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ORIGINAL ARTICLE

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Constancy of nitrogen turnover kinetics in the plant cell: insights into the integration of subcellular N fluxes

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Abstract Compartmental analysis with ^{13}N was used to determine cytosolic nitrate (NO_3^-) pools, and their turnover rates, in roots of intact barley (*Hordeum vulgare* L. cv Klondike) seedlings. Influx, efflux, flux to the vacuole and assimilation, and flux to the xylem, varied as much as 300-fold over a wide range of external NO_3^- conditions. By contrast, the kinetic constant k_c describing cytosolic NO_3^- turnover varied by less than 4% from a mean value of 0.0407 min^{-1}. Accordingly, cytosolic NO_3^- pools varied linearly with influx. A literature survey showed that k_c constancy is observed with both NO_3^- and ammonium (NH_4^+) fluxes in many plant species, including *H. vulgare*, *Arabidopsis thaliana*, *Picea glauca*, and *Oryza sativa*. The regulatory system implied by this phenomenon is fundamentally different from that of potassium (K^+) fluxes, in which cytosolic pool size is held constant while k_c varies with external K^+ concentrations. We further present data showing that barley plants, grown on one steady-state concentration of NH_4^+, restore k_c within minutes of exposure to new, non-steady-state, NH_4^+ concentrations. We propose the existence of a high-fidelity mechanism governing the timing of cytosolic N turnover, and discuss its implications for attempts to improve plants biotechnologically.

Keywords Ammonium · Compartmental analysis · Homeostasis · *Hordeum* (barley) · Nitrogen-13 · Nitrate · *Oryza* (rice)

Abbreviations k_c: kinetic constant describing cytosolic NO_3^- turnover · NR: nitrate reductase

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Introduction

Nitrogen (N) is the element most extensively taken up by higher plants from the soil environment. Knowledge about its movement, compartmentation, and turnover is critical to plant physiological ecology, because N availability limits plant growth and yield more than any other nutritional factor (Vitousek and Howarth 1991; Cassman 1993; Crawford and Glass 1998). Recent genetic analyses and N-tracer studies have shown that the expression and activities of plant plasma-membrane influx systems for nitrate (NO_3^-) and ammonium (NH_4^+), the two major sources of N utilised by plants, are highly variable and are associated with similar variability in metabolic fluxes, fluxes across subcellular membranes, long-distance fluxes between root and shoot, and fluxes from the cytosol to the external medium (Glass and Siddiqi 1995; Kronzucker et al. 1995a; Crawford and Glass 1998; Krapp et al. 1998; Rawat et al. 1999). However, the regulatory processes that integrate these large variations remain unclear. For instance, recent studies involving the up-regulation of nitrate reductase (NR), thought to be the enzyme most limiting to nitrate acquisition in plants, have resulted in no gains in plant growth or N content, a phenomenon which requires explanation.

The present study was designed to quantify changes in subcellular flux partitioning of inorganic N in root tissues of higher plants, and to measure the kinetic constants for N exchange with the cytosolic compartment of cells (k_c), as functions of external NO_3^- or NH_4^+ concentrations. To obtain high-precision and highly time-resolved measurements, we used the short-lived radiotracer ^{13}N (half-life = 9.98 min). Due to its direct and non-invasive character, the technique of compartmental analysis by efflux was used to obtain estimates of subcellular fluxes and pool sizes, as well as to derive k_c (MacRobbie 1971; Walker and Pitman 1976; Siddiqi et al. 1991; Wang et al. 1993). The cytosol was the focus of our study, because it exchanges N directly

with the external environment via the plasma membrane, and because it is a metabolically critical compartment housing such key enzymes as NR and glutamine synthetase (cytosolic isoform, GS_1), and providing substrates for inorganic N flux through subcellular and tissue-level transport systems (Campbell 1990; Oaks 1994; Crawford and Glass 1998). Our experiments were conducted principally on barley (*Hordeum vulgare*), a cereal species recognised as a model system for the study of NO_3^- and NH_4^+ acquisition (Bloom and Chapin 1981; Siddiqi et al. 1991).

