition is shown for long-term comparison. All solutions contained 10 mM NH_4^+. Letters indicate significantly different means (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM. (c) Effect of channel inhibitors (10 mM) on direct NH_4^+ influx measurements using short-term (5 min) ^{13}N labelling in intact rice seedlings grown at 10 mM NH_4^+ and low or high $[K^+]_{ext}$. Letters indicate significantly different means for a given K^+ condition (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM.

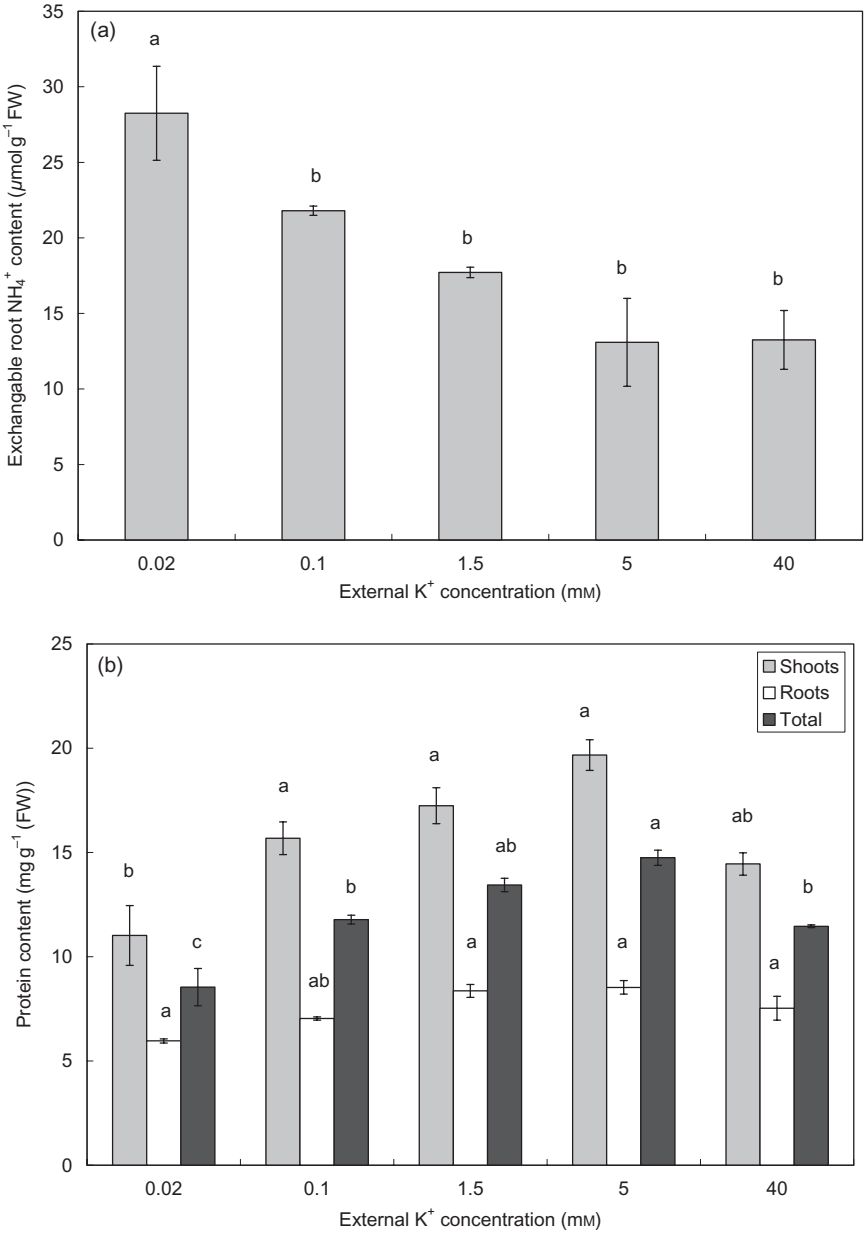

Figure 5. (a) Rapidly exchangeable root NH_4^+ measured by compartmental analysis in rice seedlings grown with 10 mM NH_4^+ and five concentrations of K^+. Letters indicate significantly different means (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM. (b) Shoot, root and total protein content in rice grown with 10 mM NH_4^+ and five concentrations of K^+. Letters indicate significantly different means for a given organ or for the whole plant (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM.

when NH_4^+ and NO_3^- sources are combined (Kronzucker *et al.* 1999).

