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RESEARCH PAPER

Futile Na^+ cycling at the root plasma membrane in rice (*Oryza sativa* L.): kinetics, energetics, and relationship to salinity tolerance

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Abstract

Globally, over one-third of irrigated land is affected by salinity, including much of the land under lowland rice cultivation in the tropics, seriously compromising yields of this most important of crop species. However, there remains an insufficient understanding of the cellular basis of salt tolerance in rice. Here, three methods of $^{24}Na^+$ tracer analysis were used to investigate primary Na^+ transport at the root plasma membrane in a salt-tolerant rice cultivar (Pokkali) and a salt-sensitive cultivar (IR29). Futile cycling of Na^+ at the plasma membrane of intact roots occurred at both low and elevated levels of steady-state Na^+ supply ($[Na^+]_{ext}$=1 mM and 25 mM) in both cultivars. At 25 mM $[Na^+]_{ext}$, a toxic condition for IR29, unidirectional influx and efflux of Na^+ in this cultivar, but not in Pokkali, became very high [>100 μmol g (root FW)$^{-1}$ h^{-1}], demonstrating an inability to restrict sodium fluxes. Current models of sodium transport energetics across the plasma membrane in root cells predict that, if the sodium efflux were mediated by Na^+/H^+ antiport, this toxic scenario would impose a substantial respiratory cost in IR29. This cost is calculated here, and compared with root respiration, which, however, comprised only ~50% of what would be required to sustain efflux by the antiporter. This suggests that either the conventional 'leak-pump' model of Na^+ transport or the energetic model of proton-linked Na^+ transport may require some revision. In addition, the lack of suppression of Na^+ influx by both K^+ and Ca^{2+}, and by the application of the channel inhibitors Cs^+, TEA^+, and Ba^{2+}, questions the participation of potassium channels and non-selective cation channels in the observed Na^+ fluxes.

Key words: Efflux, influx, ion transport, respiration, rice, sodium, salinity, salt stress.

Introduction

Soil salinization is a major problem in modern agriculture, particularly for irrigated croplands (Boyer, 1982; Yeo, 2007). Rice (*Oryza sativa* L.), the world's most important agricultural species and a crop grown under extensive irrigation regimes, is unusually susceptible to salinity stress (Yeo and Flowers, 1985; Sahi *et al*., 2006). In the past decade, impressive advances in the engineering of salt tolerance in crop plants have resulted from modifications of Na^+ transport across cell membranes (Apse *et al*., 1999; Shi *et al*., 2000; Munns, 2005; Ren *et al*., 2005). In a particularly striking example, overexpression of the *NHX1* gene, to increase sodium transport across the vacuolar, tonoplast, membrane under salt stress, has led to the production of glycophytes (such as tomato) that are able to survive and reproduce on NaCl levels as high as 200 mM (Yamaguchi and Blumwald, 2005). Despite substantial progress in the understanding of mechanisms of Na^+ tolerance and toxicity, however, current knowledge of the primary Na^+ fluxes at the plasma membrane of roots that distinguish tolerant from sensitive cultivars remains insufficient (Haro *et al*., 2005; Munns, 2005; Ren *et al*., 2005; Yeo, 2007), and indeed the question of how Na^+ enters the plant in the first place is still not satisfactorily resolved (Flowers, 2006; Wang *et al*., 2007).

Because Na^+ toxicity becomes manifest at high external Na^+ concentrations ($[Na^+]_{ext}$), low-affinity transport systems (LATS) are believed to catalyse the influx of Na^+ into the plant under salt stress. LATS are typically characterized by passive entry into the cell, a linear

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relationship between influx and the activity of the transported substrate over a wide range of concentrations, and a high concomitant efflux (Britto and Kronzucker, 2006). The molecular identity of the transporters involved in low-affinity Na^+ transport is currently under debate (Munns, 2005; Flowers, 2006; Yeo, 2007). Putative candidates include potassium channels, the low-affinity cation transporter LCT1 (Yeo, 2007), and members of the HKT family, which, while usually described as high-affinity carriers, can nevertheless display some low-affinity character (Rubio *et al*., 1995). In addition, substantial evidence suggests that non-selective cation channels may provide a pathway for Na^+ entry (Demidchik *et al*., 2002; Essah *et al*., 2003; Kader and Lindberg, 2005; Munns and Tester, 2008) and, while the genes encoding this pathway are not known at present, proposed candidates include cyclic nucleotide-gated channels (CNGs). The efflux of Na^+ appears to be mediated by an Na^+/H^+ antiport mechanism (SOS1; Shi *et al*., 2002), with a probable $Na^+:H^+$ stoichiometry of 1:1 (Darley *et al*., 2000; Shi *et al*., 2002; Pardo *et al*., 2006; Yeo, 2007), and ultimately powered by proton-translocating ATPases (Britto and Kronzucker, 2006; Yeo, 2007). Together, unidirectional influx and efflux provide the two main components of the currently accepted model of Na^+ uptake in plants, which is that of a leak-pump system (Boyer, 1982; Cheeseman, 1982; Shi *et al*., 2000; Yeo, 2007; Munns and Tester, 2008), in which Na^+ passively enters the cell, down the ion's electrochemical potential gradient, and exits the cell via a secondarily active, proton-driven sodium pump (Fig. 1).