Materials and methods

Plant culture

Plants were grown following methods previously described (Wang et al. 1993; Kronzucker et al. 1999). In brief, following germination for 2 days (barley, *Hordeum vulgare* L. cv Klondike) or 4 days (rice, *Oryza sativa* L. cv IR-72), plants were cultivated hydroponically for 7 days (barley) and 21 days (rice) in controlled-environment growth chambers. Growth solutions consisted of modified Johnson's nutrient solutions (Wang et al. 1993; Kronzucker et al. 1999) supplemented with either $Ca(NO_3)_2$ or $(NH_4)_2SO_4$.

Compartmental analysis

Procedures for compartmental analysis by efflux were as described in Siddiqi et al. (1991), and Kronzucker et al. (1995a, b, e). Briefly, roots of intact barley or rice seedlings were labelled in solutions identical to their respective nutrient solutions, except that they contained trace amounts of radioactive $^{13}NO_3^-$ or $^{13}NH_4^+$ produced by proton irradiation of water by the Tri-University Meson Facility (TRIUMF) cyclotron at the University of British Columbia (Vancouver, Canada). Labelling time was 30 min for NO_3^- experiments and 1 h for NH_4^+ experiments, allowing for substantial labelling of cytosolic (though not vacuolar) pools (Walker and Pitman 1976). Plants were transferred from labelling solutions to efflux funnels, and periodic elution of tracer from labelled roots was then performed, following a time series (Kronzucker et al. 1995b; also see Fig. 1) declining in elution frequency over the 20- to 22-min experiments. In steady-state experiments, tracer was eluted into 20-ml aliquots of non-labelled growth medium, while in perturbation experiments the external $[NH_4^+]$ was altered part way through the elution series (see below). After the final elution, roots were excised from shoots, spun for 45 s to remove excess surface-bound solution, and roots, shoots, and efflux aliquots were analysed separately for ^{13}N activity in a Packard gamma-counter (Minaxi δ, Auto-γ 5000 Series). All experiments were performed using two replicates and repeated at least three times.

Calculations of component fluxes and compartmental concentrations were as described in Siddiqi et al. (1991). All fluxes are expressed in μmol (g FW)$^{-1}$ h^{-1}. Symbols, and basic calculation methods, for fluxes are as follows:

- ϕ_{co} = efflux from the cytosol, obtained from the rate of ^{13}N release from the cytosol at time zero.
- ϕ_{net} = net flux, obtained directly from the accumulation of ^{13}N in the plants at the end of the elution period.
- ϕ_{oc} = unidirectional influx, calculated from $\phi_{net} + \phi_{co}$.
- ϕ_{xylem} = xylem flux of ^{13}N, obtained directly from count accumulation in the shoot, at the end of the elution period.
- $\phi_{met/vac}$ = combined flux to assimilation and vacuole, resulting from $\phi_{net} - \phi_{xylem}$

k_c values were obtained from the slopes of regressed semi-logarithmic (phase III) tracer elution lines. The k_c values reported here are expressed on the basis of decadal logarithms.

Cytosolic NO_3^- concentrations were calculated from the quotient of the rate of $^{13}NO_3^-$ release integrated over 5 times the half-life of cytosolic NO_3^- exchange, and the ratio of efflux to all fluxes removing $^{13}NO_3^-$ from the cytosol, and were based on the assumption that the cytosol occupies 5% of cell volume (see Siddiqi et al. 1991 for more details of calculation methods).

Perturbational experiments with barley plants followed a protocol similar to steady-state experiments, except that:

1. An initial elution series using aliquots of steady-state growth medium, containing 100 μM NH_4^+, was performed on a periodic schedule of 2, 4, 5, 6, 7, and 8 min.
2. A more rapid elution series followed, again with steady-state medium, on the schedule of 8.5, 9, 9.25, 9.5, 9.75, and 10 min.
3. The experiment was terminated with a final series of elution aliquots, containing solutions in which the 100 μM NH_4^+ was substituted by either 10 μM NH_4^+ or 10 mM NH_4^+; this series followed a schedule of 10.25, 10.5, 10.75, 11, 11.25, 11.5, 11.75, 12, 12.5, 13, 13.5, 14, 15, 16, 18, 20, 22, 24, 26, 28, 30, and 32 min.

Because the specific activity of the pool releasing tracer after change in $[NH_4^+]$ of eluates was unknown, fluxes and pool sizes were not calculable. Perturbational experiments were thus conducted principally for the observation of short-term concentration effects on k_c values.