Irrespective of N source, we found that growth of both roots and shoots of rice was optimal at 5 mM $[K^+]_{ext}$ within the range of applied K^+ conditions (Fig. 1). Interestingly, when tested in detail at 10 mM NH_4^+, the 5 mM condition was also that in which, on the one hand, the influx of NH_4^+ (when measured directly; Fig. 4a), the efflux:influx ratio for NH_4^+ (Fig. 2b), the exchangeable root NH_4^+ (Fig. 5a), and the root oxygen demand (Fig. 3) were minimized, while, on the other hand, the net influx of NH_4^+ (Fig. 2b), the plant protein content (Fig. 5b), and the activities of GS and PEP carboxylase (Fig. 6) were the highest. The tremendous increase in plant performance under this condition (657% higher growth compared with the condition at 0.02 mM $[K^+]_{ext}$, and 216% compared with the 0.1 mM $[K^+]_{ext}$ condition) suggests that a fertilization regime using 10 mM NH_4^+ and 5 mM K^+ could significantly improve, indeed optimize, rice growth in the field, at least at the seedling stage. The optimization effect on plant growth of increasing $[K^+]_{ext}$ was also seen at a lower NH_4^+ supply, and with 10 mM NO_3^-, illustrating the broader nutritional and agronomic importance of our findings. However, it must be kept in mind that extrapolation, to the field, of results obtained with hydroponically grown plants, are complicated by sorption processes and spatial and temporal heterogeneity. Nevertheless, these results appear particularly significant in the context of a century-long decline in K^+-bearing clay minerals in many rice cultivating areas of China (Li, Velde & Li 2003), and similar declines in other parts of Asia (Cassman, Peng & Dobermann 1997). Indeed, K^+ deficiency is common in many parts of the continent, including some

