

# The cytosolic $\text{Na}^+ : \text{K}^+$ ratio does not explain salinity-induced growth impairment in barley: a dual-tracer study using $^{42}\text{K}^+$ and $^{24}\text{Na}^+$

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## ABSTRACT

**It has long been believed that maintenance of low  $\text{Na}^+ : \text{K}^+$  ratios in the cytosol of plant cells is critical to the plant's ability to tolerate salinity stress. Direct measurements of such ratios, however, have been few. Here we apply the non-invasive technique of compartmental analysis, using the short-lived radiotracers  $^{42}\text{K}^+$  and  $^{24}\text{Na}^+$ , in intact seedlings of barley (*Hordeum vulgare* L.), to evaluate unidirectional plasma membrane fluxes and cytosolic concentrations of  $\text{K}^+$  and  $\text{Na}^+$  in root tissues, under eight nutritional conditions varying in levels of salinity and  $\text{K}^+$  supply. We show that  $\text{Na}^+ : \text{K}^+$  ratios in the cytosol of root cells adjust significantly across the conditions tested, and that these ratios are poor predictors of the plant's growth response to salinity. Our study further demonstrates that  $\text{Na}^+$  is subject to rapid and futile cycling at the plasma membrane at all levels of  $\text{Na}^+$  supply, independently of external  $\text{K}^+$ , while  $\text{K}^+$  influx is reduced by  $\text{Na}^+$ , from a similar baseline, and to a similar extent, at both low and high  $\text{K}^+$  supply. We compare our results to those of other groups, and conclude that the maintenance of the cytosolic  $\text{Na}^+ : \text{K}^+$  ratio is not central to plant survival under  $\text{NaCl}$  stress. We offer alternative explanations for sodium sensitivity in relation to the primary acquisition mechanisms of  $\text{Na}^+$  and  $\text{K}^+$ .**

*Key-words:* barley; cellular ion exchange; cytosolic concentration; efflux; high-affinity transport; influx, low-affinity transport, potassium, salinity stress, sodium.

*Abbreviations:* HATS, high-affinity transport system; LATS, low-affinity transport system;  $[\text{Na}^+]_{\text{cyt}}$ , cytosolic  $\text{Na}^+$  concentration;  $[\text{K}^+]_{\text{cyt}}$ , cytosolic  $\text{K}^+$  concentration;  $[\text{Na}^+]_{\text{ext}}$ , external sodium concentration;  $[\text{K}^+]_{\text{ext}}$ , external potassium concentration; SEM, standard error of the mean.

## INTRODUCTION

Salinization of agricultural soils represents one of the largest environmental challenges worldwide. Over 6% of the world's land area and 20% of the world's irrigated land are currently affected by salinity (Rhoades, Kandiah & Mashali 1992; Munns 2005). In such soils,  $\text{NaCl}$  concentrations

typically exceed 40 mM, and much higher values are frequently found (Munns 2005), creating toxic growth conditions for most plants, including all major crop species. It has long been known that  $\text{NaCl}$  toxicity is largely attributable to the effects of  $\text{Na}^+$ , and only rarely those of  $\text{Cl}^-$  (Tester & Davenport 2003), and that  $\text{Na}^+$  toxicity is linked strongly to the plant's ability to sustain the acquisition and *in planta* distribution of  $\text{K}^+$  (Rains & Epstein 1967; Warne *et al.* 1996; Zhu, Liu & Xiong 1998; Tyerman & Skerrett 1999; Kader & Lindberg 2005). At the cellular level, the relative cytosolic activities of  $\text{Na}^+$  and  $\text{K}^+$ , in particular in root tissues, have been considered a key factor in salinity tolerance (Hajibagheri, Harvey & Flowers 1987; Hajibagheri *et al.* 1989; Maathuis & Amtmann 1999; Flowers & Hajibagheri 2001; Carden *et al.* 2003). It is commonly held that cytosolic concentrations of  $\text{K}^+$  are homeostatically maintained near 100 mM, over an extensive range of  $\text{K}^+$  provision (Jeschke & Wolf 1988; Walker, Leigh & Miller 1996; Walker, Black & Miller 1998; Leigh 2001), whereas cytosolic  $\text{Na}^+$  concentrations increase with increasing salinity levels (Jeschke & Stelzer 1976; Hajibagheri *et al.* 1987; Jeschke & Wolf 1988; Schröppel-Meier & Kaiser 1988; Hajibagheri *et al.* 1989; Speer & Kaiser 1991; Flowers & Hajibagheri 2001; Carden *et al.* 2003; Halperin & Lynch 2003; Kader & Lindberg 2005). In addition, several studies have shown, or inferred, a suppression of the cytosolic  $\text{K}^+$  concentration in the presence of  $\text{Na}^+$  (Hajibagheri *et al.* 1987; Hajibagheri *et al.* 1988; Schröppel-Meier & Kaiser 1988; Speer & Kaiser 1991; Flowers & Hajibagheri 2001; Carden *et al.* 2003; cf. Jeschke & Wolf 1988).

$\text{K}^+$  is an essential activator for many enzymes located in the cytosol, and it has been postulated that  $\text{Na}^+$  cannot substitute for this biochemical function (Wyn Jones & Pollard 1983; Flowers & Dalmond 1992; Maathuis & Amtmann 1999). Nevertheless,  $\text{Na}^+$  can compete directly for  $\text{K}^+$ -binding sites on enzymes, supporting the view that the cytosolic  $\text{Na}^+ : \text{K}^+$  ratio, rather than the absolute  $\text{Na}^+$  concentration, may be critical for  $\text{NaCl}$  tolerance (Maathuis & Amtmann 1999; Carden *et al.* 2003). Methods have differed in the estimates produced for cytosolic  $[\text{Na}^+]$ , and direct measurements of both cytosolic  $[\text{Na}^+]$  and  $[\text{K}^+]$  in the same plant system, to test the hypothesis of the role of the  $\text{Na}^+ : \text{K}^+$  ratio, have been rare. The cereal crops maize and barley have been used as model systems in several key investigations (Jeschke & Stelzer 1976; Hajibagheri *et al.*

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1987, 1989; Flowers & Hajibagheri 2001; Carden *et al.* 2003), and pronounced disagreements, in regard to cytosolic Na<sup>+</sup> levels in particular, have emerged in recent literature (Flowers & Hajibagheri 2001; Carden *et al.* 2003).

