Planta (2019) 249:1037–1051
https://doi.org/10.1007/s00425-018-3059-7

ORIGINAL ARTICLE

Plasma-membrane electrical responses to salt and osmotic gradients contradict radiotracer kinetics, and reveal Na^+-transport dynamics in rice (*Oryza sativa* L.)

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Received: 9 August 2018 / Accepted: 23 November 2018 / Published online: 29 November 2018

Abstract

Main conclusion **A systematic analysis of NaCl-dependent, plasma-membrane depolarization ($\Delta\Delta\Psi$) in rice roots calls into question the current leading model of rapid membrane cycling of Na^+ under salt stress.**

To investigate the character and mechanisms of Na^+ influx into roots, Na^+-dependent changes in plasma-membrane electrical potentials ($\Delta\Delta\Psi$) were measured in root cells of intact rice (*Oryza sativa* L., cv. Pokkali) seedlings. As external sodium concentrations ($[Na^+]_{ext}$) were increased in a step gradient from 0 to 100 mM, membrane potentials depolarized in a saturable manner, fitting a Michaelis–Menten model and contradicting the linear (non-saturating) models developed from radiotracer studies. Clear differences in saturation patterns were found between plants grown under low- and high-nutrient (LN and HN) conditions, with LN plants showing greater depolarization and higher affinity for Na^+ (i.e., higher V_{max} and lower K_m) than HN plants. In addition, counterion effects on $\Delta\Delta\Psi$ were pronounced in LN plants (with $\Delta\Delta\Psi$ decreasing in the order: $Cl^- > SO_4^{2-} > HPO_4^{2-}$), but not seen in HN plants. When effects of osmotic strength, Cl^- influx, K^+ efflux, and H^+-ATPase activity on $\Delta\Delta\Psi$ were accounted for, resultant K_m and V_{max} values suggested that a single, dominant Na^+-transport mechanism was operating under each nutritional condition, with K_m values of 1.2 and 16 mM for LN and HN plants, respectively. Comparing saturating patterns of depolarization to linear patterns of $^{24}Na^+$ radiotracer influx leads to the conclusion that electrophysiological and tracer methods do not report the same phenomena and that the current model of rapid transmembrane sodium cycling may require revision.

Keywords Depolarization · Efflux · Influx · Ionic · Osmotic · Membrane potential · Radiotracer · Rapid transmembrane sodium cycling (RTSC) · Rice · Roots · Salinity stress · Sodium transport

Abbreviations

$\Delta\Delta\Psi$	Change in plasma-membrane electrical potential
HN	High nutrient
LN	Low nutrient
NMDG-Cl	*N*-Methyl-D-glucamine chloride
NSCC	Non-selective cation channel
RTSC	Rapid transmembrane sodium cycling

Introduction

Salinity and salt stress reduce global crop productivity over *c*. 25% of the earth's agricultural land, at a cost of as much as US$ 27 billion annually (Qadir et al. 2014; Munns and Gilliham 2015). While Na^+ transport mechanisms in plant roots are considered to be of fundamental importance to salt

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-018-3059-7) contains supplementary material, which is available to authorized users.

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stress and tolerance (Assaha et al. 2017), the "low-affinity" plasma-membrane Na^+ transport systems that operate under saline conditions (i.e., at high external Na^+ concentrations, $[Na^+]_{ext}$), in root epidermal and cortical cells, remain poorly understood, even after decades of intense study (Britto and Kronzucker 2009, 2015; Kronzucker and Britto 2011; Cheeseman 2013; Deinlein et al. 2014; Nieves-Cordones et al. 2016; Santa-María and Rubio 2018).

Nevertheless, it is widely assumed, on the basis of radiotracer studies, that Na^+ enters root cells at very high rates under saline conditions (Kronzucker and Britto 2011), and electrophysiological studies suggest that this entry is mediated by ion channels (e.g., K^+ channels or non-selective cation channels, NSCCs, which may include glutamate receptors, cyclic nucleotide-gated channels, and aquaporins; Plett and Møller 2010; Deinlein et al. 2014; Assaha et al. 2017; Byrt et al. 2017). Because values of unidirectional Na^+ influx into roots, as measured with radiotracers over short time scales ($\leq$ 5 min) and under saline conditions, typically exceed net whole-plant Na^+ accumulation rates by an order of magnitude or more, it is also generally accepted that the great majority of incoming Na^+ must be rapidly returned from root cells back to the external medium, at the expense of considerable cellular energy (Munns and Tester 2008; Britto and Kronzucker 2013; Nieves-Cordones et al. 2016). We have recently suggested that this energy-intensive influx–efflux cycle of Na^+ at the plasma membrane of root cells be referred to as "Rapid Transmembrane Sodium Cycling" (RTSC; Britto and Kronzucker 2015; Assaha et al. 2017; Flam-Shepherd et al. 2018; Santa-María and Rubio 2018).