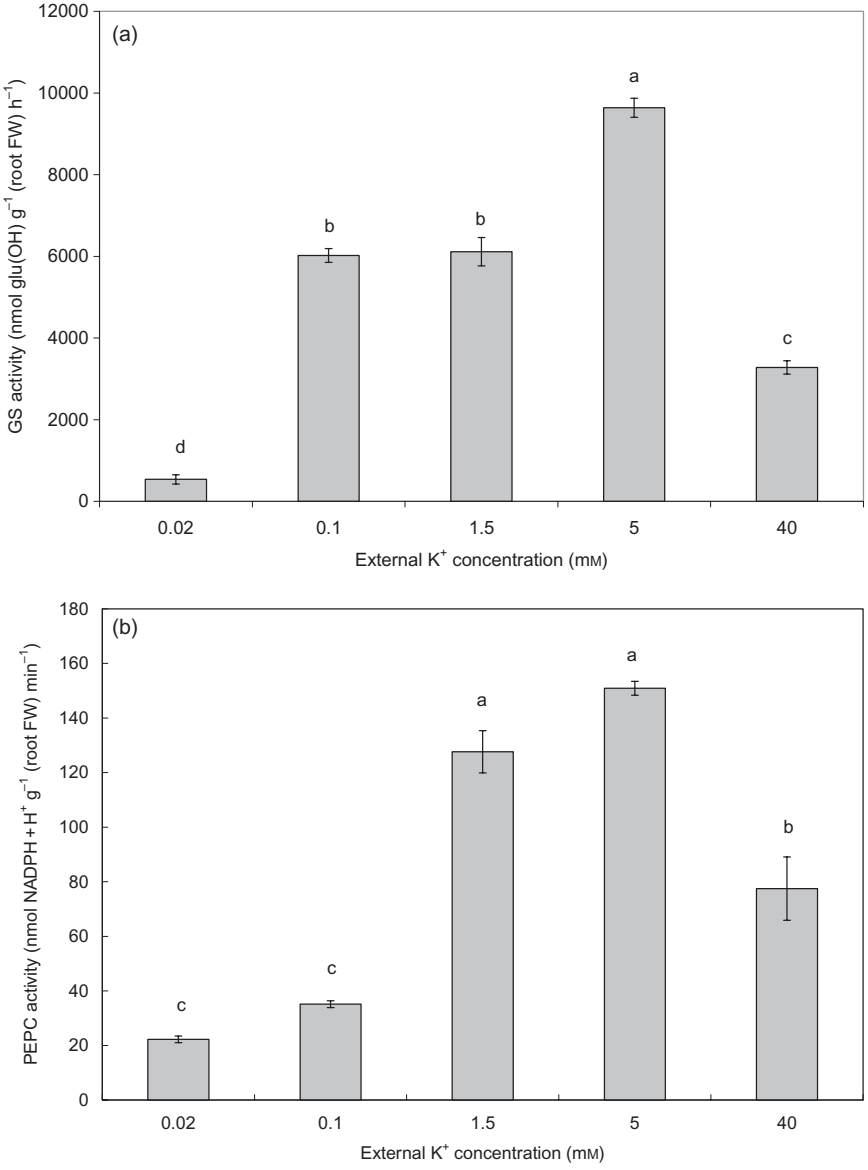

Figure 6. (a) *In vitro* glutamine synthetase (GS) activities in roots of rice grown with 10 mM NH_4^+ and five concentrations of K^+. Letters indicate significantly different means (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM. (b) *In vitro* phosphoenolpyruvate carboxylase (PEPC) activities in roots of rice grown with 10 mM NH_4^+ and five concentrations of K^+. Letters indicate significantly different means (one-way analysis of variance with Tukey-Kramer Multiple Comparison Test, $P < 0.05$). Error bars indicate ± SEM.

70% of rice paddies in southeast China (Yang *et al.* 2005), and much of the Indo-Gangetic plains (Bijay-Singh, Imas & Jian-chang 2003) and K^+ has been singled out as the most limiting nutrient for rice yields (Yang *et al.* 2005). Increasing fertilizer input will likely be required for such optimization in the field, in conjunction with newer practices such as the incorporation of rice straw into the soil at harvest time, which can return most of the absorbed K back to the paddy (Yoshinori *et al.* 2003).

NH_4^+ fluxes: K^+ dependence and pharmacological tests

Varying the K^+ provision to rice plants resulted in profound changes in unidirectional NH_4^+ fluxes (Figs 2 & 4), with elevated K^+ reducing NH_4^+ influx and efflux, and the degree of futile NH_4^+ cycling across the plasma membrane of root cells (measured here as a reduction of the efflux:influx ratio). In large part due to the lack of suitable tracers, there are few literature precedents for or against these results. However, two important exceptions are found in Mengel, Viro & Hehl (1976), who found that net $^{15}NH_4^+$ acquisition in rice was stimulated by $[K^+]_{ext}$ (see below), and Wang, Siddiqi & Glass (1996), who showed that K^+ could reduce the unidirectional uptake of $^{13}NH_4^+$ in rice grown under high-affinity uptake conditions for the two cations (as opposed to the low-affinity conditions examined here).

Our previous work (Britto *et al.* 2001) suggested that rice, unlike barley, is resistant to the respiratory drain caused by futile NH_4^+ cycling, and the broad similarity in oxygen depletion among K^+ treatments (Fig. 3) supports this, indicating that relief from respiratory excess is not a major cause of the growth optimization at 5 mM K^+ in this species. This may be because of a passive, Nernstian distribution of NH_4^+ across the plasma membrane in rice, in contrast to a

condition requiring active efflux in barley (Britto *et al.* 2001; see below). Despite this potentially critical difference between the energetics of NH_4^+ transport in barley and rice, however, the two species clearly have in common a two-component mechanism of low-affinity NH_4^+ influx: a K^+-sensitive component (the dominant of the two), and a K^+-insensitive one (see Szczerba *et al.* 2008a).