In this study, we add to this discussion by providing an extensive investigation of cytosolic Na<sup>+</sup>:K<sup>+</sup> ratios in roots of intact barley, using the non-invasive method of steady-state compartmental analysis by tracer efflux. We examined eight nutritional conditions characterized by edaphically realistic levels of salinity (1–100 mM NaCl) and K<sup>+</sup> supply (0.1 and 1.5 mM, representing the LATS mode), with the objective of testing, within one genotype, the extent to which the cytosolic Na<sup>+</sup>:K<sup>+</sup> ratio is a determinant of growth under saline conditions.

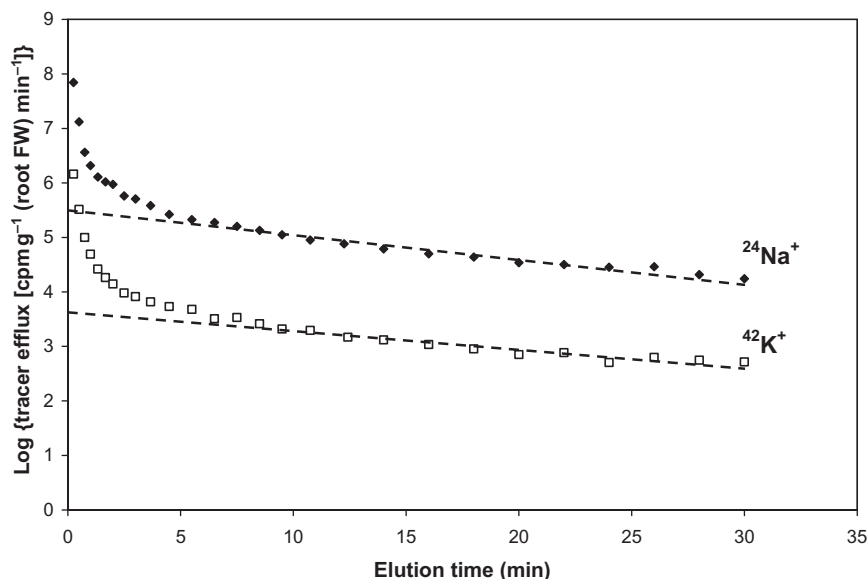
## MATERIALS AND METHODS

### Plant culture

Seeds of barley (*Hordeum vulgare* L. cv. 'Klondike') were surface-sterilized for 10 min in 1% sodium hypochlorite and germinated under acid-washed sand for 3 d prior to placement in 4-L vessels containing aerated hydroponic growth medium (modified one-fourth-strength Johnson's solution, pH 6.3–6.5) for an additional 4 d. The solution was modified to provide NO<sub>3</sub><sup>-</sup> [as Ca(NO<sub>3</sub>)<sub>2</sub>] at 10 mM, two concentrations of potassium (as K<sub>2</sub>SO<sub>4</sub>), at 0.1 and 1.5 mM, and four concentrations of sodium (as NaCl), at 1, 25, 50 and 100 mM. Solutions were exchanged frequently (daily, or often enough to prevent more than 25% depletion of any nutrient) to ensure that plants were at a nutritional steady state. Plants were cultured in walk-in growth chambers under fluorescent lights (Philips Econ-o-watt, F96T12; Phillips Lighting Co., Somerset, NJ, USA), with an irradiation of 200 μmol photons m<sup>-2</sup> s<sup>-1</sup> at plant height, for 16 h d<sup>-1</sup>. Daytime temperature was 20 °C; nighttime temperature was 15 °C, and relative humidity was approximately 70%.

### Flux and compartmentation experiments

Compartmental analysis by tracer efflux was used to estimate subcellular fluxes and compartmental concentrations (described here in brief; for details, see Lee & Clarkson 1986; Siddiqi, Glass & Ruth 1991; Kronzucker, Siddiqi & Glass 1995; Britto *et al.* 2001; Kronzucker, Szczerba & Britto 2003; Britto, Szczerba & Kronzucker 2006). Each replicate consisted of five plants held together at the shoot base by a plastic collar. Intact roots of these plants were labelled for 60 min in solution identical to growth solution but containing the radiotracer <sup>42</sup>K ( $t_{1/2}=12.36\text{h}$ ) or <sup>24</sup>Na ( $t_{1/2}=14.96\text{h}$ ), provided by the McMaster University Nuclear Reactor (Hamilton, Ontario, Canada). Labelled seedlings were attached to efflux funnels and washed successively with 13-mL aliquots of 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), 1.0 min (five times), 1.25 min (once), 1.5 min (once), 1.75 min (once) and 2.0 min (eight times). Solutions were mixed using a fine stream of air bubbles. After elution, roots were detached from shoots and spun in a low-speed centrifuge for 30 s, to remove surface water, then weighed. Radioactivity in eluates, roots, shoots and centrifugates was measured by gamma counting (Canberra-Packard, Quantum Cobra Series II, model 5003; Packard Instrument Co., Meriden, CT, USA). Linear regression of the function  $\ln\phi_{\text{co}(t)}^* = \ln\phi_{\text{co}(t)}^* - kt$  [in which  $\phi_{\text{co}(t)}^*$  is the tracer efflux at elution time  $t$ ;  $\phi_{\text{co}(t)}^*$  is the initial radioactive tracer efflux, and  $k$  is the rate constant of the exponential decline in radioactive tracer efflux, found from the slope of the tracer release rate; see Fig. 1] was used to resolve the kinetics of the slowest-exchanging phase in these experiments, which represents tracer exchange with the cytosolic compartment (see Results). Cytosolic exchange half-times ( $t_{1/2}$ ) were found from the inverse of the rate constant  $k$ , using the formula  $t_{1/2} = (\ln 2)/k$ . Efflux of K<sup>+</sup> and Na<sup>+</sup> was determined from the



**Figure 1.** Representative semi-logarithmic plots of <sup>24</sup>Na<sup>+</sup> (filled diamonds) and <sup>42</sup>K<sup>+</sup> (open squares) and radiotracer efflux from roots of barley seedlings grown at 1.5 mM [K<sup>+</sup>]<sub>ext</sub> and 100 mM [Na<sup>+</sup>]<sub>ext</sub>. Plots have been normalized for specific activity (to the arbitrary value of 2 × 10<sup>5</sup> cpm μmol<sup>-1</sup>) allowing direct comparison of initial efflux rates by inspection of y-intercepts of regression lines (representing the cytosolic compartment). FW, fresh weight.

