Subcellular NH_4^+ flux analysis in leaf segments of wheat (*Triticum aestivum*)

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Summary

• We report the first use of tracer $^{13}NH_4^+$ (^{13}N-ammonium) efflux and retention data to analyse subcellular fluxes and compartmentation of NH_4^+ in the leaves of a higher plant (wheat, *Triticum aestivum*).

• Leaf segments, 1–2 mm, were obtained from 8-d-old seedlings. The viability of the segments, and stability of NH_4^+ acquisition over time, were confirmed using oxygen-exchange and NH_4^+-depletion measurements. Fluxes of NH_4^+ and compartment sizes were estimated using tracer efflux kinetics and retention data.

• Influx and efflux across the plasma membrane, half-lives of exchange and cytosolic pool sizes were broadly similar to those in root systems. As the external concentration of NH_4^+ ($[NH_4^+]_o$) increased from 10 μM to 10 mM, both influx and efflux greatly increased, with a sixfold increase in the ratio of efflux to influx. Half-lives were similar among treatments, except at $[NH_4^+]_o$ = 10 mM, where they declined. Concentrations of NH_4^+ in the cytosol ($[NH_4^+]_c$) increased from 2.6 to 400 mM.

• Although $[NH_4^+]_c$ became large as $[NH_4^+]_o$ increased, the ratio of $[NH_4^+]_c$ to $[NH_4^+]_o$ decreased more than sixfold. The apparently futile cycling of NH_4^+ at high $[NH_4^+]_o$ suggested by the large fluxes of NH_4^+ in both directions across the membrane indicate that leaf cells respond to potentially toxic NH_4^+ concentrations in a manner similar to root cells.

Key words: nitrogen, ammonium, compartmental analysis, efflux, wheat (*Triticum aestivum*), leaves, membrane fluxes, ion transport.

Introduction

Numerous processes assimilate and liberate the ammonium ion (NH_4^+) in plant leaves (Joy, 1988; Feng *et al.*, 1998). Ammonium is assimilated in leaf cells mainly through the plastidic isoform of glutamine synthetase (Lam *et al.*, 1996), and liberated mainly through the serine synthase reaction of the photorespiratory nitrogen cycle (Givan *et al.*, 1988; Leegood *et al.*, 1995), amino acid catabolism (Joy, 1988) and phenylalanine ammonia lyase activity (Nakashima *et al.*, 1997). On a whole-plant scale, NH_4^+ fluxes are a component of the translocation of nitrogen from root to shoot in the xylem stream (van Beusichem *et al.*, 1988; Finnemann & Schjoerring, 1999), and are involved in the exchange of NH_3 with the atmosphere (Farquhar *et al.*, 1980; Mattsson *et al.*, 1997; Hanstein & Felle, 1999).

The involvement of NH_4^+ in such diverse processes implies that there are specific and well-regulated mechanisms for its transport across leaf cell membranes. Raven & Farquhar (1981) used the NH_4^+ analogue ^{14}C-methylammonium in leaf segments of *Phaseolus vulgaris* to provide evidence that NH_4^+ transport across the plasma membrane of leaf cells is mediated by an electrogenic uniport mechanism, as has been postulated for low-affinity transport in roots of rice (Wang *et al.*, 1994). More recently, Nielsen & Schjoerring (1998) observed large, concentration-dependent, bidirectional fluxes in leaf discs of *Brassica napus*, using $^{15}NH_4^+$ and $^{14}NH_4^+$ infiltration techniques, resulting in well-regulated concentrations

of NH_4^+ in the apoplasm. The physiological evidence for specific NH_4^+ transport in leaves is augmented by the finding that several of the genes encoding putative NH_4^+ transporter proteins in roots are also present in leaves (von Wiren *et al.*, 2000).

Despite these advances, knowledge of NH_4^+ membrane fluxes and subcellular compartmentation in leaf tissues remains scant. For other ions, numerous studies have been made of fluxes and compartmentation in leaf segments, protoplasts, and cells of *Asparagus* cladophylls (Smith & Epstein, 1964; Rains, 1967, 1968; Jeschke, 1976; Bown, 1982; Leonard & Rader, 1985).

In the present study, we use NH_4^+ labeled with the short-lived positron-emitting radiotracer ^{13}N and the compartmental analysis by efflux method (MacRobbie, 1971; Walker & Pitman, 1976; Lee & Clarkson, 1986; Siddiqi *et al.*, 1991; Britto & Kronzucker, 2001a,b) to measure unidirectional fluxes across the plasma membrane (influx and efflux), cytosolic turnover constants, and cytosolic concentrations of NH_4^+ in leaf segments of wheat. This methodology has recently been used to measure fluxes and subcellular compartmentation of NH_4^+ in plant roots (Kronzucker *et al.*, 1999; Britto *et al.*, 2001a). The present work seeks to extend its application to leaves.

Materials and Methods

Plant culture

Seedlings of wheat (*Triticum aestivum* L. cv. 'Max Red'), were grown for 8 d in hydroponic tanks in climate-controlled walk-in chambers (relative humidity 70%, temperature 20 ± 2°C). Nutrients were provided as modified 1/10 strength Johnson's solution containing 1 mM NH_4^+ as $(NH_4)_2SO_4$ (for details see Kronzucker *et al.*, 1995a,b). A photon flux of approximately 200 μmol m^{-2} s^{-1} was provided by wide-spectrum fluorescent tubes (Vita-Lite Duro-Test, Phillips, Abbotsford, BC, Canada) with a 16-h photoperiod.