Results and discussion

Figure 1 shows representative semi-logarithmic plots of radiotracer efflux from the roots of intact barley and rice seedlings that had been labelled with $^{13}NO_3^-$ under steady-state nutritional conditions. Phase III in these curves represents $^{13}NO_3^-$ released specifically from the root cytosol (Kronzucker et al. 1995e) of the two species. Phase-III slopes yield exchange constants k_c (expressed as min^{-1}) that describe the decline in the rate of cytosolic $^{13}NO_3^-$ release, which obeys first-order kinetics (MacRobbie and Dainty 1958; MacRobbie

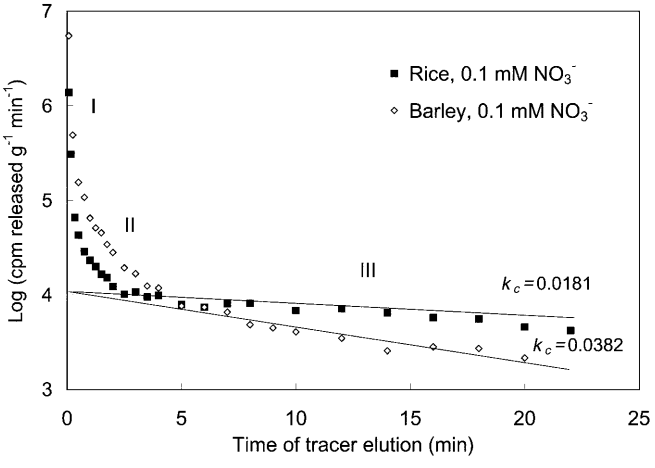

Fig. 1 Representative plots of $^{13}NO_3^-$ (tracer) efflux from roots of intact barley (*Hordeum vulgare* L. cv. Klondike) and rice (*Oryza sativa* L. cv. IR-72) seedlings grown, labelled, and eluted in nutrient solution containing 0.1 mM $^{14}NO_3^-$. Linear regression on semi-logarithmic plots was used to resolve phases I, II and III, which represent tracer elution from the surface film, cell wall and cytosol of roots, respectively (Kronzucker et al. 1995e). Kinetic exchange constants for phase III (k_c) are as indicated

1971, Walker and Pitman 1976), determined by the rapidity of turnover of the cytosolic NO_3^- pool. Hence, k_c reflects the activities of all fluxes introducing and removing NO_3^- to and from this pool, and provides a foundation for direct and inclusive evaluation of cytosolic NO_3^- turnover (Lee and Clarkson 1986; Siddiqi et al. 1991).

To obtain information on how plants adjust their cellular exchange kinetics to variations in nutritional conditions, we cultured seedlings of barley under regimes of NO_3^- provision that varied in external concentration over four orders of magnitude and, in one case, we maintained an uninduced condition by withholding NO_3^- from the growth medium (see Kronzucker et al. 1995a). Table 1 shows that the component fluxes (influx, efflux, flux to assimilation and the vacuole, flux to the xylem) estimated using compartmental analysis vary as much as 300-fold over the conditions examined, and that the ratios among fluxes also vary substantially from one condition to the next. However, in stark contrast to these flux differences, and despite the operation of entirely distinct transport systems under the different nutrient regimes (Glass and Siddiqi 1995), the essentially uniform slopes of the regression lines in Fig. 2 show that k_c for NO_3^- exchange varied by less than 4% from a mean value of 0.0407 min^{-1} across the spectrum of tested conditions. As k_c is essentially equivalent to a frequency term (expressed in min^{-1}), its relative constancy in the presence of substantial changes in individual N fluxes suggests the existence of a co-ordinating mechanism, or system of mechanisms, which set(s) the timing of the entire complex of cytosolic N fluxes involved in NO_3^- acquisition.

The quantity Q of a traced steady-state pool is related (by a proportionality constant Ω) to the traced flux ϕ into that pool and to the exchange constant as follows:

$$Q = \Omega \cdot \frac{\phi}{k} \quad (1)$$

(MacRobbie 1971; Walker and Pitman 1976). In the case of cytosolic NO_3^- pools, a major consequence of the constancy of k_c is therefore a condition in which the size of the pool is linearly related to the steady-state influx of NO_3^- to the cytosol from the external medium. Because many influx values are possible under nutrient regimes varying over four orders of magnitude (see above), the size of the cytosolic NO_3^- pool is accordingly adjusted. Hence the proposed timing mechanism not only controls the rate of turnover, but also determines pool size.