efflux of their respective tracers [ $\phi_{\text{co(i)}}^*$ ], divided by the respective specific activity of the cytosol at the end of the 60 min labelling period (please see references at the start of this section). *Net flux* was found using total-plant  $^{42}\text{K}^+$  or  $^{24}\text{Na}^+$  retention after desorption, divided by the specific activity of the external supply of the respective tracer (Kronzucker *et al.* 2003). *Influx*,  $\phi_{\text{oc}}$ , was calculated from the sum of net flux and influx. *Cytosolic  $\text{K}^+$  and  $\text{Na}^+$  concentrations* were determined using the flux-turnover equation,  $[\text{Na}^+ \text{ or } \text{K}^+]_{\text{cyt}} = \Omega \cdot \phi_{\text{oc}}/k$ , where  $\Omega$  is a proportionality constant correcting for the cytosolic volume being approximately 5% of total tissue (Kronzucker *et al.* 2003). *Shoot translocation* of  $\text{Na}^+$  and  $\text{K}^+$  was traced by  $^{24}\text{Na}^+$  and  $^{42}\text{K}^+$  accumulation in the shoot at the end of desorption.

## Statistics

Efflux plots were analysed using linear regression and an  $R^2$  maximization procedure, as described previously (Siddiqi *et al.* 1991). All results are given as means  $\pm$  SE (SEM); errors in cytosolic ratios of  $[\text{Na}^+]$  to  $[\text{K}^+]$  were found using a standard analysis of error propagation. Significance analyses are as indicated in the text.

## RESULTS

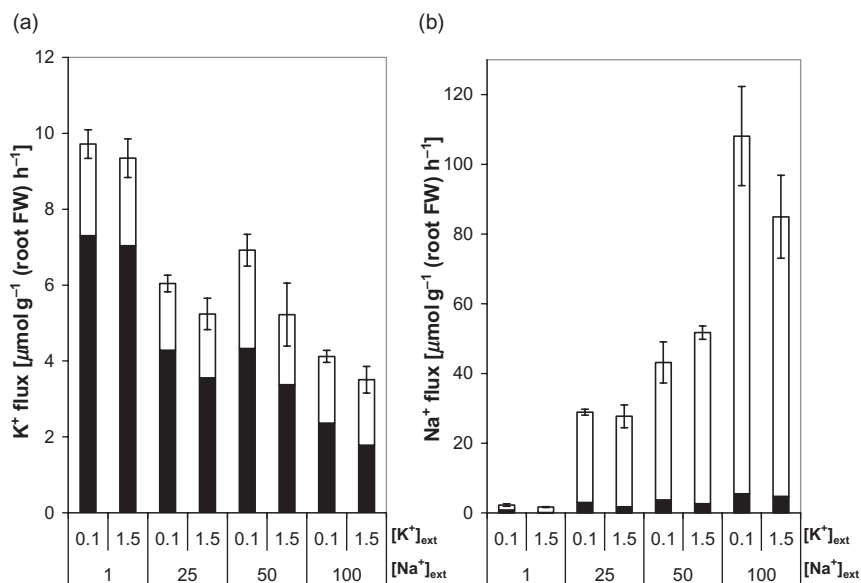
Figure 1 shows representative semi-logarithmic  $^{42}\text{K}^+$  and  $^{24}\text{Na}^+$  efflux plots from one of the eight nutritional conditions examined in this study (specifically, the 1.5 mM  $[\text{K}^+]/100.0$  mM  $[\text{Na}^+]$  condition). All plots had a similarly high quality of data resolution. Efflux of  $^{42}\text{K}^+$  and  $^{24}\text{Na}^+$  radiotracers from labelled roots of 7-day-old barley seedlings displayed compoundly exponential kinetics that were resolvable into three kinetically distinct phases, with high correlation (in most cases,  $R^2 > 0.9$ ). The slowest of these phases was identified as tracer released from the cytosolic compartment, by reference to earlier work with potassium

(Memon, Saccomani & Glass 1985; Hajibagheri *et al.* 1988; Kronzucker *et al.* 2003) and sodium (Yeo 1981; Cheeseman 1982; Binzel *et al.* 1988).

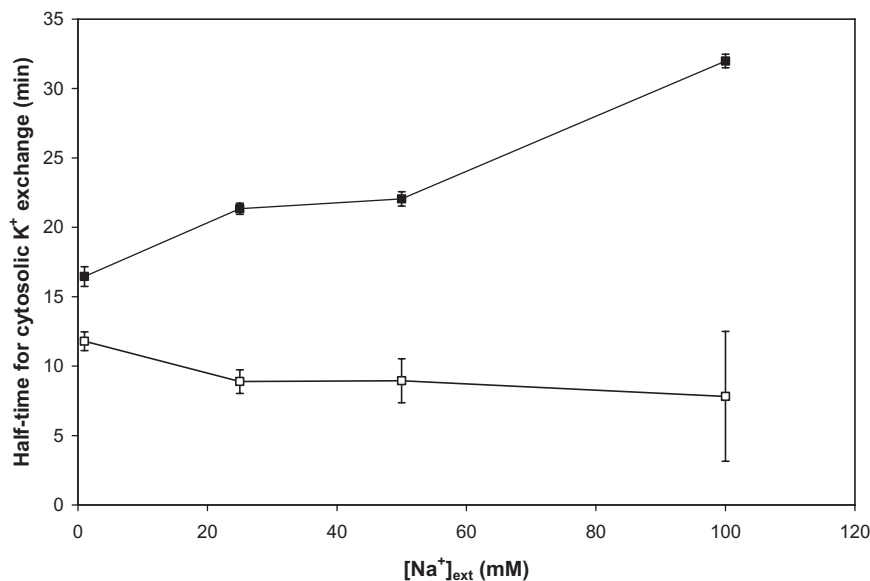
Using these kinetic patterns of efflux, in combination with tracer retention data, the net absorption (net flux), and the unidirectional influx and efflux of  $\text{K}^+$  and  $\text{Na}^+$  were estimated under the eight conditions tested (Fig. 2). Influx and net flux of  $\text{K}^+$  were inhibited by  $\text{Na}^+$  to a similar extent at both external  $\text{K}^+$  concentrations, while  $\text{K}^+$  efflux was relatively unaffected by  $[\text{Na}^+]_{\text{ext}}$  (Fig. 2a). Within a given sodium condition,  $\text{K}^+$  supply did not influence either sodium or potassium fluxes ( $P < 0.05$ ). By contrast, increases in  $\text{Na}^+$  supply culminated in a more than 50-fold increase in  $\text{Na}^+$  efflux, and  $\text{Na}^+$  influx and net flux also rose substantially (Fig. 2b). Under all conditions, sodium efflux accounted for a large fraction of the total incoming sodium, ranging from a minimum value of 0.6 (at 0.1 mM  $[\text{K}^+]_{\text{ext}}$  and 1.0 mM  $[\text{Na}^+]_{\text{ext}}$ ), to a maximal value of 0.95 (at 0.1 mM  $[\text{K}^+]_{\text{ext}}$  and 100.0 mM  $[\text{Na}^+]_{\text{ext}}$ ). While always high, the ratio of efflux to influx of sodium generally increased with increasing  $[\text{Na}^+]_{\text{ext}}$ .