Preparation of leaf segments

Leaves were detached above the basal sheath and cut transversely on a sheet of glass, using frequently changed razor blades to produce 1–2 mm segments. Batches (0.5–1.0 g fresh weight (f. wt)) of segments were weighed and placed in a sidearm Erlenmeyer flask containing uptake solution, which was identical to the solution the plants were grown in, except that the external NH_4^+ concentration, $[NH_4^+]_o$ (as $(NH_4)_2SO_4$), was varied. To reduce buoyancy, leaf segments were degassed under vacuum for 10–15 s using a tap aspirator. After two washes with uptake solution, the segments were either used for tracer uptake experiments following a 3-h pretreatment or used immediately for depletion studies. In the pretreatment, the segments were steeped in unlabelled uptake solution in the growth chamber, with the solution stirred by aeration. The solution volumes were sufficient to ensure that the leaf segments removed less than 30% of the nutrients.

Depletion studies

Samples (0.5–1.0 g f. wt) of segments were placed in 10–20 ml of uptake solution containing 0.1 mM NH_4^+ in 25-ml glass beakers at the above irradiance, relative humidity, and temperature. The solutions were circulated by aeration. At 10-min intervals, 1-ml aliquots of external solution were removed and analysed for NH_4^+ (Solorzano, 1969). In shorter-term experiments the solution was replaced every 30 min with new solution and in longer-term experiments leaf segments were kept in 1-l solution so that $[NH_4^+]_o$ was essentially constant.

Gas exchange

Oxygen production and consumption by leaf segments were monitored over several hours following excision, by placing 50–100 mg tissue in an oxygen electrode chamber (Hansatech DW-1; Hansatech Instruments Ltd., Kings Lynn, Norfolk, UK) containing 2.5 ml of well-stirred uptake solution at the same irradiance, relative humidity and temperature as above.

Application of the photosynthetic electron flow blocker 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) caused an immediate and pronounced drop in oxygen evolution from leaf segments, indicating that diffusion of solutes to or within the segments did not limit the uptake of solutes from the external solution.

Efflux analysis

Samples of $^{13}NO_3^-$, provided by the Tri-University Meson Facility (TRIUMF) on the University of British Columbia campus, Vancouver, Canada, were reduced to $^{13}NH_4^+$ with Devarda's alloy, purified by distillation and used immediately (Wang *et al.*, 1993; Kronzucker *et al.*, 1995a,b, 1999). Samples (1.00 g f. wt) were transferred to uptake solutions containing radioactivity at known specific activity (cpm μmol^{-1}) for 1 h. At the end of this period the individual samples were placed in 100-ml glass funnels equipped with nylon mesh to prevent loss of plant material, and with clamped Tygon (R-3603; Saint-Gobain Performance Plastics, Akron, OH, USA) spouts to control elution. Over the course of the experiment, 20-ml aliquots of an unlabeled washing solution, chemically identical to the labeling solution, were poured into the clamped funnel, released from it after gradually increasing time intervals, and immediately replaced with a subsequent aliquot (Siddiqi *et al.*, 1991; Wang *et al.*, 1993). The schedule followed was 10 s (3×), 15 s (6×), 30 s (4×), 1 min (2×), 2 min (10×); 2-min washings were occasionally extended beyond 10 times when the activity of the tracer was sufficiently large, to avoid

premature truncation of the plots of tracer elution. Tracer captured in the eluates was determined by γ-emission, as was tracer remaining in the tissue after the final elution.

Variations on this basic procedure included treatment of leaf tissue with a 1% solution of the membrane-solubilizing detergent sodium dodecyl sulfate (SDS) during the labeling period, and use of a cation exchange resin to determine the charge of label in the eluate (Kronzucker *et al.*, 1995b). The latter showed that 90% of the tracer released into efflux solutions was positively charged, indicating that only a small proportion was in the form of amino acids.

Treatment of efflux data was based on methods described in detail in Siddiqi *et al.* (1991) and on the theoretical considerations given in Lee & Clarkson (1986). Briefly, the key parameters were determined as follows:

- *Efflux* from the phase considered to be the cytosol (see the Discussion section) was obtained from the rate of ^{13}N release from this phase extrapolated to time = 0.
- *Net flux* to the vacuole and metabolism was obtained from the accumulation of ^{13}N in the plant material at the end of the elution period.
- *Influx* to the cytosol was obtained from the sum of efflux and net flux.
- *Half-lives of NH_4^+ exchange* ($t_{1/2}$) were obtained from the slopes of semi-logarithmic plots of the rate of release of ^{13}N in log (cpm released g^{-1} min^{-1}) against the time of elution from leaf segments.
- Cytosolic NH_4^+ concentrations were calculated from the quotient of the rate of $^{13}NH_4^+$ release integrated over five times the half-life of cytosolic NH_4^+ exchange, the ratio of efflux to all fluxes removing $^{13}NH_4^+$ from the cytosol, and the assumption that the cytosol occupies 5% of cell volume.