Direct measurements of NH_4^+ influx (Fig. 4) agreed well with those determined using efflux analysis (Fig. 2), and provided important additional information. As shown in Fig. 4b, a switch from 0.1 to 40 mM $[K^+]_{ext}$ just prior to tracer addition resulted in a sudden (within 1 min) and substantial (by about 35%) drop in NH_4^+ influx, as was seen in barley in prior work (Szczerba *et al.* 2008a). Conversely, removal of K^+ from growth medium previously containing 5 mM K^+ stimulated NH_4^+ influx within 5 min (not shown). The immediacy and reversibility of this response indicates that the differences in NH_4^+ influx among K^+ conditions are not necessarily a result of differential expression of genes encoding NH_4^+ transporters over the long term. Rather, this phenomenon argues in favour of a more direct effect of K^+ on the NH_4^+ transport system(s), either through competition for entry into the cell via a common transport mechanism, through a regulatory binding step that may allosterically alter NH_4^+ transport proteins, or through a reduction in driving force into the cell resulting from K^+-dependent depolarization of the membrane (Mertz & Higinbotham 1974; Cheeseman & Hanson 1979).

The means by which NH_4^+ enters the plant cell in the low-affinity range is not fully resolved. However, because of the high capacity of both components of the flux, they are likely to be channel-mediated, possibly by non-selective cation channels (NSCCs; White 1999; Demidchik, Davenport & Tester 2002), inward-rectifying K^+ channels (Bertl *et al.* 1997) such as AKT1, or aquaporins (Jahn *et al.* 2004). Thus, it was of interest in the present study to further characterize, by use of channel-blocking agents, the K^+-sensitive and -insensitive components of low-affinity NH_4^+ transport. At the low-K^+ condition (0.02 mM), application of all blockers caused significant reductions in NH_4^+ influx (Fig. 4c). The broad-spectrum blocker La^{3+}, the NSCC blocker Zn^{2+}, and the K^+-channel blocker Cs^+ reduced the flux by about 23%, while the K^+-channel and aquaporin blocker tetraethylammonium (TEA^+) had a significantly stronger effect, showing a 50% suppression of the flux. At higher K^+ (5 mM), Cs^+ no longer had an effect, but the other three blockers significantly reduced the NH_4^+ flux. These results suggest that there are multiple channel types mediating NH_4^+ entry into the cell (non-selective, K^+-specific, and possibly aquaporin), and there may be both K^+-sensitive and K^+-insensitive components associated with each of them. This finding contrasts with those obtained in barley in our previous work (Szczerba *et al.* 2008a), in which La^{3+}-sensitivity data revealed that weakly voltage-dependent, NSCCs were likely candidates for the K^+-sensitive component. By contrast, the engagement of K^+-specific channels in NH_4^+ transport were considered unlikely in barley roots, because both Cs^+ and TEA^+ enhanced the NH_4^+ flux, rather than suppressing it as in the present study.