A pronounced difference between the  $^{42}\text{K}^+$  tracer-release characteristics of the two potassium treatments was seen in the relative changes, with changing  $[\text{Na}^+]_{\text{ext}}$ , in turnover of the cytosolic  $\text{K}^+$  pool (Fig. 3). Turnover rates are described by the exponential rate constant  $k$  ( $= 0.693/t_{1/2}$ , where  $t_{1/2}$  is the exchange half-time) of the cytosolic phase of tracer efflux, found from the slopes of the cytosolic regression lines (Fig. 1). Thus, increasing sodium had the effect of slowing down cytosolic  $\text{K}^+$  turnover in the low- $\text{K}^+$  condition, while slightly speeding up that of the higher- $\text{K}^+$  condition.  $\text{Na}^+$  turnover rates, by contrast, were affected neither by  $[\text{K}^+]_{\text{ext}}$  nor by  $[\text{Na}^+]_{\text{ext}}$  (not shown). At all values of  $[\text{Na}^+]_{\text{ext}}$ , exchange half-times were significantly shorter in the high-potassium condition ( $P < 0.05$ ).

Influx and turnover data were combined to estimate the cytosolic concentrations of both  $\text{K}^+$  and  $\text{Na}^+$  ( $[\text{K}^+]_{\text{cyt}}$  and  $[\text{Na}^+]_{\text{cyt}}$ , respectively; see flux-turnover equation in



**Figure 2.** Comparison of  $\text{K}^+$  (a) and  $\text{Na}^+$  (b) component fluxes, determined at two external  $\text{K}^+$  concentrations and four  $\text{Na}^+$  conditions. Bars are divided into net flux (filled segments) and efflux (clear segments), which together comprise the influx term. Error bars refer to  $\pm$  SEM of 4–12 replicates. FW, fresh weight.



**Figure 3.** Plots of the half-time for cytosolic  $\text{K}^+$  exchange for seedlings grown at two  $[\text{K}^+]_{\text{ext}}$ , 0.1 mM (filled squares) and 1.5 mM (clear squares), and four  $[\text{Na}^+]_{\text{ext}}$ . Error bars refer to  $\pm$  SEM of 4–12 replicates.

Materials and Methods). In the low-potassium plants, the tendency for half-times of  $\text{K}^+$  exchange to increase with increasing  $[\text{Na}^+]_{\text{ext}}$  counteracted the trend of decreasing  $\text{K}^+$  influx along this gradient, resulting in a rather constant  $[\text{K}^+]_{\text{cyt}}$  value (Fig. 4a). By contrast, the decreasing  $\text{K}^+$  influx in the high-potassium condition had no such offsetting trend in  $\text{K}^+$  exchange rate, and this resulted in a strong decrease of  $[\text{K}^+]_{\text{cyt}}$  as external sodium supply increased. On the other hand, the cytosolic sodium concentration increased steadily with  $[\text{Na}^+]_{\text{ext}}$ , resulting in very high values of  $[\text{Na}^+]_{\text{cyt}}$  (308–311 mM) at the highest external  $\text{Na}^+$  concentration of 100 mM (Fig. 4b). These patterns of  $[\text{K}^+]_{\text{cyt}}$  and  $[\text{Na}^+]_{\text{cyt}}$  variations can be expressed in the form of cytosolic  $\text{Na}^+ : \text{K}^+$  ratios (Fig. 4c), and for both potassium treatments, this ratio increased with increasing  $[\text{Na}^+]_{\text{ext}}$ . The extent of the increase was much greater with the high- $\text{K}^+$  plants, which had higher  $\text{Na}^+ : \text{K}^+$  ratios at all  $[\text{Na}^+]_{\text{ext}} > 1$  mM ( $P < 0.05$ ). This trend culminated at 100 mM  $[\text{Na}^+]_{\text{ext}}$ , which yielded a fivefold higher  $\text{Na}^+ : \text{K}^+$  ratio compared with low- $\text{K}^+$  plants.

Figure 5 shows the accumulation of  $^{42}\text{K}^+$  (Fig. 5a) and  $^{24}\text{Na}^+$  (Fig. 5b) tracers in the shoots of barley plants, as a function of  $[\text{K}^+]_{\text{ext}}$  and  $[\text{Na}^+]_{\text{ext}}$ , after isotope exposure for 60 min, followed by desorption for 30 min. Accumulation of both tracers was strongly dependent on  $[\text{Na}^+]_{\text{ext}}$ , but independent of  $[\text{K}^+]_{\text{ext}}$ , with an approximate eightfold decrease in  $^{42}\text{K}^+$  translocation between 1 and 100 mM  $[\text{Na}^+]_{\text{ext}}$ , and an approximate 40-fold increase in  $^{24}\text{Na}^+$  translocation between these conditions. For each  $[\text{Na}^+]_{\text{ext}}$ , no significant difference was seen between low and high  $[\text{K}^+]_{\text{ext}}$ , in terms of tracer translocation ( $P < 0.05$ ).