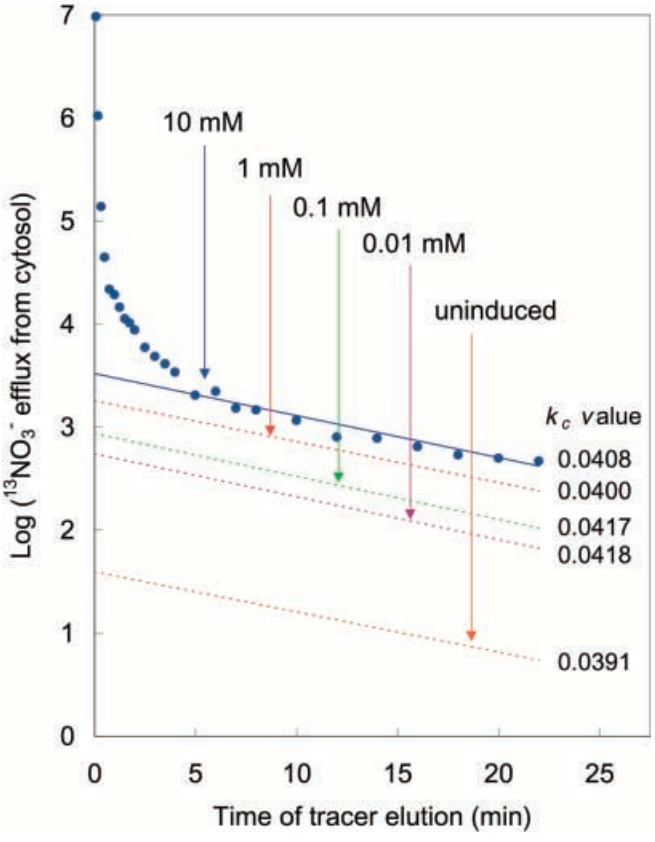

Fig. 2 Comparison of cytosolic $^{13}NO_3^-$ efflux patterns in roots of barley seedlings grown and tested under conditions of external NO_3^- supply varying over four orders of magnitude, and under an uninduced condition in which seedlings were deprived of NO_3^- until the time of labelling. Plots have been corrected for differences in root mass, tracer activity, and efflux-to-influx ratio (see Kronzucker et al. 2000), so that *y*-intercepts of regression lines directly indicate relative sizes of cytosolic NO_3^- pools. k_c values, averaged over several experiments (standard errors were within 15% of the mean), are indicated next to respective regression lines

Table 2, based upon compartmental analysis (performed essentially as described here) in a large number of plant systems, shows that the condition of unchang-

Table 1 Cellular and long-distance fluxes of $^{13}NO_3^-$ in barley roots grown under various nitrogen regimes. Uninduced plants were grown without N and measured at 0.01 mM NO_3^-; in the remaining experiments, plants were grown and measured at 0.01, 0.1, 1, and 10 mM NO_3^-, respectively

Flux[a]	Uninduced (0–0.01 mM)	0.01 mM[a]	0.1 mM[a]	1 mM[a]	10 mM[a]
ϕ_{oc}	0.26 ± 0.01	3.62 ± 0.53	5.69 ± 0.40	11.90 ± 1.02	21.67 ± 1.89
ϕ_{co}	0.03 ± 0.00	0.42 ± 0.05	0.36 ± 0.11	1.89 ± 0.49	6.59 ± 1.97
ϕ_{net}	0.23 ± 0.01	3.20 ± 0.48	5.33 ± 0.30	10.01 ± 0.79	15.07 ± 0.55
$\phi_{met/vac}$	0.21 ± 0.01	2.58 ± 0.36	4.20 ± 0.26	7.30 ± 0.72	8.75 ± 1.09
ϕ_{xylem}	0.02 ± 0.01	0.62 ± 0.11	1.13 ± 0.00	2.71 ± 0.11	6.32 ± 1.12

[a] Fluxes are expressed as μmol (g FW)$^{-1}$ h^{-1} ± SE. ϕ_{oc} influx, ϕ_{co} efflux, ϕ_{net} net flux, $\phi_{met/vac}$ flux to metabolism and vacuole, ϕ_{xylem} flux to shoot