The energy required to drive ion transport across plant root membranes can comprise the majority of cellular energy budgets (Poorter *et al*., 1991; Scheurwater, 1999; Britto *et al*., 2001; Kronzucker *et al*., 2001; Kurimoto *et al*., 2004), but the energetics of Na^+ transport across the membrane have received limited attention. The objectives of the present study were (i) to measure the unidirectional Na^+ fluxes across the plasma membrane of roots of intact rice seedlings of two genotypes differing in salt tolerance; (ii) to evaluate the involvement of various transporter candidates to such fluxes *in planta*; and (iii) to assess the energetic consequences of these fluxes in relation to Na^+ toxicity. To accomplish this, we used three methods of $^{24}Na^+$ tracer analysis to determine unidirectional Na^+ influx and efflux across the plasma membrane of root cells, and translocation of Na^+ to the shoot. Tracer studies were complemented by a thermodynamic analysis based on current models and on measurements of root respiration and plasma membrane electrical potential differences.

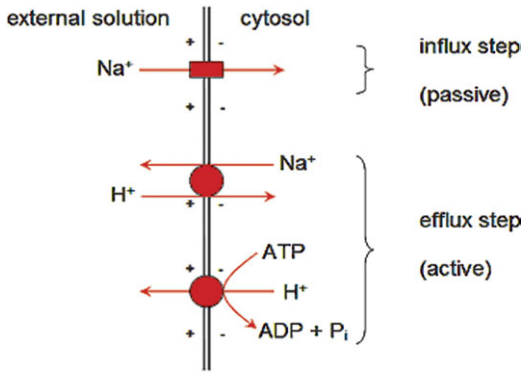

Fig. 1. Accepted 'pump-leak' mechanism of sodium transport across the plasma membrane in plant cells.

Materials and methods

Plant growth

Two cultivars of tropical lowland (Indica) rice (*O. sativa* L.), one salt-tolerant (cv. 'Pokkali') and one salt-sensitive (cv. 'IR29'), were grown hydroponically with aerated, modified quarter-strength Johnson's solution (including 1 mM $CaCl_2$), as described previously (Kronzucker *et al*., 1999), except that growth solutions were supplemented with either 1 mM or 25 mM NaCl. Plants were grown for 3 weeks in the same walk-in growth chamber in which experiments were conducted, with a 12 h light/12 h dark cycle, an irradiance of 300 μmol m^{-2} s^{-1} (provided by cool-white fluorescent tubes, Sylvania F96T12/CW/VHO), a relative humidity of 70%, and daytime/nighttime temperatures of 30 °C and 20 °C, respectively. During the growth period, solutions were regularly exchanged to prevent depletion of any nutrient by >20%. For silicate experiments, Na_4SiO_4 was added in sufficient quantity (3 mM) to give an effective concentration of ~0.1 mM silicate (Yeo *et al*., 1999). For sodium sulphate experiments, IR29 seedlings were grown for 3 weeks with 25 mM Na^+ applied as Na_2SO_4, instead of NaCl.

Flux analysis

Unidirectional fluxes of Na^+ were measured as described in detail previously (Siddiqi *et al*., 1991; Kronzucker *et al*., 1999; Britto *et al*., 2001, 2006). Briefly, roots of intact plants were first immersed for 1 h in a nutrient solution identical to growth solution, except that it contained $^{24}Na^+$ in addition to non-radioactive $^{23}Na^+$. Roots were desorbed of radioactivity in tracer-free solutions for the monitoring of $^{24}Na^+$ efflux and retention by gamma counting (Canberra-Packard Quantum Cobra Series II, Model 5003), using either of two methods: (i) periodic elution into a timed series of non-radioactive aliquots of nutrient solution (Fig. 2A, Method 1 in Table 1) (Siddiqi *et al*., 1991; Kronzucker *et al*., 1999; Britto *et al*., 2001); or (ii) periodic subsampling (every 30 s) of non-radioactive nutrient solution in which labelled roots were placed for 25 min (Fig. 2B, Method 2 in Table 1) (Britto *et al*., 2006). For periodic elutions, the time series was arranged as follows (also see Fig. 2A): 15 s (4×), 20 s (3×), 30 s (2×), 40 s (1×), 50 s (1×), 1 min (5×), 1.25 min (1×), 1.5 min (1×), 1.75 min (1×), and 2 min (8x) min (8×). The subsampling method was employed to minimize errors potentially resulting from physical disturbance of the root system, which may enhance efflux, in particular under high-salt conditions (for details, see Britto *et al*., 2006).