Results

It was important to establish the viability of the leaf-segment system, especially with respect to NH_4^+ fluxes. Changes in NH_4^+ acquisition over the duration of the tracer experiments would invalidate the assumption of steady-state conditions inherent in the compartmental analysis (see the Discussion section). Accordingly, we assayed net uptake of unlabeled NH_4^+ (0.1 mM) over an extended period following excision of tissue (Fig. 1). The rate of decrease in $[NH_4^+]_o$ after the first 20–60 min (0.92 ± 0.07 μmol g^{-1} f. wt h^{-1}) following cutting was not significantly different from that 30 h later (0.94 ± 0.11 μmol g^{-1} f. wt h^{-1}). The average net uptake (i.e. rate of NH_4^+ assimilation and storage) was 1.00 ± 0.15 μmol g^{-1} f. wt h^{-1}. In a further experiment, photosynthetic oxygen evolution and respiratory oxygen consumption were monitored, and neither was found to change significantly over 6 h following the preparation of segments (not shown).

Figure 2 shows a representative semi-logarithmic plot of the change with time in the rate of $^{13}NH_4^+$ (tracer) release from labeled leaf segments of 8-d-old wheat seedlings. Three distinct phases of exponentially declining efflux of the tracer could be identified (see Fig. 2). Linear regressions of the data for the three phases gave high coefficients of determination ($r^2 > 0.9$, typically). Figure 2 also shows the effects of SDS on efflux (Fig. 2, inset).

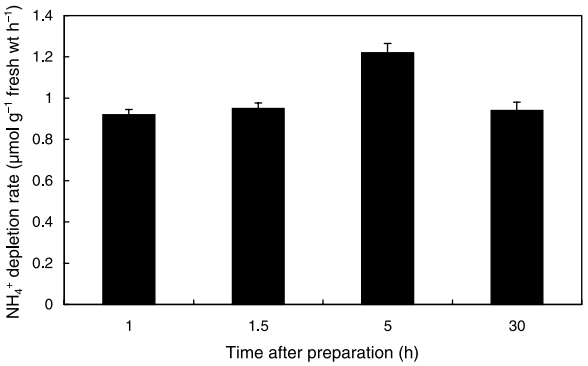

Fig. 1 Ammonium depletion rates of wheat leaf segments at 0.1 mM $[NH_4^+]_o$ over 30 h following preparation of segments. Times of solution sampling are as indicated on the *x*-axis. Error bars indicate ± SEM.

Table 1 Half-lives ($t_{1/2}$) of cytosolic NH_4^+ exchange and cytosolic NH_4^+ pool sizes ($[NH_4^+]_c$) in leaf segments of wheat, as estimated by compartmental analysis

	External NH_4^+ concentration (mM)			
	0.01	0.1	1	10
$t_{1/2}$ (min)	14.5 ± 1.6	14.7 ± 1.5	12.2 ± 1	9.3 ± 0.9
$[NH_4^+]_c$ (mM)	2.6 ± 0.4	13.5 ± 2.8	65 ± 3	400 ± 33

Values are means ± SEM of four to eight experiments.

Figure 3 gives values for NH_4^+ influx and efflux over a wide range of external NH_4^+ concentrations ($[NH_4^+]_o$), obtained from the data on efflux kinetics and tracer retention, with the assumption that the compartment responsible for phase III in Fig. 2 corresponds to the collective leaf cytosol (see the Discussion section). A strong trend of increasing NH_4^+ flux was observed as $[NH_4^+]_o$ increased from 0.01 to 10 mM, influx at the highest concentration being 260 times that at the lowest. The most pronounced change, however, was in the efflux, which increased 1700-fold with this change in $[NH_4^+]_o$. The disparity between the rates of change of efflux and influx underlies the dramatic increase in the efflux–influx ratio, from a low of 0.14 at 0.01 mM to a peak of 0.91 at 10 mM (Fig. 3).

Table 1 shows half-lives of cytosolic NH_4^+ exchange and cytosolic NH_4^+ concentrations over the treatment range. Values of $t_{1/2}$ were nearly identical as $[NH_4^+]_o$ increased from 0.01 to 1 mM and cytosolic NH_4^+ concentration, $[NH_4^+]_c$, increased from 2.6 to 65 mM. At the largest $[NH_4^+]_o$ (10 mM, which is known to be toxic to many species when supplied to the root), $t_{1/2}$ decreased substantially, as it did with SDS pretreatment (Fig. 2, inset). The 10 mM