Table 2 Kinetic constants (k_c, in $min^{-1} \pm SE$) for steady-state cytosolic exchange of NO_3^- and NH_4^+ in selected plant systems

	NO_3^-[a] (mM)						Reference
	0–0.01[b]	0.01	0.1	1	1.5	10	
Hordeum vulgare (cv. Klondike)	0.0391 ± 0.0014	0.0418 ± 0.0064	0.0418 ± 0.0035	0.0400 ± 0.0054	–	0.0408 ± 0.0027	Siddiqi et al. 1991
Hordeum vulgare (cv. CM-72)	–	–	0.0275 ± 0.0014	0.0353 ± 0.0017	–	0.0388 ± 0.0019	Kronzucker et al. 1999
Populus tremuloides	–	–	0.0303 ± 0.0006	–	0.0302 ± 0.0031	–	Min et al. 1999
Picea glauca	0.0412 ± 0.0132	0.0401 ± 0.0025	0.0356 ± 0.0044	–	0.0436 ± 0.0046	–	Kronzucker et al. 1995b
Pinus contorta	–	–	0.0427 ± 0.0016	–	0.0414 ± 0.0018	–	Min et al. 1999
Pseudotsuga menziesii	–	–	0.0371 ± 0.0017	–	0.0439 ± 0.0016	–	Min et al. 1999
	NO_3^- development of induction[c]						
	0–0.1 mM[b]	1 day	2 days	3 days	4 days	5 days	
Picea glauca	0.0492 ± 0.0031	0.0472 ± 0.0058	0.0357 ± 0.0012	0.0402 ± 0.0038	0.0401 ± 0.0038	0.0453 ± 0.0033	Kronzucker et al. 1995a
	NH_4^+[a] (mM)						
	0–0.01[b]	0.002	0.01	0.1	1	1.5	
Oryza sativa	–	0.0433 ± 0.0071	–	0.0409 ± 0.0007	0.0361 ± 0.0026	–	Wang et al. 1993
Hordeum vulgare (cv. Midas)	–	–	–	0.0257 ± 0.0007	–	0.0268 ± 0.0011	[d]
Triticum aestivum (leaf slices)	–	–	0.0209 ± 0.0058	0.0206 ± 0.0059	0.0248 ± 0.0043	–	[d]
Arabidopsis thaliana	–	–	–	0.0385 ± 0.0032	0.0415 ± 0.0049	–	Rawat et al. 1999
Populus tremuloides	–	–	–	0.0330 ± 0.0063	0.0323 ± 0.0017	–	Min et al. 1999
Picea glauca	0.0220 ± 0.0024	–	0.0172 ± 0.0014	0.0204 ± 0.002	–	0.0286 ± 0.0011	Kronzucker et al. 1998
Pinus contorta	–	–	–	0.0234 ± 0.0044	–	0.0308 ± 0.0036	Min et al. 1999
Pseudotsuga menziesii	–	–	–	0.0385 ± 0.0032	–	0.0415 ± 0.0049	Min et al. 1999

[a] Steady-state N provision as indicated

[b] 0–0.01 mM and 0–0.1 mM treatments refer to growth on zero N and provision of 0.01 or 0.1 mM N, respectively, during the compartmental-analysis protocol

[c] Induction of the NO_3^--acquisition pathway was monitored, with plants grown without NO_3^- exposed for the first time to 0.1 mM NO_3^- for 0–5 days and assayed at this concentration after the given period

[d] Our unpublished data. Experimental procedures for *H. vulgare* were as outlined in *Materials and methods*. Leaves of *T. aestivum* were sliced with razor blades to a width of 1–2 mm, vacuum-infiltrated for 10 s, and prior to the onset of labelling, pretreated in uptake solution for 3 h. Time courses and protocols for labelling and elution were as for barley and rice roots