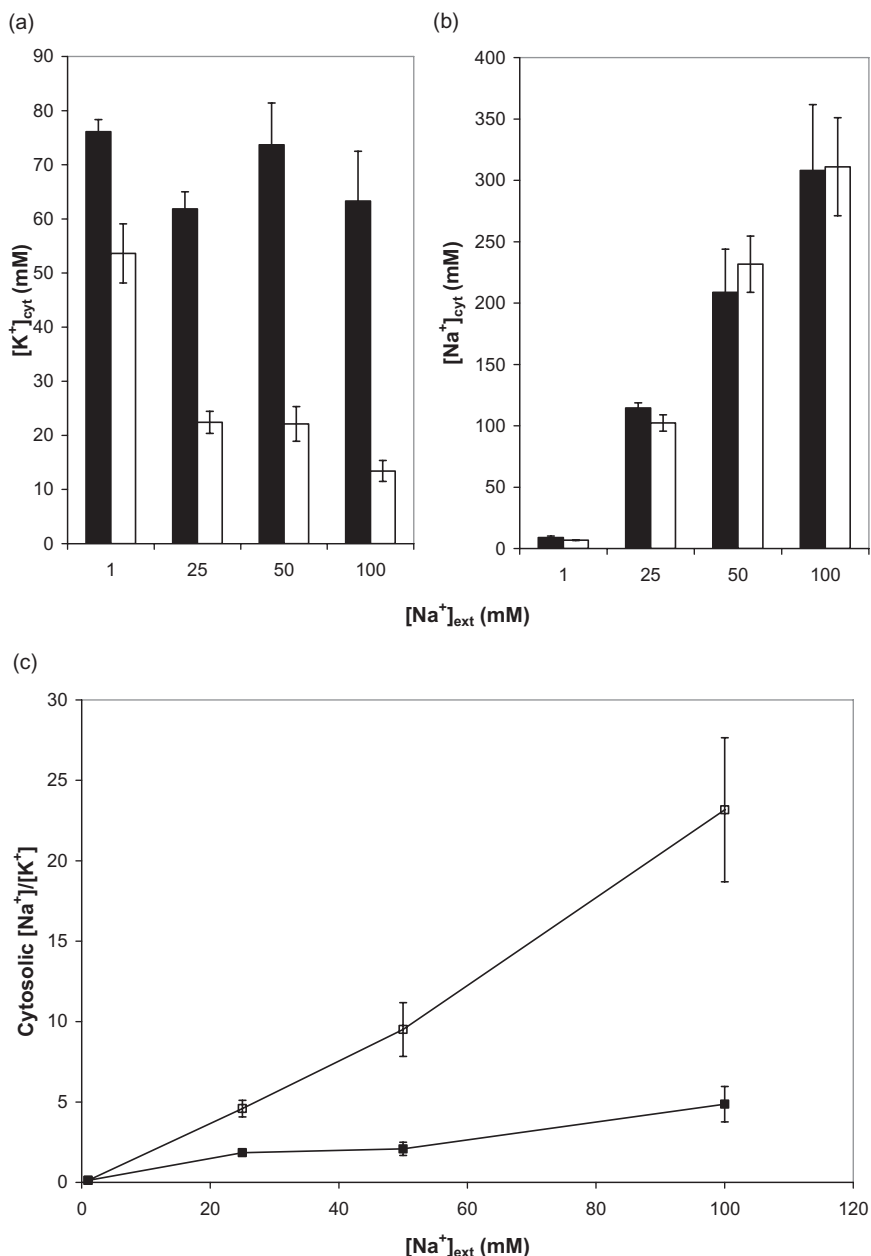
Plant growth at 7 d was measured as fresh weight (FW) for the eight conditions (Fig. 6). No significant difference ( $P < 0.05$ ) in growth was seen between low- $\text{K}^+$  and high- $\text{K}^+$  conditions, with increased growth suppression following increased  $[\text{Na}^+]_{\text{ext}}$ . This was true for both roots and shoots individually, as well as for the whole plant. The trend in

Fig. 6 was also observed using dry weight measurements (not shown).

## DISCUSSION

### Potassium fluxes

One of the best-known effects of sodium stress on plant nutrition is a suppression of potassium uptake. This effect has been demonstrated for plant systems growing both at low  $[\text{K}^+]_{\text{ext}}$ , at which the HATS operates (Santa-María *et al.* 1997; Fu & Luan 1998; Rubio, Santa-María & Rodríguez-Navarro 2000; Martínez-Cordero, Martínez & Rubio 2005), and at high  $[\text{K}^+]_{\text{ext}}$ , at which the high-affinity transport system (HATS) predominates (Rains & Epstein 1967). Our study examines both ranges of potassium transport, using a HATS concentration of 0.1 mM  $[\text{K}^+]_{\text{ext}}$  and a LATS concentration of 1.5 mM  $[\text{K}^+]_{\text{ext}}$ , both of which are edaphically realistic (Ashley, Grant & Grabov 2006), and confirms the effect of  $\text{Na}^+$  on  $\text{K}^+$  fluxes, by showing similar  $\text{Na}^+$ -dependent suppression at both low and high  $[\text{K}^+]_{\text{ext}}$  (Fig. 2a). This is in contrast to the effects of  $\text{NH}_4^+$  upon  $\text{K}^+$  fluxes, which are pronounced in the potassium-HATS range, but are absent in the LATS range (Hirsch *et al.* 1998; Santa-María, Danna & Czibener 2000; Kronzucker *et al.* 2003). In both HATS and LATS systems, 100 mM  $[\text{Na}^+]_{\text{ext}}$  reduced potassium influx by more than 50% relative to the 1 mM  $[\text{Na}^+]_{\text{ext}}$  condition, and reduced the net  $\text{K}^+$  flux to an even greater extent. The suppression of  $\text{K}^+$  influx with increasing  $[\text{Na}^+]_{\text{ext}}$  may be related to changes in osmotic conditions within the cell, particularly in the internal sodium concentration, which is shown to influence  $\text{K}^+$ -channel activity (Qi & Spalding 2004). Interestingly, the efflux of  $\text{K}^+$  is fairly constant across the range of  $\text{Na}^+$  supply, indicating that the mechanisms of sodium and potassium efflux are distinct and independent. Potassium provision does not strongly influence  $\text{K}^+$  fluxes, indicating that the plants are acclimated