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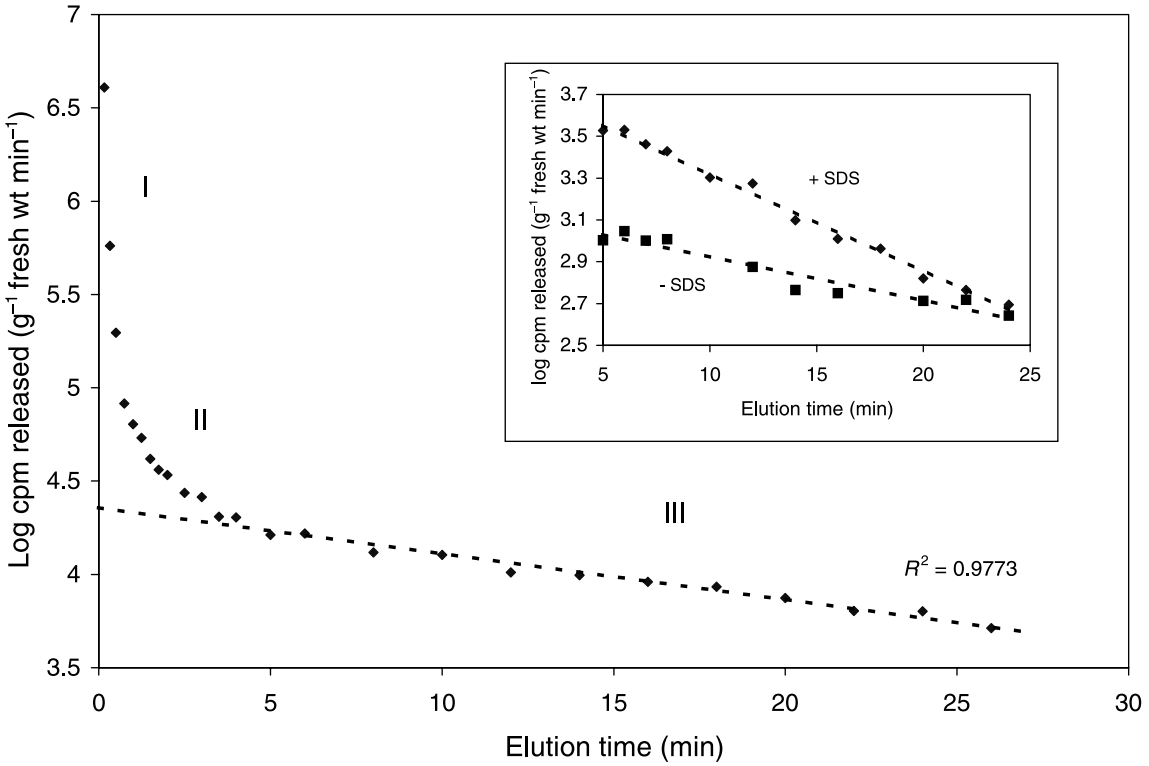

Fig. 2 Representative semi-logarithmic plot of $^{13}NH_4^+$ efflux from leaf segments of wheat. Segments were pretreated for 3 h in nonradioactive uptake medium containing 0.1 mM $[NH_4^+]_o$ prior to exposure to $^{13}NH_4^+$, in solution of identical chemical composition, for a subsequent hour. In agreement with extensive previous studies (see Kronzucker *et al*., 1995a, and Discussion section in text), the dashed regression line represents tracer released from the cytosolic phase (phase III) of the labeled preparation, while phases I and II are assigned to the extracellular components of surface film/water free space and Donnan free space, respectively. Inset, effect on phase-III efflux plot of membrane solubilization by 1% sodium dodecyl sulfate (SDS) (at 0.1 mM $[NH_4^+]_o$). The *y*-axis here is not directly comparable to that in the main graph because the data came from a different experiment, with a different specific activity of ^{13}N.

$[NH_4^+]_o$ treatment resulted in a cytosolic concentration of 400 mM.

That the compartment responsible for phase III in Fig. 2 was membrane bound was confirmed using the membrane-dissolving detergent SDS. Treatment of leaf segments with 1% SDS at 0.1 mM $[NH_4^+]_o$ during labeling decreased influx by 20%, increased the efflux–influx ratio to 0.92 (compared with 0.29 in controls), decreased the size of the compartment responsible for phase III by 40%, and increased the slope of the phase-III line nearly threefold (data not shown).

Discussion

Two important requirements to be satisfied in compartmental analysis by efflux are: (1) that the experimental system is at, or near, steady state with respect to the processes being measured; and (2) that the assignment of compartments releasing tracer is correct. Two lines of evidence suggest that the plant material used in the present work were at a steady state in terms of NH_4^+ acquisition, despite disturbances caused by excision. First, the high coefficients of determination (r^2) for phase III of the ^{13}N efflux (as in Fig. 2) show that efflux is stable over the elution time. Second, the depletion of $^{14}NH_4^+$ by leaf segments, incubated at 0.1 mM $[NH_4^+]_o$, remained uniform and substantial much longer than the time required for efflux experiments (Fig. 1), indicating that the sum of pertinent rates, i.e. influx and efflux, as well as vacuolar and metabolic fluxes, was steady over this period. Comparison of net uptake rates in depletion experiments with net fluxes observed at these concentrations in tracer experiments (i.e. the differences between influx and efflux in Fig. 3), however, showed moderately higher estimates with the tracer experiments. This apparent discrepancy is attributable to the fact that, while net fluxes consist of assimilatory and vacuolar fluxes, the assimilate pool and the vacuole remained essentially unlabeled during the loading period (Britto & Kronzucker, 2001a) and therefore fluxes from these pools did not contribute to $^{13}NH_4^+$ efflux (MacRobbie, 1971; Feng *et al*., 1998).