ing k_c, and implicitly its association with pool-size variation, applies with equal stringency to other plant species, and to the case of NH_4^+, the second major inorganic N source for plants. As in the case of NO_3^-, the rate of NH_4^+ turnover is held within relatively narrow limits in the presence of large changes in component fluxes resulting from variations in external N (Wang et al. 1993; Kronzucker et al. 1995c). Most strikingly, as illustrated in Fig. 3, we found that sudden deviations in NH_4^+ supply from the steady state, when imposed midway through the elution period, deflected tracer efflux decline patterns from their initial courses only momentarily. Within minutes, k_c was re-established, even though the whole organism was still hours to days from attaining a steady state in uptake, metabolism, storage or growth (see Kronzucker et al. 1998). Such an unexpectedly rapid return to a set flux equilibrium emphasises the fidelity of the control mechanism(s) integrating N fluxes at the cellular level, which is only gradually transduced into a whole-organism response. Transient perturbations in other key cellular parameters, such as membrane electrical potential and cytosolic pH, are also known to rapidly follow changes in extracellular N provision of the magnitude imposed here (Wang et al. 1994; Kosegarten et al. 1997), suggesting that they might be involved in the rectification of N-turnover kinetics. However, in many cases these processes require significantly longer times to achieve new resting states, when compared to k_c rectification (Wang et al. 1994; Kosegarten et al. 1997).

As seen in Table 2, k_c approaches uniformity for a given N form and plant system, from domesticated cereals to wild tree species to the genetic-model-system plant *Arabidopsis thaliana*. In fact, every organism we have studied using compartmental analysis has yielded genotype-specific k_c constancy. Even studies examining $^{13}NH_4^+$ efflux kinetics in leaf slices of wheat (*Triticum aestivum* L. cv Max Red, our unpublished data) have yielded k_c values closely similar across a wide range of external N conditions, indicating that this trait (i) can be detected among distinct cell types; and (ii) is highly resilient even under conditions of substantial tissue disturbance (i.e., leaf-slice preparation).

It must be emphasised that k_c constancy could not have been anticipated a priori, and is, in fact, not observed for potassium (K^+). Table 3 shows that cytosolic K^+ is exchanged in a manner fundamentally different from that of NH_4^+ and NO_3^-. With K^+, k_c is not constant, but increases with external concentration and with associated changes in component fluxes (Memon et al. 1985), such that the Q term in Eq. 1 is instead held constant. Unlike with inorganic N, then, cytosolic K^+ turnover is modified from state to state, whereas the size of the K^+ pool is maintained at approximately 100 mM, as confirmed by several independent measurement systems (Memon et al. 1985, and references therein). Clearly, pools and fluxes of ions in plant cells are subject to high-level integration owing to the regulatory activity of at least two fundamentally differing types of mechanisms within the plant, but

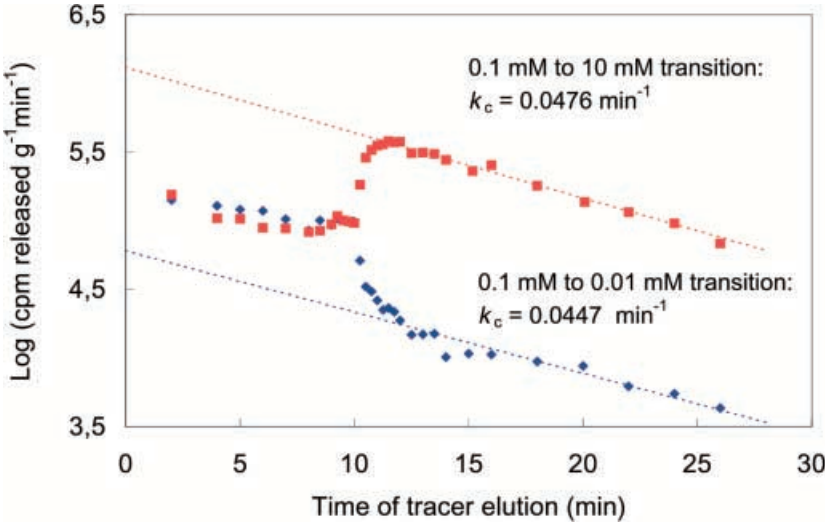

Fig. 3 Efflux of $^{13}NH_4^+$ from roots of barley seedlings subjected to transitions in eluate [NH_4^+] from 0.1 to 10 mM (*upper series*), and from 0.1 to 0.01 mM (*lower series*), following 10 min of steady-state tracer elution. Unlike data in Fig. 1, time resolution increased immediately before and after the onset of transition. Kinetic constants (k_c) are as indicated for post-transition regression lines, whose coefficients of determination (r^2) were >0.9 in both cases