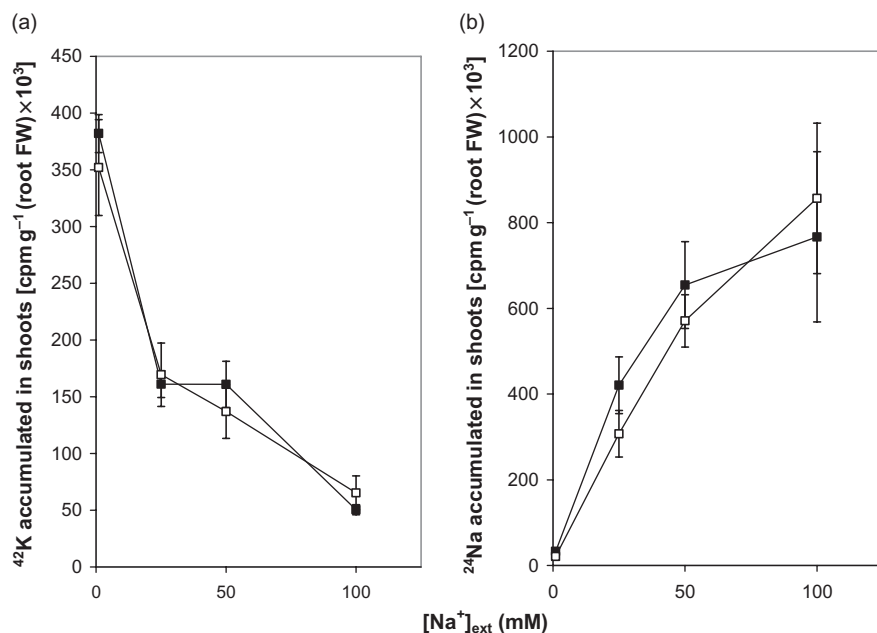


**Figure 4.** (a)  $K^+$  and (b)  $Na^+$  concentrations of the root cytosolic compartment for barley seedlings grown at two  $[K^+]_{\text{ext}}$  and four  $[Na^+]_{\text{ext}}$ . Filled bars represent seedlings grown at 0.1 mM  $[K^+]_{\text{ext}}$ , and clear bars represent seedlings grown at 1.5 mM  $[K^+]_{\text{ext}}$ . (c) The ratio of cytosolic  $[Na^+]$  to  $[K^+]$  is shown for the two  $K^+$  conditions (filled squares, 0.1 mM; clear squares, 1.5 mM) at each of the four  $[Na^+]_{\text{ext}}$ . Error bars refer to  $\pm$  SEM of 4–12 replicates.

to steady-state conditions (see Asher & Ozanne 1966). Nevertheless, despite these similarities in potassium fluxes in HATS and LATS ranges, we observed strong distinctions in cytosolic  $K^+$  turnover between these two conditions, with accelerated turnover in the LATS, in agreement with previous work (Kochian & Lucas 1982; Memon *et al.* 1985; Kronzucker *et al.* 2003; Britto *et al.* 2006). What has not been previously demonstrated is that the  $K^+$  turnover differences between HATS and LATS are accentuated by increasing  $[Na^+]_{\text{ext}}$  (Fig. 3). These differential  $Na^+$  effects upon  $K^+$  turnover underscore the fundamental differences between HATS and LATS transport modes that we have previously demonstrated (Kronzucker *et al.* 2003; Britto *et al.* 2006), and have important consequences for cytosolic  $K^+$  concentration estimates (see subsequent discussion).

### Sodium fluxes

Varying the potassium supply had no strong or consistent effect on sodium fluxes, with plants showing similar responses in the  $K^+$  HATS and LATS ranges. By contrast,  $[Na^+]_{\text{ext}}$  had a pronounced influence on sodium fluxes, particularly efflux, which at 100 mM  $[Na^+]_{\text{ext}}$  showed values from 50- to 70-fold higher than at 1 mM. Nevertheless, large ratios of efflux to influx were observed for  $Na^+$  even at the lowest  $[Na^+]_{\text{ext}}$ , indicating futile cycling of this ion in barley roots, under all  $Na^+$  conditions tested. Although not systematically studied until now, futile cycling of  $Na^+$  at the plasma membrane has been previously observed by others (Cheeseman 1982; Jacoby & Hanson 1985; Lazof & Cheeseman 1988a,b; Schubert & Läuchli 1990; Essah,



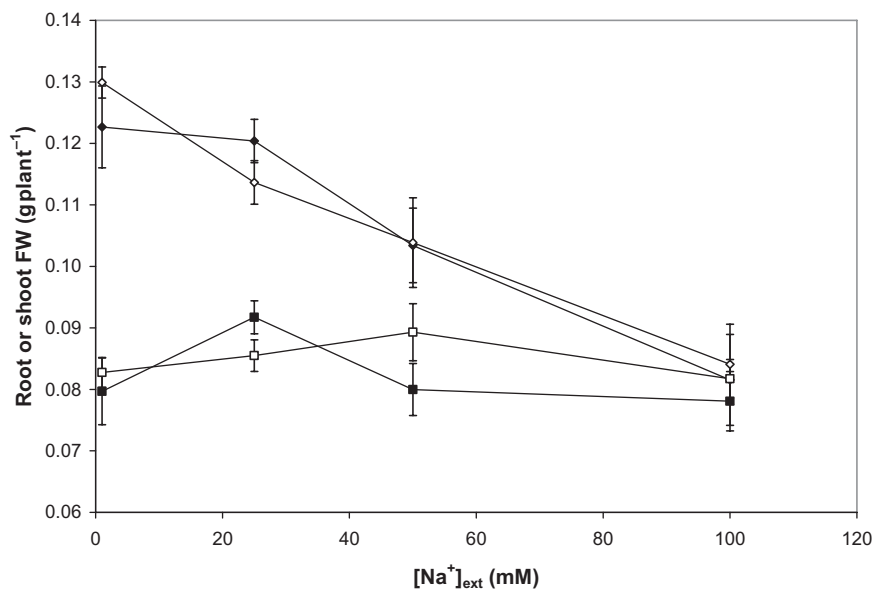
**Figure 5.** Accumulation of  $^{42}\text{K}^+$  and  $^{24}\text{Na}^+$  in shoots after 60 min labelling and 30 min desorption, determined at two  $[\text{K}^+]_{\text{ext}}$  and four  $[\text{Na}^+]_{\text{ext}}$  values. Filled symbols refer to seedlings grown at 0.1 mM  $[\text{K}^+]_{\text{ext}}$ , and clear symbols refer to seedlings grown at 1.5 mM  $[\text{K}^+]_{\text{ext}}$ . Plots have been normalized for specific activity (to the arbitrary value of  $2 \times 10^5$  cpm  $\mu\text{mol}^{-1}$ ). Error bars refer to  $\pm$  SEM of 4–12 replicates. FW, fresh weight.