Unidirectional Na^+ fluxes were determined from these methods as follows:

(i) Efflux was calculated from the initial rate of $^{24}Na^+$ release from the cytosol, divided by the initial specific activity of $^{24}Na^+$ in

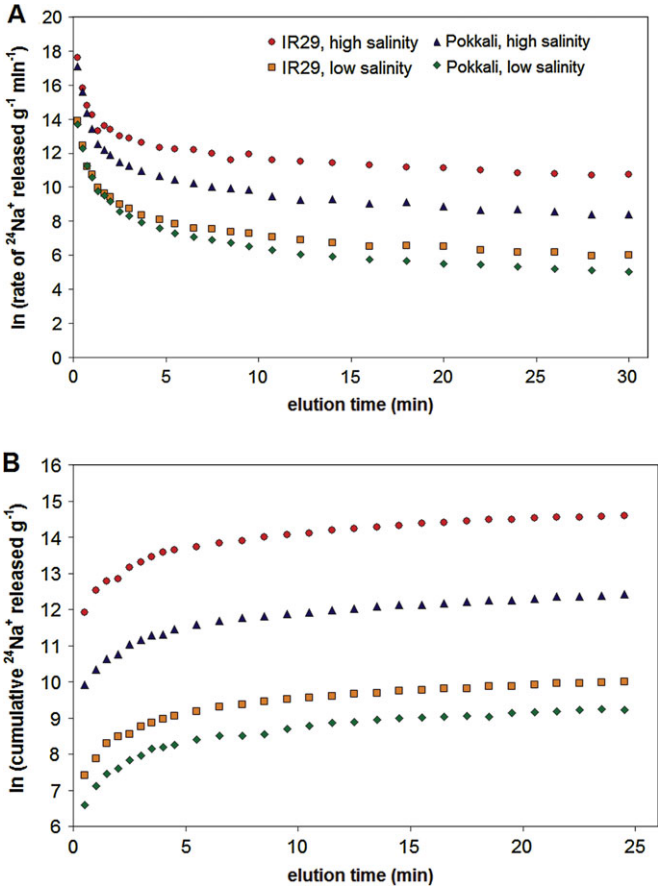

Fig. 2. Efflux of $^{24}Na^+$ from roots of intact seedlings of two cultivars of rice. (A and B) Representative plots of efflux measured using (A) a periodic elution protocol (Method 1) or (B) a subsampling procedure (Method 2).

this compartment, which is a function of the labelling time in the external medium of known specific activity (S_{ext}), and the kinetic constant k that describes the exponential rate of change of tracer efflux from the cytosol (Walker and Pitman, 1976). This constant was determined from the slope of the cytosolic line obtained by regression of data plots such as shown in Fig. 2A (for Method 1), and from the first derivative of curves such as shown in Fig. 2B (for Method 2).

(ii) Net Na^+ flux was determined from retention of tracer in root and shoot at the end of the desorption protocol, divided by S_{ext}, while influx was calculated from the sum of Na^+ efflux and the net Na^+ flux.

(iii) Translocation of sodium from root to shoot was estimated from S_{ext}, k, the duration of root labelling and elution, and shoot tracer retention, as described in Kronzucker *et al.* (2003).

Influx of Na^+ was also measured in short-term experiments, in which intact roots were labelled in solutions chemically identical to the growth solution, but containing $^{24}Na^+$, for 1, 2, 3, 4, 5, 10, 20, or 30 min, followed by a 5 s dip (to remove radioisotope adhering to the root surface), and then a 5 min desorption in non-radioactive growth solution (to remove radioisotope residual in the Donnan-free space component of the cell wall) prior to gamma counting, as described previously (Szczerba *et al.*, 2006). In addition, 5 min short-term labelling experiments were conducted in the presence of CsCl (5 mM), tetraethylammonium chloride (TEA, 20 mM), $BaCl_2$ (5 mM), variable K^+ (as K_2SO_4, at 0.3, 1.3, and 40 mM [K^+]), and variable Ca^{2+} concentrations (as $CaSO_4$, at 0.03, 1, and 3 mM). These agents were applied as 10 min pre-treatments and during subsequent 5 min labelling in radioactive solutions, but were absent from dip and desorption steps. Concentrations of inhibitors were chosen according to published protocols (White and Lemtiri-Chlieh, 1995; Demidchik and Tester, 2002; Wang *et al.*, 2006).

Energetics analysis

Root respiration was determined by immersing roots of intact plants in a cuvette containing nutrient solution, in which oxygen depletion was monitored by use of a Clark-type O_2 electrode system (Hansatech Instruments Ltd). Membrane electrical potentials were measured in epidermal and cortical cells in the midsection of the root (midway between the root cap and the root–shoot interface), using borosilicate glass microelectrodes filled with 3 M KCl and connected to an electrometer (World Precision Instruments, Duo 773). Both types of measurements were conducted as described previously (Britto *et al.*, 2001; Szczerba *et al.*, 2006). The theoretical respiratory cost of ion efflux was determined using the relationship $\phi_{O_2} = 0.2\,\phi_{co}$, where ϕ_{O_2} represents the oxygen consumption by roots, and ϕ_{co} represents efflux. This analysis is based on the electroneutral Na^+/H^+ exchange mechanism shown in Fig. 1, a stoichiometry of 1 H^+ pumped per ATP hydrolysed (Britto and Kronzucker 2006), and a phosphorylation efficiency (P/O_2 ratio) of 5 (Kurimoto *et al.*, 2004; Britto and Kronzucker, 2006).