Table 3 Kinetic constants (k_c, in $min^{-1} \pm SE$) for steady-state cytosolic exchange of K^+ in three cultivars of barley

	K^+ [a] (mM)		Reference
	0.01	0.1	
Hordeum vulgare (cv. Fergus)	0.0032 ± 0.0001	0.0089 ± 0.0003	Memon et al. 1985
Hordeum vulgare (cv. Compana)	0.0040 ± 0.0002	0.0092 ± 0.0004	Memon et al. 1985
Hordeum vulgare (cv. Betzes)	0.0054 ± 0.0005	0.0080 ± 0.0005	Memon et al. 1985

[a] Steady-state K^+ provision as indicated

questions as to why such divergent strategies may have evolved remain open.

The apparent ubiquity of k_c constancy redefines our view of N homeostasis at the cellular level, and we suggest that it could profoundly influence the outcome of initiatives to improve plant growth and yield by biotechnological means. Although overexpression of enzymes involved in N metabolism has in special cases been reported to increase growth and yield (Gallardo et al. 1999; Migge et al. 2000), it has failed to do so in most instances (Foyer et al. 1994; Quillère et al. 1994; Ferrario et al. 1995; Gojon et al. 1998; Limami et al. 1999). Most pertinently, in the case of NR, the enzyme widely assumed to limit nitrate acquisition (Campbell 1990; Oaks 1994), efforts by leading laboratories to engineer improved plant performance through NR overexpression have, perplexingly, resulted in little or no effect upon either growth or yield (Crawford 1995, and references cited above). This outcome has been difficult to explain. It is unknown whether cytosolic N exchange rates are held constant in the event of over- or under-expression of components, such as NR, of the acquisition pathway, but given the data presented in this paper, we predict that they are. This inference is supported by the extensively documented observation that plants adapt to changing N conditions, displaying substantial excess capacity of key enzymes and transporters involved in N acquisition. A particularly dramatic example is that of the conifer white spruce (*Picea glauca*), which, although exhibiting an impaired NO_3^--influx capacity (Kronzucker et al. 1997), nevertheless exhibits a substantial overshooting of that capacity during the induction of its NO_3^--acquisition pathway (Kronzucker et al. 1995a, d). Similar excess capacity has been observed in the time course of NR induction itself (Kronzucker et al. 1995a, and references therein). Clearly, any increase in plant N demand must be sustained by increased N supply from the external medium, but no enhancement of N uptake has ever been observed in NR over-expressers. Indeed, the reverse appears to be the case (Gojon et al. 1998). We propose that a consequence of k_c homeostasis might be that other components of the acquisition pathway will counteract the imposed manipulation (for example, through enhanced efflux, diminished influx, altered tonoplast fluxes, or through down-regulation of potentially overactive enzymes such as NR (Kronzucker et al. 1995a; Gojon et al. 1998), thereby maintaining constancy of cytosolic turnover. Such compensatory shifts in component fluxes are readily observed, perhaps most dramatically in the aforementioned case of *P. glauca* plants undergoing the transition from an uninduced to a steady state of NO_3^- acquisition. Intriguingly, even here, cytosolic k_c appeared not to change over the transitional period (Table 2), indicating the stringency of its regulation. However, as Fig. 1 and Table 2 illustrate, distinct differences in k_c for cytosolic N exchange do exist between and even within plant species. Thus, it is not impossible for a coordinated complex of flux processes to evolve towards, or to be bred for, different set points, reflecting the plant's growth strategy (Chapin 1980). In this light, we believe it to be much more likely that N acquisition in crop species will be improved by means of classical breeding, which, by selecting for a complex of features rather than for isolated components, may circumvent the limitations imposed by k_c constancy.

While individual fluxes, pool sizes and turnover rates involved in plant N acquisition are reliably measurable and must have a genetic basis (see Table 2), the factors integrating them are unknown at the molecular level. Metabolites such as amino acids, as well as NO_3^- and NH_4^+ themselves, are known to regulate activities of transporters and enzymes of N metabolism (Imsande and Touraine 1994; Oaks 1994; Crawford and Glass 1998; Rawat et al. 1999), but in all likelihood these pools are only single components of a much more complex network of regulating factors, both genetic and environmental. We conclude that flux regulation in plant systems is much more stringent and complex than previously assumed and may generate hitherto unforeseen obstacles for prospects to improve plant N acquisition.

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