Davenport & Tester 2003; Wang *et al.* 2006). The phenomenon of futile ion cycling has been characterized for other toxic ions such as  $\text{NH}_4^+$  (Britto *et al.* 2001) and  $\text{Cl}^-$  (Britto *et al.* 2004), and has also been seen with relatively non-toxic ions including  $\text{K}^+$  (Britto *et al.* 2006) and  $\text{NO}_3^-$  (Scheurwater *et al.* 1999; Kronzucker, Glass & Siddiqi 1999). For all ions, it is now recognized that this condition is energetically burdensome to the plant (Schubert & Läuchli 1990; Poorter *et al.* 1991; Yeo 1998; Scheurwater *et al.* 1999; Kronzucker *et al.* 2001; Tester & Davenport 2003), and the high cost of futile cycling has been calculated for some ions (Kronzucker *et al.* 2001). Therefore, when photosynthetic energy or carbon capture is compromised, such as under conditions of substantial  $\text{NH}_4^+$  or  $\text{Na}^+$  infiltration into leaf tissues (Marschner 1995), futile cycling at the plasma

membrane may be a significant contributor to toxicity of these ions (Yeo 1998; Kronzucker *et al.* 2003; Tester & Davenport 2003).

### Cytosolic sodium and potassium concentrations and ratios

From the data in Fig. 4a, we conclude that the effect of varying external sodium on  $[\text{K}^+]_{\text{cyt}}$  depends strongly on  $[\text{K}^+]_{\text{ext}}$ . Under low  $[\text{K}^+]_{\text{ext}}$ , increasing  $[\text{Na}^+]_{\text{ext}}$  from 1 to 100 mM has no effect on  $[\text{K}^+]_{\text{cyt}}$ , while at high  $[\text{K}^+]_{\text{ext}}$ , the same sodium gradient results in a fourfold suppression of  $[\text{K}^+]_{\text{cyt}}$ . Our finding of  $\text{Na}^+$ -dependent  $[\text{K}^+]_{\text{cyt}}$  suppression at higher  $[\text{K}^+]_{\text{ext}}$  is in agreement with several previous studies employing various methods (Hajibagheri *et al.* 1987, 1988;



**Figure 6.** Root and shoot fresh weights (FW) for individual plants grown with either 0.1 mM  $[\text{K}^+]_{\text{ext}}$  (filled symbols) or 1.5 mM  $[\text{K}^+]_{\text{ext}}$  (clear symbols) at four  $\text{Na}^+$  conditions. Squares represent root masses, while diamonds represent shoot masses. Error bars refer to  $\pm$  SEM of 4–24 replicates.

Schröppel-Meier & Kaiser 1988; Speer & Kaiser 1991; Flowers & Hajibagheri 2001; Carden *et al.* 2003). However, this is the first demonstration that, at low  $[K^+]_{\text{ext}}$ , the homeostasis of  $[K^+]_{\text{cyt}}$  is resistant to external  $Na^+$ . This is an interesting contrast to the response of  $[K^+]_{\text{cyt}}$  to applications of  $NH_4^+$ , where suppression is seen in the low- $[K^+]_{\text{ext}}$  condition, but not in the high- $[K^+]_{\text{ext}}$  condition (Kronzucker *et al.* 2003). The  $[K^+]_{\text{ext}}$ -dependent response to  $[Na^+]_{\text{ext}}$  presented here reveals an additional distinction between the HATS and LATS modes of  $K^+$  transport (previously discussed in the context of cytosolic  $K^+$  turnover rates).

In contrast to the strong relationship between  $K^+$  transport mode and  $Na^+$  sensitivity of  $[K^+]_{\text{cyt}}$ , no relationship between  $K^+$  transport mode and  $[Na^+]_{\text{cyt}}$  is apparent. Rather,  $[Na^+]_{\text{cyt}}$  is a function only of  $[Na^+]_{\text{ext}}$  (Fig. 4b). Because (1) cytosolic  $[Na^+]_{\text{cyt}}$  values are virtually identical between HATS and LATS modes of  $K^+$  transport, and (2) cytosolic  $[K^+]_{\text{cyt}}$  values differ strongly between these modes, the pattern of  $[Na^+]_{\text{cyt}} : [K^+]_{\text{cyt}}$  ratios differs substantially (up to nearly fivefold) between modes as the external  $[Na^+]_{\text{ext}}$  is increased from 1 to 100 mM, the ratio being more suppressed in the HATS-conditioned plants (Fig. 4c). One might therefore predict that a greater impairment of growth should be seen with LATS-conditioned plants. However, this is not borne out; the growth responses to the test range of 1–100 mM  $[Na^+]_{\text{ext}}$  are very similar with both LATS- and HATS-conditioned plants (Fig. 6). This shows that the  $[Na^+]_{\text{cyt}} : [K^+]_{\text{cyt}}$  ratio of root cells cannot strictly be the cause of growth suppression, or even a reliable diagnostic of  $Na^+$  toxicity, even though there is a negative relationship between plant growth and this ratio *within* a specific mode.

This conclusion depends on the accuracy of the cytosolic concentration estimates provided. On the one hand, our values for  $[K^+]_{\text{cyt}}$  agree remarkably well with estimates made using other systems, such as microelectrodes (Walker *et al.* 1996; Carden *et al.* 2003), X-ray microanalysis (Hajibagheri *et al.* 1988) and longitudinal ion profiling (Jeschke & Stelzer 1976; Hajibagheri *et al.* 1988), whereas our  $[Na^+]_{\text{cyt}}$  values agree with some studies but not others, reflecting the considerable variability that exists in the literature. In general, studies using sodium-selective microelectrodes (Carden *et al.* 2003), sodium-sensitive dyes (Halperin & Lynch 2003; Kader & Lindberg 2005) and chloroplast concentrations (assumed in some studies to reflect cytosolic concentrations; Schröppel-Meier & Kaiser 1988; Speer & Kaiser 1991) tend to yield lower readings (typically < 100 mM), while X-ray microanalysis (Hajibagheri *et al.* 1988; Flowers & Hajibagheri 2001) and compartmental analysis by tracer efflux (Hajibagheri *et al.* 1988) tend to show higher values (typically > 100 mM). However, cytosolic  $Na^+$  activities as high as 295 mM have been detected using sodium-selective microelectrodes (in *Azobiscyanoacrylate* exposed to artificial seawater; Amtmann & Gradmann 1994), and chloroplast concentrations as high as 165 mM have been measured in *Spinacia oleracea* (at 200 mM  $[Na^+]_{\text{ext}}$ ; Robinson, Downton & Millhouse 1983), and 257 mM in *Sueda maritima* (at 340 mM  $[Na^+]_{\text{ext}}$ ;

Harvey *et al.* 1981). By contrast, compartmental analysis has, in some cases, yielded moderate values, near 10 mM at 25 mM  $[Na^+]_{\text{ext}}$  in maize (Schubert & Läubli 1990), and 54 mM at 428 mM  $[Na^+]_{\text{ext}}$  in tobacco cells (Binzel *et al.* 1988). This indicates that differences in cytosolic values are not necessarily technique driven, and that compartmental analysis can yield values spanning a large range of cytosolic  $[Na^+]_{\text{cyt}}$ , depending on the plant system.