Statistical analysis

All experiments (including flux experiments, respiration, and electrophysiology) were performed with 3–34 replicates. For respiration and electrophysiology, replicates consisted of a single plant; for flux measurements, replicates consisted of a bundle of five plants attached together at the shoot base by a collar made from plastic tubing. The resulting variation in the measurements was expressed as the mean ±SEM. Means were compared using analysis of variance (ANOVA, P <0.05).

Results and discussion

The direct measurement of low-affinity unidirectional Na^+ influx in intact plants is challenging both because of the high extent of efflux and because of the rapid exchange of the ion between root cells and the external medium (Cheeseman, 1982; Britto and Kronzucker, 2001; Essah *et al.*, 2003; Wang *et al.*, 2006). In the present study, unidirectional influx and efflux across the plasma membrane of roots of two rice cultivars, salt-tolerant Pokkali and salt-sensitive IR29, have been quantified using three methods of $^{24}Na^+$ tracer analysis, two of which employed compartmental analysis by tracer efflux (Siddiqi *et al.*, 1991; Britto *et al.*, 2006), and one of which directly measured apparent influx (the term 'apparent' refers to the fact that such measurements do not take into account the effect of simultaneous efflux; see Szczerba *et al.*, 2006). At the outset, it should be noted that the two cultivars, in addition to differing in salt tolerance, have different morphologies (IR29 is a dwarf variety, unlike Pokkali), and that caution should be applied when extrapolating these results to all genotypes of rice.

Table 1. *Fresh weights, membrane potentials, and Na^+ fluxes in roots of intact seedlings of salt-tolerant (Pokkali) and salt-sensitive (IR29) rice, under 1 mM and 25 mM external NaCl ($[Na^+]_{ext}$)*

Na^+ fluxes were measured using two methods of tracer analysis (see Materials and methods).

$[Na^+]_{ext}$ (mM)	Rice cultivar	Whole-plant FW (g)	Membrane electrical potential (mV)	Tracer method	Na^+ fluxes [μmol (g FW)$^{-1}$ h^{-1}]		
					Influx	Efflux	Root-to-shoot flux
1	Pokkali	0.44±0.01	–82.8±3.2	1	0.64±0.06	0.44±0.04	0.11±0.03
				2	1.26±0.30	1.05±0.30	0.07±0.01
	IR29	0.38±0.03	–78.7±2.6	1	1.49±0.17	1.26±0.15	0.16±0.02
				2	1.64±0.25	1.31±0.19	0.12±0.02
25	Pokkali	0.41±0.02	–89.9±11.2	1	19.5±1.8	14.8±1.9	2.93±0.21
				2	22.1±2.3	18.4±2.2	2.08±0.37
	IR29	0.13±0.01	–80.6±8.4	1	118±9	107±9	9.20±1.03
				2	153±23	131±23	9.10±2.77

The two types of efflux experiment showed that, in both cultivars, the influx, efflux, and shoot translocation of Na^+ were more than an order of magnitude greater under salt stress ($[Na^+]_{ext}$=25 mM) than at the low, non-toxic level of Na^+ supply (1 mM; Fig. 2A, B; Table 1). The high magnitudes of influx estimated by these analyses and the pronounced differences between cultivars were confirmed by the direct method (Figs 3, 4), which monitored $^{24}Na^+$ accumulation in tissue following short-term (1–30 min) exposure. The rapidly declining apparent influx over the time course in Fig. 3 is indicative of some free space carryover of tracer in the shortest (1 min) trials, and of simultaneous tracer efflux in the longer trials (see Szczerba *et al*., 2006). While Na^+ influx in salt-sensitive IR29 rose to extremely high values of 118–153 μmol g (root FW)$^{-1}$ h^{-1} at 25 mM $[Na^+]_{ext}$, even higher values have been reported previously in the literature: in the halophyte *Spergularia maritima* Na^+ efflux was as high as 600 μmol g (root FW)$^{-1}$ h^{-1} at 90 mM $[Na^+]_{ext}$ (influx was presumed to be slightly higher due to a small net flux component; Lazof and Cheeseman, 1986); in japonica rice, Na^+ influx reached ~240 μmol g (root FW)$^{-1}$ h^{-1} at 5 mM $[Na^+]_{ext}$ (Horie *et al*., 2007); and in *Arabidopsis thaliana*, Na^+ influx at 100 mM $[Na^+]_{ext}$ was as high as 180 μmol g (root FW)$^{-1}$ h^{-1}, when calcium supply was low (Essah *et al*., 2003).