The broad similarities between the results shown here, and those routinely observed using efflux analysis with intact roots (Lee & Ratcliffe, 1991; Wang *et al*., 1993; Kronzucker *et al*., 1995a,b, 1999; Min *et al*., 1999; Britto *et al*., 2001a,b), is further evidence that leaf NH_4^+ flux systems and compartmentation were near steady state. Magnitudes of unidirectional fluxes across the plasma membrane, half-lives of cytosolic NH_4^+ exchange and their low variability over a wide

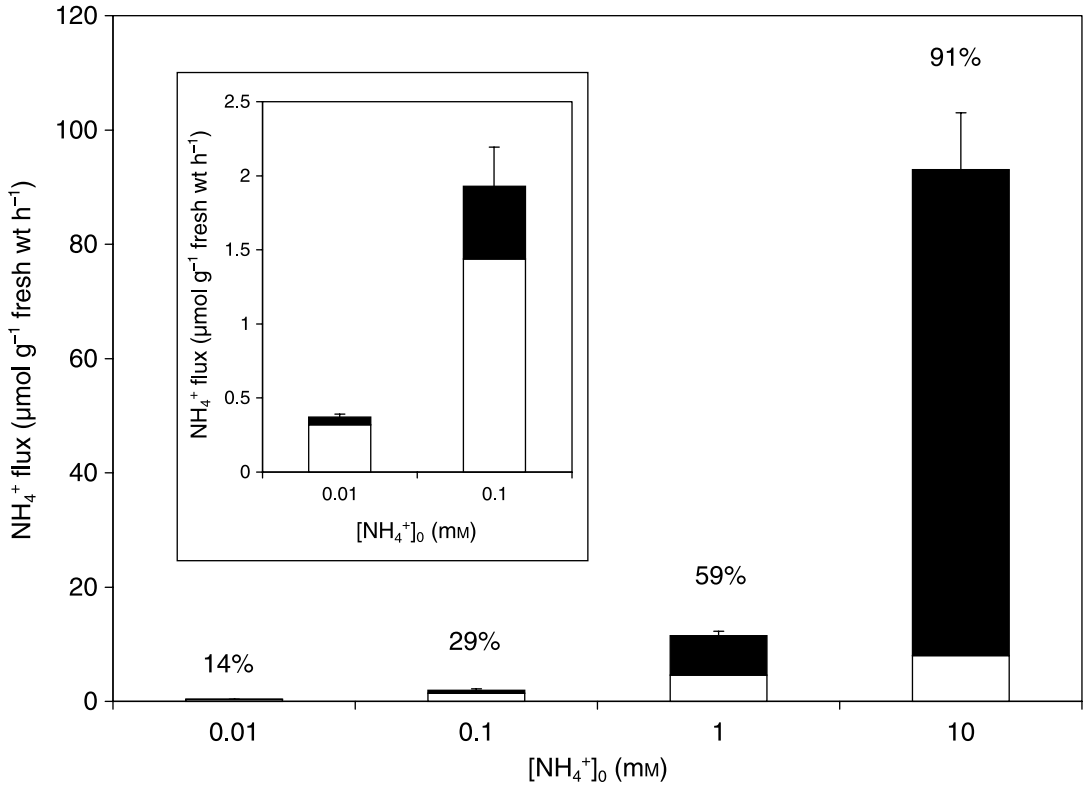

Fig. 3 Comparison of NH_4^+ component fluxes in wheat leaf segments at four external ammonium concentrations. Bars are broken up into efflux (black segments) and net flux (flux to vacuole plus assimilatory fluxes, clear segments), which together comprise the influx term. Error bars indicate ± SEM. Ratios of efflux to influx are indicated above the bars. Inset, magnified view of fluxes at 0.01 mM and 0.1 mM $[NH_4^+]_o$.

concentration range, and increasing efflux–influx ratios with increasing $[NH_4^+]_o$ are all quantitatively similar to those measured in root systems (Lee & Ratcliffe, 1991; Wang *et al.*, 1993; Kronzucker *et al.*, 1995a,b, 1999; Min *et al.*, 1999; Britto *et al.*, 2001a,b). Such similarities in cellular N-exchange parameters reflect the common N-flux and N-assimilatory processes operating in root and leaf cells (Joy, 1988; Lam *et al.*, 1996; Feng *et al.*, 1998; von Wiren *et al.*, 2000). Root systems in studies of this nature are at physiological steady state. Thus, similarities with leaf segments in essential flux and compartmentation parameters at comparable $[NH_4^+]_o$ values suggest that the leaf tissue was also at or near steady state. Also, when $^{13}NH_4^+$-labeled barley root systems are exposed to physical disturbance and non-steady-state NH_4^+ concentrations during elution, phase-III efflux lines return to the predisturbance slope within approximately 10 min (Britto & Kronzucker, 2001a,b). This is evidence that plant systems rapidly adjust to short-term changes, suggesting that leaf segments may be able to achieve new physiological steady states in the short term, despite trauma incurred by slicing and pretreatment.

In addition to corroborating the assumption that the leaf segments were at a physiological steady state, the similarities to results for root systems further support the assignment of compartments to efflux phases in the present study. The identity of phase III as the collective cytosol of the leaf tissue was also confirmed by treatment of leaf segments with SDS. The substantial decrease in $t_{1/2}$ and increase in efflux following treatment with SDS in Fig. 2 is in agreement with results using SDS in roots, which have helped establish the cytosolic identity of phase III (Siddiqi *et al.*, 1991; Kronzucker *et al.*, 1995a). More importantly, the maintenance of $t_{1/2}$ under widely varying external NH_4^+ concentrations (and associated variations in fluxes and internal pool sizes) can only be explained by the compartment in question being able to adjust delivery and removal of NH_4^+ (Britto & Kronzucker, 2001b). This cannot be done by the vacuole, which is the only other sizable membrane-bound compartment that could give rise to the SDS effect depicted in Fig. 2 (inset).