Interestingly, a recent study focusing on two varieties of barley that differed in  $NaCl$  tolerance (Carden *et al.* 2003) presented cytosolic  $Na^+$  activity values, obtained using sodium-selective microelectrodes that were one to two orders of magnitude lower than  $[Na^+]_{\text{cyt}}$  values obtained in a preceding study using X-ray microanalysis in the same varieties (Flowers & Hajibagheri 2001). The authors concluded that microelectrodes provided more accurate readings, because X-ray microanalysis depends on several assumptions, such as the approximation of cytosolic water content. Nevertheless, the microelectrode values obtained by Carden *et al.* (2003) contradict several currently established models of  $NaCl$  stress tolerance. In particular, in the tolerant variety of barley investigated by this group, a 14-fold increase in cytosolic  $Na^+$  activity was measured between days 5 and 8 in a 200 mM  $NaCl$  solution, coincident with a measured decline in vacuolar  $Na^+$  activity, which suggests an offloading of sodium from vacuole to cytosol. This finding disagrees with the widely held concept of vacuolar sequestration of  $Na^+$  in the service of maintaining low  $[Na^+]_{\text{cyt}}$ , as a strategy of  $NaCl$  tolerance (Apse *et al.* 1999; Apse & Blumwald 2002). By contrast, the sensitive variety of barley displayed a constancy of cytosolic  $Na^+$  activity at 19 mM (and of vacuolar activity at 32 mM). This cytosolic value was substantially lower than the value achieved, by the end of the time course, in the tolerant variety, in contradiction with several studies that have observed higher cytosolic accumulation in sodium-sensitive cultivars (Hajibagheri *et al.* 1987, 1989; Kader & Lindberg 2005; our unpublished results; see also Tester & Davenport 2003). The results shown in our present contribution to this debate agree not with the microelectrode results, but much more closely with X-ray microanalysis. We suggest that there has perhaps been too much readiness to accept microelectrode readings at the expense of other methodologies (also see Carden, Diamond & Miller 2001; Shabala *et al.* 2005).

## CONCLUSION

Although it is widely believed that the ratio of  $[Na^+]_{\text{cyt}}$  to  $[K^+]_{\text{cyt}}$  is a key determinant of plant  $NaCl$  tolerance, few studies in the literature have actually measured these parameters, and none has investigated their  $Na^+$  and  $K^+$  concentration dependence and relationship to growth within a single genotype. Contrary to the prevailing view, this study shows that the growth response of a cultivar can be identical in the presence of  $[Na^+]_{\text{cyt}} : [K^+]_{\text{cyt}}$  ratios that differ by as much as fivefold. It is therefore worth considering which variables change similarly when similar degrees of growth suppression are observed. In our study, these

factors are (1) the absolute value of [Na<sup>+</sup>]<sub>cyt</sub> (as opposed to a ratio of this value with [K<sup>+</sup>]<sub>cyt</sub>) (Fig. 4b); (2) the magnitude of the Na<sup>+</sup> fluxes, particularly efflux (Fig. 2b); (3) the extent of Na<sup>+</sup> translocation to the shoot (Fig. 5b); and (4) the suppression, by Na<sup>+</sup>, of K<sup>+</sup> influx, net flux and translocation from root to shoot (Figs 2a and 5a). In our view, these primary sodium- and potassium-acquisition parameters potentially combine to produce the toxicity syndrome (Fig. 6). Another important toxicity factor is likely to be osmotic stress, although it is instructive to note that high (up to 40 mM) external [K<sup>+</sup>] failed to inhibit growth in barley seedlings (Szczerba, Britto & Kronzucker 2006). It should also be noted that in this study, we used relatively high Ca<sup>2+</sup> concentrations (10 mM) in the growth media, which are known to attenuate Na<sup>+</sup> toxicity (Rains & Epstein 1967), via effects on non-selective cation channels (Tester & Davenport 2003); nevertheless, even with the protective presence of Ca<sup>2+</sup>, substantial growth suppression was observed. The large, relatively uncontrolled Na<sup>+</sup> influxes (Fig. 2b) lead to substantial accumulation of Na<sup>+</sup> in the cytosol (Fig. 4b), which, in turn, provides a steep gradient for Na<sup>+</sup> infiltration into the xylem stream and the shoot (Fig. 5). Simultaneously, potassium acquisition and translocation to the shoot are reduced by Na<sup>+</sup>. Both effects on translocation are known to impair photosynthetic function and, thus, energy supply (Marschner 1995). Under such conditions, the high Na<sup>+</sup> effluxes (Figs 1 and 2b) that occur against a thermodynamic gradient (Cheeseman 1982; Lazof & Cheeseman 1988a,b; Schubert & Läuchli 1990; Essah *et al.* 2003; Tester & Davenport 2003; Wang *et al.* 2006) may constitute a detrimental energy sink, aggravating the compromised energy status of the plant. We have previously shown, in the context of NH<sub>4</sub><sup>+</sup> toxicity, that energy-demanding efflux can be pivotal to plant survival under stress (Britto *et al.* 2001; Kronzucker *et al.* 2001; Britto & Kronzucker 2002), and propose that a similar mechanism may hold in the case of Na<sup>+</sup> stress. The ions Na<sup>+</sup> and NH<sub>4</sub><sup>+</sup> appear to share the hallmarks of relatively unrestricted, channel-mediated influx, accompanied by high rates of active efflux (futile cycling), high levels of cytosolic ion build-up and increasing shoot infiltration at increasing external Na<sup>+</sup> and NH<sub>4</sub><sup>+</sup> concentrations. Furthermore, in both cases, K<sup>+</sup> relations (in particular cytosolic concentrations and root–shoot translocation rates) are affected, although Na<sup>+</sup> and NH<sub>4</sub><sup>+</sup> differ in the extent to they influence high-affinity, as opposed to low-affinity K<sup>+</sup> transport. It is intriguing to speculate whether cultivars tolerant of one toxicant may also be tolerant of the other, opening the possibility of breeding and biotechnologically engineering for cross-tolerance to these important environmental stressors.

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