Much debate currently surrounds the identity, at the molecular level, of the transporters mediating low-affinity, toxic Na^+ influx into plant roots. The chief candidates are K^+-specific channels (Kader and Lindberg, 2005; Wang *et al*., 2007), the low-affinity cation transporter LCT1 (Amtmann *et al*., 2001; Apse and Blumwald, 2007), and the HKT transporter family (Rubio *et al*., 1995; Golldack *et al*., 2002; Garciadeblás *et al*., 2003; Rodriguez-Navarro and Rubio, 2006; Horie *et al*., 2007; Huang *et al*., 2008). In addition, non-selective cation channels (NSCCs) have been strongly implicated in toxic sodium influx into plant roots (Demidchik and Tester, 2002; Essah *et al*., 2003; Kader and Lindberg, 2005; Munns and Tester, 2008), although no genes encoding these channels have yet been identified. To test whether any of these transporter types were involved in the influx of Na^+ into roots of the two rice cultivars, a range of chemical treatments expected to inhibit their activities was applied (Fig. 4). These treatments were as follows: (i) K^+ was applied externally from 0.3 mM to 40 mM, to test for competitive interactions that may be expected if Na^+ enters via pathways also available to K^+ [i.e. K^+-specific channels, NSCCs, LCT1; e.g. competitive inhibition of *Arabidopsis* K^+ channels by Na^+ has been shown by Quintero and Blatt (1997), K^+ channels in *Chara* are inhibited by Na^+ while not transporting it, as shown by Bertl (1989), and competition between K^+ and Na^+ has been observed in human K^+ channels by Korn and Ikeda (1995); see also Klieber and Gradmann (1993) for a more general treatment of this topic]; (ii) Ca^{2+} was applied externally from 0.03 mM to 3 mM, to test for the involvement of NSCCs, which are known to be inhibited by calcium (Amtmann, 2001; Demidchik and Tester, 2002; Kader and Lindberg, 2005, Apse and Blumwald, 2007; LCT1 is also inhibited by Ca^{2+}, but at higher concentrations; see Schachtman *et al*., 1997); and (iii) the well-known K^+

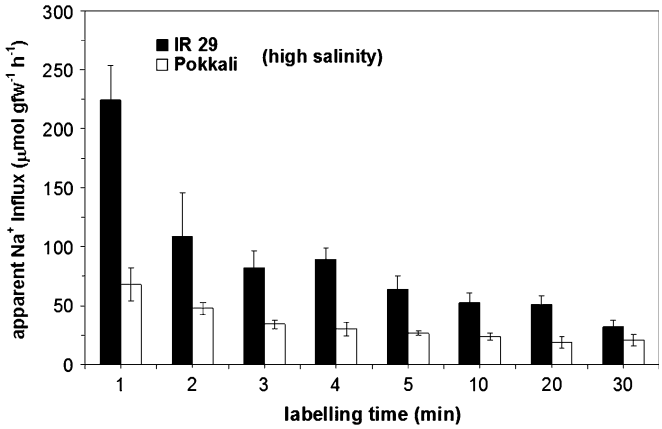

Fig. 3. Time course of apparent Na^+ influx into roots of intact seedlings of two cultivars of rice, at 25 mM $[Na^+]_{ext}$. Declining influx over time is an indication of the extent of simultaneously occurring efflux.