The characteristics of influx and efflux for leaf segments across a range of external concentrations shown in Fig. 3 indicate that the capacity of leaf tissue to process NH_4^+ is extensive and highly concentration-dependent. The external NH_4^+ concentrations were chosen to cover the range of apoplastic concentrations that leaf cells are likely to be exposed to under natural circumstances (Husted & Schjoerring, 1995; Mattsson *et al.*, 1997; Finnemann & Schjoerring, 1999). Figure 3 shows that influx continues to change over a wide range of $[NH_4^+]_o$, increasing 259-fold when $[NH_4^+]_o$ increases 1000-fold. However, efflux increases across this range by a much greater factor (1700), and the different responses of influx

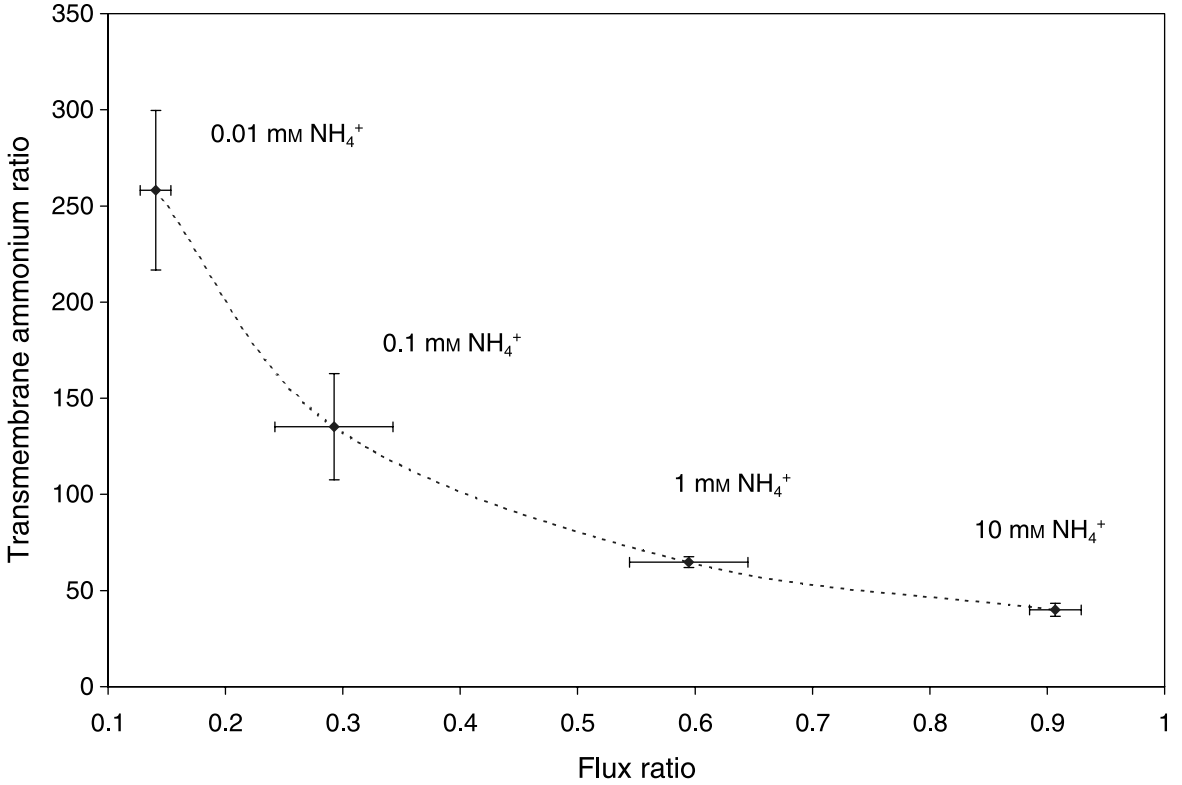

Fig. 4 Ratio of NH_4^+ concentrations inside the cytosol to that in the external medium, as plotted against efflux–influx ratios as a function of $[NH_4^+]_o$ in wheat leaf segments. External ammonium concentrations are as noted for each point. Error bars indicate ± SEM for both parameter sets.

and efflux to $[NH_4^+]_o$ suggest that independent regulatory systems exist for these opposed processes. The trend of increasing efflux–influx ratio as $[NH_4^+]_o$ increases indicates that most of the nitrogen taken up at high $[NH_4^+]_o$ is lost through efflux by the leaf cells. This futile cycling is reminiscent of the response of plant roots to NH_4^+ toxicity (Britto *et al.*, 2001a), and Nielsen & Schjoerring (1998) have obtained similar results in leaf tissue using an entirely different technique (isotopic N-infiltration). Nielsen & Schjoerring (1998) argue that such fluxes provide a mechanism by which deflections from a homeostatic set-point of apoplastic $[NH_4^+]$ may be rectified. It is interesting to speculate whether high efflux–influx ratios are a feature of the low-affinity transport system (LATS) for NH_4^+, given that N losses greater than 50% of influx become obvious at $[NH_4^+]_o$ above 1 mM, which is regarded as the cut-off point for the operation of LATS (Wang *et al.*, 1993; von Wiren *et al.*, 2000). Evidence that cation channels (as opposed to systems with Michaelis–Menten kinetics in the high-affinity, low-concentration, range) mediate NH_4^+ influx in both roots and shoots at high $[NH_4^+]_o$ has been provided by several studies (Nielsen & Schjoerring, 1998; Kronzucker *et al.*, 2001), and we propose that the less stringent regulation of such transporters is the cause of futile NH_4^+ cycling (see Britto *et al.*, 2001a).