Tissue NH_4^+ and its localization

Comparison of Fig. 5a and Table 1 indicates a surprisingly good agreement between rapidly exchangeable ($t_{1/2}$ = 11–16 min) root tissue NH_4^+, as measured by ^{13}N tracer analysis (Fig. 5a), and total root tissue NH_4^+ as measured by chemical (OPA) analysis (Table 1); this agreement suggests that all tissue NH_4^+ is rapidly exchangeable. However, because the NH_4^+ released in this rapid phase of efflux is thought to be cytosolic in origin based on previous work (Kronzucker *et al.* 1995; Britto & Kronzucker 2003), this would mean that virtually all tissue NH_4^+ is cytosolically located, and that vacuolar and organellar NH_4^+ (which is rarely, if ever, measured directly) is very low. Alternatively, this could mean that the compartment from which the $^{13}NH_4^+$ flux originates has been wrongly assigned, and that internal membranes (e.g. the tonoplast) enclosing subcellular NH_4^+ pools are extremely permeable to NH_4^+ (or its conjugate base NH_3). Under such a condition, the entire cell might behave as a single compartment with release kinetics dictated by the plasma membrane efflux apparatus. However, there is no evidence that this occurs, while evidence to support the cytosolic localization of the exchangeable/tissue ammonium can be found in the lack of a strong respiratory response to large changes in NH_4^+ fluxes (compare Figs. 2–4). In a previous study with the same cultivar of rice, examined under similar conditions (Britto *et al.* 2001), this lack of response was attributed to the establishment of a passive distribution of NH_4^+ across the plasma membrane, based on data from compartmental analysis and elecrophysiological ($\Delta\Psi$) measurements. As in the present study, assigning the rapidly exchanging NH_4^+ to a cytosolic pool yields the approximately Nernstian distribution that could explain the overall uniformity of respiration. By contrast, recalculating the pool on a whole-cell basis would result in a much lower (approximately 20-fold) intracellular activity, in turn changing the thermodynamic picture to one in which the efflux of NH_4^+ is energy-dependent, a condition seen with NH_4^+-sensitive barley in previous work (Britto & Kronzucker 2001), but not borne out in Fig. 3. Additional experimentation (such as longer-term labelling using the stable isotope ^{15}N) will be required to resolve this issue.

Glutamine synthetase, PEP carboxylase, and protein

Because the ability of plants to tolerate growth on high NH_4^+ depends in large part on their N assimilation capacity (Givan 1979; Magalhäes & Huber 1991; Gerendás *et al.* 1997), we investigated the activities of two key enzymes involved in this process: glutamine synthetase, which

catalyses the primary incorporation of NH_4^+ into the organic N pool, and PEP carboxylase, which fixes bicarbonate and results in the anaplerotic production of organic acid skeletons for subsequent amino acid synthesis (Britto & Kronzucker 2005). We found that the activities of both enzymes increased with increasing $[K^+]_{ext}$, reaching maxima at 5 mM (Fig. 6), corresponding to the observed growth maximum (Fig. 1). The powerful response of GS, in particular, may be an important key to the adaptation of rice to NH_4^+ (Magalhäes & Huber 1989): in cucumber, an ammonium-sensitive species, the response of GS to rising $[K^+]_{ext}$ was not as dramatic as seen here (Roosta & Schjoerring 2008), although it did lead to improved NH_4^+ tolerance.

Consistent with the rise in assimilation capacity with increasing $[K^+]_{ext}$ was the substantial decline in exchangeable and tissue NH_4^+ (Fig. 5a, Table 1). This decline cannot be attributed to the reduced flux of NH_4^+ into the plant, because the net flux of NH_4^+ (black bar segments in Fig. 2b) shows no such declining pattern (cf. Mengel *et al.* 1976). On the other hand, the protein content of plants showed an increasing trend, peaking at the growth-optimizing $[K^+]_{ext}$ of 5 mM (Fig. 5b). This trend is even stronger than that depicted in Fig. 5b, if one compares treatments in terms of protein content per plant, considering that increasing protein per gram parallels the trend in growth with increasing $[K^+]_{ext}$ (such an analysis shows that the 5 mM K^+ condition exceeds the 0.02, 0.1 and 1.5 mM conditions by 1134, 271 and 140%, respectively). This evidence strongly indicates that the upregulation of enzymes involved in the assimilation of NH_4^+ can account for the reduced tissue NH_4^+, and for the greater partitioning of the net NH_4^+ flux towards amino acids and protein, both of which may be key factors in the ability of plants to thrive on high NH_4^+ as a sole N source (Givan 1979).

In conclusion, the optimization of NH_4^+ assimilation by K^+, in conjunction with the K^+-dependent curtailment of excessive NH_4^+ fluxes at the plasma membrane, mirror and may underlie the growth optimization seen at 10 mM NH_4^+ and 5 mM K^+. Given the prevalence of NH_4^+-N in paddy soils, and their increasing paucity of K^+, this finding is likely to be of high agronomic significance to the cultivation of rice.

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