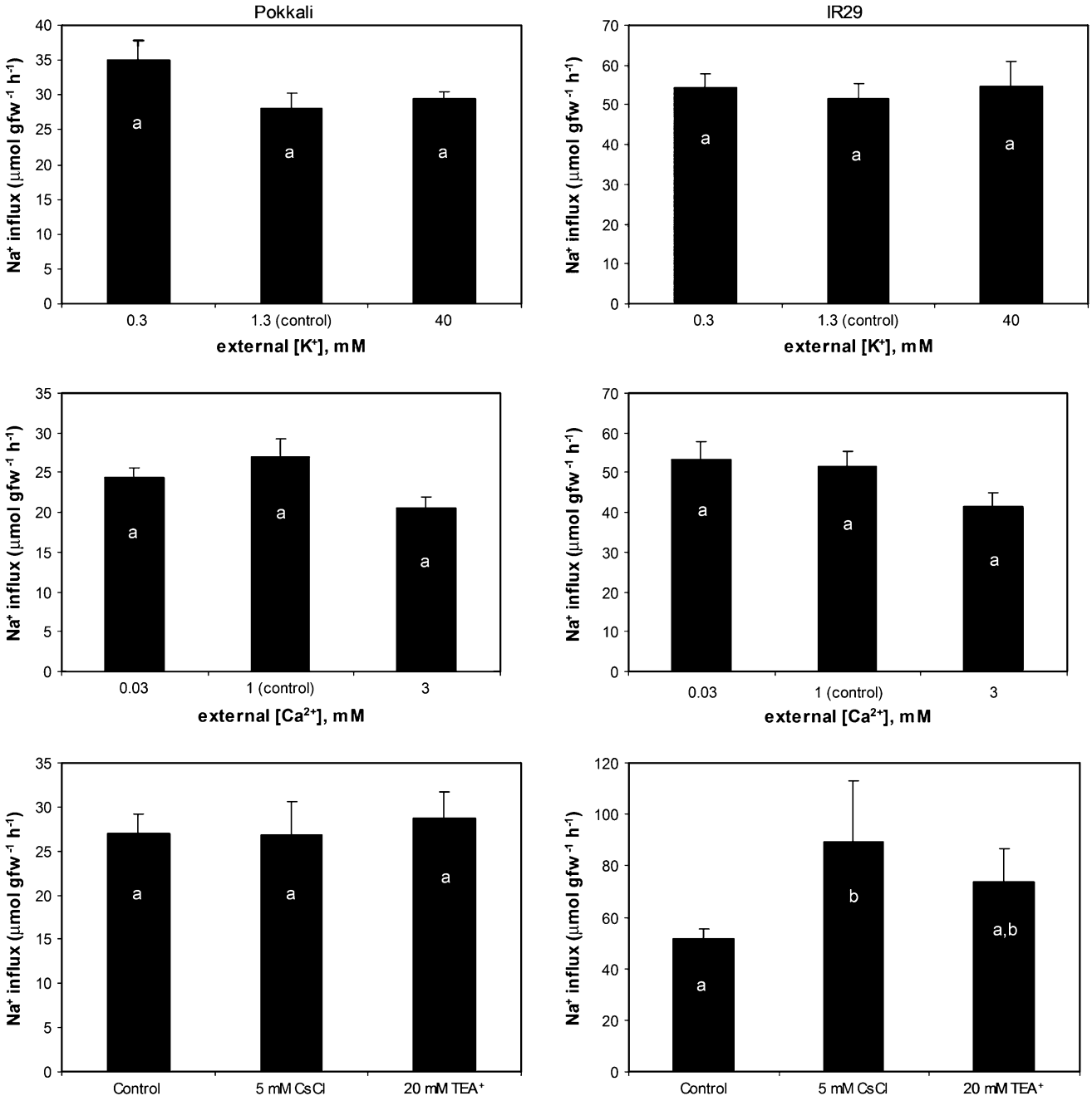

Fig. 4. Five minute 'direct' influx into roots of intact seedlings of two cultivars of rice (Pokkali, left side; IR29, right side) at 25 mM $[Na^+]_{ext}$, under variable ionic conditions and in the presence of established channel-modifying agents (see Materials and methods). Different letters (a, b) indicate significant differences between means within a given cultivar, and a given individual graph (P <0.05, n >3).

channel blockers Ba^{2+}, Cs^+, and TEA^+ were applied to test for the involvement of K^+ channels (Kader and Lindberg, 2005; Volkov and Amtmann, 2006).

Figure 4 shows the results of these influx experiments, which revealed similar patterns between rice cultivars. When K^+ was varied, during the uptake assay, over a range exceeding two orders of magnitude, no significant differences in influx were observed in either Pokkali or IR29. Similarly, a 100-fold gradient of Ca^{2+} supply also failed to produce significant differences in the flux in either cultivar. In addition, influx assays with Ba^{2+} (another well-known NSCC blocker; White and Lemtiri-Chlieh, 1995) did not alter Na^+ influx (data not shown). In Pokkali, Cs^+ and TEA^+ also had no influence on sodium influx, whereas it was significantly increased by Cs^+ in IR29, a striking effect previously seen in *A. thaliana* and *Thellungiella halophila* by Wang et al. (2006). This effect may be due to membrane hyperpolarization caused by the

blockage of inward-rectifying K^+ channels by Cs^+ (Ichida *et al*. 1999).

Taken together, these results indicate that neither K^+ channels, NSCCs, nor LCT1 are strong candidates for mediation of toxic Na^+ fluxes into intact rice plants of either cultivar. In addition, the participation of HKT transporters is unlikely here, since the Na^+-transporting properties of some members of this family are greatly suppressed in the presence of K^+ (Golldack *et al*., 2002; Garciadeblás *et al*., 2003; Rodriguez-Navarro and Rubio, 2006; Horie *et al*., 2007), whereas no significant differences in Na^+ influx were seen as a result of changes in K^+ supply. Wang *et al*. (2007) have also recently questioned the involvement of NSCCs in *in planta* sodium fluxes, while suggesting that K^+ channels may be involved at very high $[Na^+]_{ext}$ (150 mM) in *Suaeda maritima*. At that concentration, sodium fluxes were sensitive to TEA^+, Cs^+, and Ba^{2+}, and were also inhibited by K^+, although not necessarily in a competitive manner.

The conclusion that none of the major transport systems put forward in the literature participates in toxic sodium influx in the intact rice plant agrees with a previous conclusion drawn in barley (Kronzucker *et al*., 2008), but is at odds with a number of studies on Na^+ transport in rice. In particular, the conclusion by Kader and Lindberg (2005), based upon a study of protoplast preparations and the use of the Na^+-sensitive fluorescent dye SBFI, that Pokkali is more salt tolerant than other rice cultivars because Na^+ enters via non-selective cation channels rather than K^+ channels is not supported in the present *in planta* study. Similarly, very large sodium fluxes in the presence of a wide range of external K^+ supply questions the importance of K^+-sensitive HKT transporters that have been implicated in sodium stress and tolerance in rice (Golldack *et al*., 2002; Garciadeblás *et al*., 2003). The calcium dependence of sodium transport in rice protoplasts (Kader and Lindberg, 2005), suggesting the involvement of NSCCs, and the Ca^{2+} dependence of root-to-shoot translocation in intact rice plants (Anil *et al*., 2005) are also not supported by the present study, which agrees with the lack of Ca^+ influence on Na^+ transport in studies by Yeo and Flowers (1985) and Song and Fujiyama (1996). A pioneering study by Rains and Epstein (1967*b*) also showed that Ca^{2+} provision from 0 mM to 10 mM had almost no effect on Na^+ influx (measured at 50 mM external Na^+) in barley plants, although some inhibition was observed beyond the relatively high 10 mM supply.