The half-lives of cytosolic NH_4^+ exchange in the leaf segments were maintained within a narrow range (Table 1). In conjunction with continuously increasing influx with $[NH_4^+]_o$, this resulted in commensurately increasing cytosolic pool sizes (see Britto & Kronzucker, 2001b, for a discussion of the linearity between unidirectional influx and cytosolic pool size under conditions of constant $t_{1/2}$). Constant $t_{1/2}$ and increasing pool sizes with varying $[NH_4^+]_o$ have also been observed in root systems (Wang *et al.*, 1993; Kronzucker *et al.*, 1995a,b, 1999; Britto *et al.*, 2001a). While $[NH_4^+]_c$ is moderate at 0.01 mM $[NH_4^+]_o$ and exceptionally high at 10 mM $[NH_4^+]_o$, it is also important to consider the influence of the transmembrane electrical potential (typically from −90 to −150 mV; Elzenga *et al.*, 1995) on the fluxes and compartmentation of NH_4^+. Figure 4 shows how the transmembrane ammonium ratio (and, therefore the Nernst potential for a given membrane potential) decreases with increasing $[NH_4^+]_o$, indicating that the observed cytosolic concentrations are much easier to achieve, energetically, as $[NH_4^+]_o$ increases. In other words, the electrochemical potential gradients favoring NH_4^+ influx become substantially steeper at the greater external NH_4^+ concentrations. That this trend appears to translate into greater fluxes at higher $[NH_4^+]_o$ further substantiates the prevailing view that channel-type transport, which is driven by the electrochemical potential gradient, is responsible for high-velocity, low-affinity NH_4^+ transport in both root and leaf cells. The strong inverse correlation between the transmembrane NH_4^+ ratio and the flux ratio (Fig. 4),

however, supports the idea that such increased fluxes become increasingly futile with higher $[NH_4^+]_o$.

In summary, the tracer method that has previously been applied to NH_4^+ fluxes and compartmentation in root systems has been successfully extended to leaf segments. The concentration dependence of unidirectional fluxes, efflux–influx ratios, cytosolic concentrations and cytosolic half-lives of exchange observed in wheat are indicative of high permeability and accumulation capacity of leaf cells for this important metabolite. The analysis detailed here opens new possibilities for the study of ionic relations in leaf tissue, and should clearly be applied to the study of other major ions, including nitrate, potassium and sodium, particularly in the context of the numerous transporters now being identified at the molecular level.

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References

van Beusichem ML, Kirkby EA, Baas R. 1988. Influence of nitrate and ammonium nutrition on the uptake, assimilation, and distribution of nutrients in *Ricinus communis*. *Plant Physiology* **86**: 914–921.

Bown AW. 1982. An investigation into the roles of photosynthesis and respiration in H^+ efflux from aerated suspensions of *Asparagus* mesophyll cells. *Plant Physiology* **70**: 803–810.

Britto DT, Kronzucker HJ. 2001a. Can unidirectional influx be measured in higher plants? A mathematical approach using parameters from efflux analysis. *New Phytologist* **150**: 37–47.

Britto DT, Kronzucker HJ. 2001b. Constancy of nitrogen turnover kinetics in the plant cell: Insights into the integration of subcellular N fluxes. *Planta* **213**: 175–181.

Britto DT, Siddiqi MY, Glass ADM, Kronzucker HJ. 2001a. Futile transmembrane NH_4^+ cycling: a cellular hypothesis to explain ammonium toxicity in plants. *Proceedings of the National Academy of Sciences of the USA* **98**: 4255–4258.

Britto DT, Glass ADM, Kronzucker HJ, Siddiqi MY. 2001b. Cytosolic concentrations and transmembrane fluxes of NH_4^+/NH_3. An evaluation of recent proposals. *Plant Physiology* **125**: 523–526.

Elzenga JTM, Hidde BAP, van Volkenburgh E. 1995. Light-induced membrane potential changes of epidermal and mesophyll cells in growing leaves of *Pisum sativum*. *Planta* **197**: 127–134.

Farquhar GD, Firth PM, Wetselaar R, Weir B. 1980. On the gaseous exchange of ammonia between leaves and the environment. Determination of the ammonia compensation point. *Plant Physiology* **66**: 710–714.

Feng J, Volk RJ, Jackson WA. 1998. Source and magnitude of ammonium generation in maize roots. *Plant Physiology* **118**: 835–841.

Finnemann J, Schjoerring JK. 1999. Translocation of NH_4^+ in oilseed rape plants in relation to glutamine synthetase isogene expression and activity. *Physiologia Plantarum* **105**: 469–477.

Givan CV, Joy KW, Kleczkowski A. 1988. A decade of photorespiratory nitrogen cycling. *Trends in Biochemical Sciences* **13**: 433–437.

Hanstein S, Felle H. 1999. The influence of atmospheric NH_3 on the apoplastic pH of green leaves: a non-invasive approach with pH-sensitive microelectrodes. *New Phytologist* **143**: 333–338.