Two other treatments that were of interest are shown in Fig. 5. First, changes in the anion accompanying Na^+ have been shown to cause changes in Na^+ influx in barley roots (Rains and Epstein, 1967*a*; see also Britto and Kronzucker, 2008 for this effect with K^+ fluxes), but no differences in Na^+ fluxes were found when the counterion for Na^+ was changed from Cl^- to SO_4^{2-}. Secondly, rice

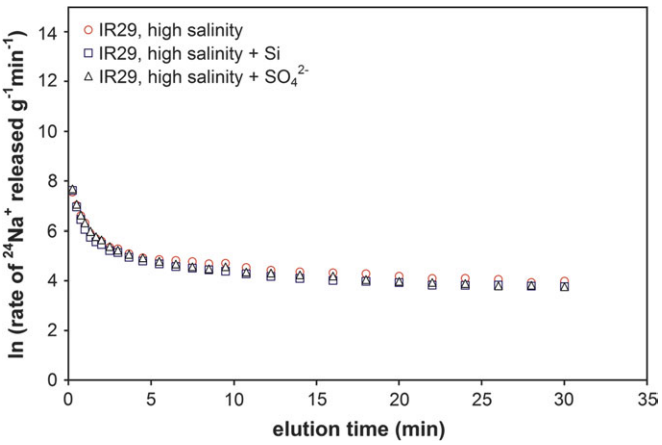

Fig. 5. $^{24}Na^+$ efflux from roots of intact seedlings of IR29 rice, grown at 25 mM $[Na^+]_{ext}$, as sodium sulphate (triangles), or as sodium chloride with (squares) or without (circles) added silicate.

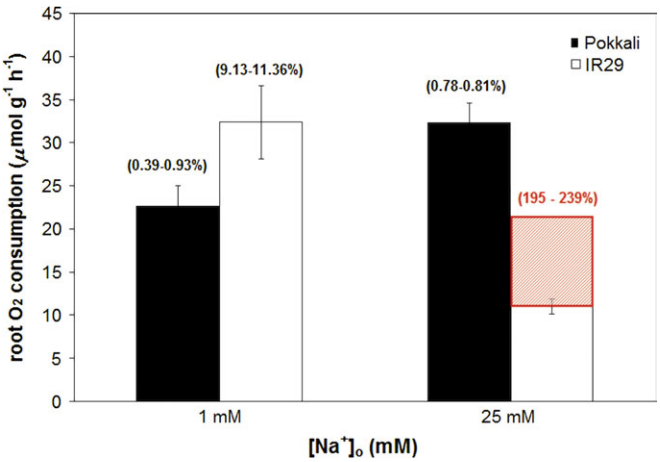

Fig. 6. Oxygen consumption by roots of two rice cultivars, under low and high sodium provision. Percentage figures above the bars indicate the proportion of the O_2 flux that would be required to power the unidirectional efflux of Na^+ from roots (as measured by Method 1 in Table 1), assuming that this flux is energetically active, and powered by an electroneutral H^+/Na^+ exchange (see Fig. 1). The hatched area above the 25 mM condition in IR29 indicates the amount by which the O_2 flux falls short of what would be required for active Na^+ efflux.

plants were grown with the addition of silicate to assess the involvement of an apoplastic bypass component of Na^+ influx, as characterized by the elegant work, in rice, of Flowers and co-workers (Yeo *et al*., 1999; Gong *et al*., 2006). In the present study, however, no significant reduction in toxic Na^+ fluxes was found when plants were grown on silicate, indicating that the silicate-dependent bypass flow does not contribute significantly to Na^+ transport measurements in the system used here. Indeed, silicate effects on bypass flow of Na^+ appear to be cultivar dependent in rice (Yeo *et al*., 1999).

Under all conditions examined, and in agreement with other studies (Cheeseman, 1982; Lazof and Cheeseman, 1986; Essah *et al*., 2003; Wang *et al*., 2006), the very

high rates of Na^+ influx in IR29 were accompanied by efflux of a nearly equal magnitude. This can be seen both in the results of the compartmental analysis (Table 1), and in the strong and rapid decline in apparent influx over a time course of labelling (Fig. 3). The high ratio of Na^+ efflux to its influx is indicative of a futile cycling of ions at the plasma membrane that has been observed with many other ions (Britto and Kronzucker, 2006). Interestingly, in the case of Na^+, futile cycling was also observed at the lower, non-toxic concentration of 1 mM.

The membrane electrical potentials measured here (Table 1) predict a cytosolic Na^+ activity of >500 mM in root cells, according to the Nernst equation, assuming a passive distribution of Na^+ across the plasma membrane at 25 mM $[Na^+]_{ext}$ (see Britto *et al*., 2001). Because such a high value is very unlikely in a glycophyte (Munns and Tester, 2008), it is reasonable to assume that an electrochemical equilibrium of Na^+ is not achieved, and that the leak-pump model of active Na^+ extrusion from root cells may therefore be operative in the toxic flux scenario with IR29 (as well as in all other scenarios reported here). The large efflux of sodium observed under these conditions, therefore, is expected to be linked to a substantial energy drain as Na^+ cycles across the membrane (see Kronzucker *et al*., 2001; Yeo, 2007). The current models of active sodium efflux (Fig. 1) and ion transport energetics (for details, see Poorter *et al*., 1991; Scheurwater, 1999; Kurimoto *et al*., 2004; Kronzucker *et al*., 2001; Britto and Kronzucker, 2006) allow one to calculate the minimal respiratory cost of this active Na^+ efflux (see Materials and methods). Three assumptions underlie this model: first, that Na^+ efflux is powered by an Na^+/H^+ antiport mechanism with a stoichiometry of 1:1 (Shi *et al*., 2002; Pardo *et al*., 2006; Yeo, 2007); secondly, that the hydrolysis of one ATP molecule by the plasma membrane H^+ ATPase leads to the active pumping of one proton across the membrane (Palmgren, 2001); and thirdly, that the respiratory phosphorylation efficiency (P/O_2 ratio) is ~5 (Poorter *et al*., 1991; Scheurwater, 1999; Kurimoto *et al*., 2004).

Applying this model to the four scenarios examined in the present study reveals that, in the three non-toxic conditions, the respiratory oxygen consumption rate required to drive the efflux estimated in Table 1 ranges from 0.088 to 3.68 μmol O_2 g (root FW)$^{-1}$ h^{-1}. When compared with measured respiratory rates in intact roots of these non-stressed plants, these predicted rates comprise 0.39–11.36% of total root respiration. In each case, total root respiration is well in excess of what would be required to sustain an active Na^+ efflux, consistent with the classic leak-pump model of Na^+ exchange across the plasma membrane. In contrast, in IR29 at 25 mM $[Na^+]_{ext}$, the toxic condition, the minimal oxygen consumption rate required to sustain an active efflux of Na^+ was found to be 21.4–26.3 μmol O_2 g (root FW)$^{-1}$ h^{-1}. When compared with measured root respiration rates of 10.7 μmol O_2 g (root FW)$^{-1}$ h^{-1} in these plants, it appears that a leak-pump mechanism cannot operate according to the above assumptions, under this special condition of sodium toxicity (Fig. 4). The discrepancy between the rate of O_2 consumption and Na^+ efflux may be even more pronounced, if the efficiency of mitochondrial coupling is reduced under salt stress, as has been demonstrated in wheat (Trono *et al*., 2004). One arrives at similar conclusions when examining the Na^+ fluxes reported in Lazof and Cheeseman (1986), Essah *et al*. (2003), and Horie *et al*. (2007), even though respiratory rates were not determined in those studies.

This mismatch between predicted and measured respiration rates in connection with high Na^+ is starkly remniscent of the early work of Ussing in amphibian systems (see Ussing, 1994); there, the energy predicted to drive active Na^+ efflux from frog sartorius muscle was also much greater than what was available to the organism, leading Ussing to conclude that a previously unknown, energy-coupling, transport mechanism was in operation. Similarly, the present observations suggest the operation of an Na^+ transport mechanism *in planta* that is different from currently held models, at least in the case of the IR29 cultivar of rice. Such a mechanism could take several possible forms: (i) a system in which active Na^+ efflux is either energized differently from what is suggested by current models, possibly via its coupling to passive fluxes of ions other than protons (or even to the passive influx of Na^+ itself); such coupled transporters are common in animal systems, and, interestingly, a sodium–potassium–chloride transporter has recently been discovered in *A. thaliana* (Colmenero-Flores *et al*., 2007); (ii) a vesicle-based system, as has been suggested by Amtmann and Gradmann (1994) for Na^+ fluxes in *Acetabularia*, and has recently been shown to operate in manganese transport in *Arabidopsis* and *Populus* (Peiter *et al*., 2007), as well as in chloride transport in the halophyte *Suaeda altissima* (Balnokin *et al*., 2007); (iii) a silicate-independent apoplastic contribution, that is not easily discernible from plasma membrane contributions. Further, the possible involvement of more Na^+-specific transport systems, as are very common in animal systems, should not be entirely discounted, as it may explain the observed recalcitrance of Na^+ influx to a wide array of externally applied agents in the present study. However, such transporters have not as yet emerged from genomic analyses, at least in *A. thaliana* (Hua *et al*., 2003).

It is clear that the fundamental question of the mediation of toxic Na^+ fluxes into plant roots remains unresolved, and that a significant gap remains between recent exciting discoveries of Na^+ transporters using molecular and electrophysiological techniques, and the involvement of these transporters in the intact plant under salt stress. It is hoped that a combination of experimental approaches, that will continue to complement more reductionistic methods

with physiological studies at the whole-organism level, will lead to the necessary breakthroughs in this area.

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