Husted S, Schjoerring JK. 1995. Apoplastic pH and ammonium concentration in leaves of *Brassica napus* L. *Plant Physiology* **109**: 1453–1460.

Jeschke WD. 1976. Ionic relations of leaf cells. In: Lüttge U, Pitman MG, eds. *Encyclopedia of plant physiology*, Vol. 2, *part a*. Springer-Verlag, Berlin. 160–194.

Joy KW. 1988. Ammonia, glutamine, and asparagine: a carbon–nitrogen interface. *Canadian Journal of Botany* **66**: 2103–2109.

Kronzucker HJ, Siddiqi MY, Glass ADM. 1995a. Analysis of $^{13}NH_4^+$ efflux in spruce roots. A test case for phase identification in compartmental analysis. *Plant Physiology* **109**: 481–490.

Kronzucker HJ, Siddiqi MY, Glass ADM. 1995b. Compartmentation and flux characteristics of ammonium in spruce. *Planta* **196**: 691–698.

Kronzucker HJ, Siddiqi MY, Glass ADM, Kirk GJD. 1999. Nitrate-ammonium synergism in rice: a subcellular flux analysis. *Plant Physiology* **119**: 1041–1046.

Kronzucker HJ, Britto DT, Davenport R, Tester M. 2001. Ammonium toxicity and the real cost of transport. *Trends in Plant Science* **6**: 335–337.

Lam HM, Coschigano K, Schultz C, Oliveira R, Melo-Oliveira R, Coruzzi GM. 1996. The molecular genetics of nitrogen assimilation into amino acids in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**: 569–593.

Lee RB, Clarkson DT. 1986. Nitrogen-13 studies of nitrate fluxes in barley roots. I. Compartmental analysis from measurements of ^{13}N efflux. *Journal of Experimental Botany* **37**: 1753–1756.

Lee RB, Ratcliffe RG. 1991. Observations on the subcellular distribution of the ammonium ion in maize root tissue using *in vivo* ^{14}N-nuclear magnetic resonance spectroscopy. *Planta* **183**: 359–367.

Leegood RC, Lea PJ, Adcock MD, Hausler RE. 1995. The regulation and control of photorespiration. *Journal of Experimental Botany* **46**: 1397–1414.

Leonard RT, Rader L. 1985. The use of protoplasts for studies on membrane transport in plants. In: Fowke LC, Constabel F, eds. *Plant protoplasts*. Boca Raton, FL, USA: CRC Press, 105–118.

MacRobbie EAC. 1971. Fluxes and compartmentation in plant cells. *Annual Review of Plant Physiology*. **22**: 75–96.

Mattsson M, Hausler RE, Leegood RC, Lea P, Schjoerring JK. 1997. Leaf-atmosphere ammonia exchange in barley mutants with reduced activities of glutamine synthetase. *Plant Physiology* **114**: 1307–1312.

Min X, Siddiqi MY, Guy RD, Glass ADM, Kronzucker HJ. 1999. A comparative study of fluxes and compartmentation of nitrate and ammonium in early-successional tree species. *Plant, Cell & Environment* **22**: 821–830.

Nakashima J, Awano T, Takabe K, Fujita M, Siki H. 1997. Immunocytochemical localization of phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase in differentiating tracheary elements derived from *Zinnia* mesophyll cells. *Plant and Cell Physiology* **38**: 113–123.

Nielsen KH, Schjoerring JK. 1998. Regulation of apoplastic NH_4^+ concentration in leaves of oilseed rape. *Plant Physiology* **118**: 1361–1368.

Rains DW. 1967. Light-enhanced potassium absorption by corn leaf tissue. *Science* **156**: 1382–1383.

Rains DW. 1968. Kinetics and energetics of light-enhanced potassium absorption by corn leaf tissue. *Plant Physiology* **43**: 394–400.

Raven JA, Farquhar GD. 1981. Methylammonium transport in *Phaseolus vulgaris* leaf segments. *Plant Physiology* **67**: 859–863.

Siddiqi MY, Glass ADM, Ruth TJ. 1991. Studies of the uptake of nitrate in barley. III. Compartmentation of NO_3^-. *Journal of Experimental Botany* **42**: 1455–1463.

Smith FA, Epstein E. 1964. Ion absorption by shoot tissue: kinetics of potassium and rubidium absorption by corn leaf tissue. *Plant Physiology* **39**: 338–341.

Solorzano L. 1969. Determination of ammonia in natural waters by the phenol-hypochlorite method. *Limnology and Oceanography* **14**: 799–801.

Walker NA, Pitman MG. 1976. Measurement of fluxes across membranes. In: Lüttge U, Pitman MG, eds. *Encyclopedia of plant physiology*, Vol. 2, *part a*. Berlin, Germany: Springer-Verlag, 93–126.

Wang MY, Siddiqi MY, Ruth TJ, Glass ADM. 1993. Ammonium uptake by rice roots. I. Fluxes and subcellular distribution of $^{13}NH_4^+$. *Plant Physiology* **103**: 1249–1258.

Wang MY, Glass ADM, Shaff JE, Kochian LV. 1994. Ammonium uptake by rice roots. III. Electrophysiology. *Plant Physiology* **104**: 899–906.

von Wiren N, Gazzarrini S, Gojon A, Frommer WB. 2000. The molecular physiology of ammonium uptake and retrieval. *Current Opinion in Plant Biology* **3**: 254–261.