There are good reasons to question the validity of the RTSC model, however. Most fundamentally, there is a lack of unequivocal evidence for the claim that very high rates of steady-state Na^+ influx and, especially, Na^+ efflux, take place across root plasma membranes (Kronzucker and Britto 2011; Britto and Kronzucker 2015; Coskun et al. 2016; Flam-Shepherd et al. 2018). Moreover, such high fluxes (were they to exist) would appear to require unrealistically large root energy expenditures, both for the restoration of the membrane electrical potential after its depolarization by Na^+ influx, and to drive the subsequent Na^+ efflux step (Britto and Kronzucker 2009, 2015). In addition, problematic is the persistently linear relationship that is typically observed between $[Na^+]_{ext}$ and reported values of Na^+ influx under saline conditions (Britto and Kronzucker 2006; Kronzucker and Britto 2011). Linearly rising Na^+ influx appears to have no defined upper limit (short of $[Na^+]_{ext}$ values high enough to destroy cells by osmotic forces alone; see Kochian and Lucas 1989), and it contrasts sharply with the saturating patterns that are seen both in ion fluxes at low substrate ("high-affinity") concentrations, and in ion-dependent, plasma-membrane depolarization measurements taken across a wide concentration range (Kochian and Lucas 1989; Davenport and Tester 2000; Kronzucker and Britto 2011; Coskun et al. 2016). Experiments with protoplasts addressing this question have also produced mixed results, with a fluorescent-dye study showing a linear transport response (Kader and Lindberg 2005), and an electrophysiological study showing a saturating one (Demidchik and Tester 2002). We note, however, that interpretation of transport phenomena in protoplasts can be problematic, because they are partially denatured systems, due to removal of the cell wall and wall-associated functions (Lew 2007). We have suggested that problems with the RTSC model might be better resolved if it were recognized that the fidelity of radiotracer-based unidirectional flux measurements can become greatly diminished when made at high substrate concentrations, such as those found under salinity, or over very short time scales (Britto and Kronzucker 2013). With similar considerations in mind for potassium fluxes, we recently showed that a breakdown in $^{42}K^+$-radiotracer fidelity occurs when K^+ transport is measured at high $[K^+]_{ext}$ (20–100 mM), and have suggested that this is due to large extracellular fluxes of tracer into, and out of, the root apoplast, but not across the plasma membrane; a similar scenario may be playing out in the case of Na^+ (Coskun et al. 2016).

Only a few alternatives to radiotracer methods are available for the precise monitoring of unidirectional ion fluxes into (or out of) plant roots (Britto and Kronzucker 2013). One involves the use of sharp micro-electrodes to measure the electrical depolarization across the plasma membranes of root cells that results from the transmembrane movement of ions such as Na^+ or K^+ (Jefferies 1973; Maathuis et al. 1996; Hirsch et al. 1998; Spalding et al. 1999; Mian et al. 2011; Kavitha et al. 2012; Coskun et al. 2016; Flam-Shepherd et al. 2018). In the case of Na^+, sharp electrodes have been used to study Na^+ fluxes in, among other systems, root cells of the halophyte *Triglochin maritima* L. (sea arrowgrass; Jefferies 1973), wild-type and *HvHKT2;1*-overexpressing barley (*Hordeum vulgare*; Mian et al. 2011), and IR20 and Pokkali cultivars of *indica* rice (*Oryza sativa* L; Kavitha et al. 2012; Flam-Shepherd et al. 2018). Here, we have used this method to examine Na^+-dependent plasma-membrane depolarizations in cells of intact rice roots, to compare them with radiotracer studies for evaluation of the RTSC model, and to better understand low-affinity Na^+ transport mechanisms operating under saline conditions. We have refined this electrophysiological approach by taking into account simultaneous Cl^- fluxes (when Na^+ is provided as NaCl) in the determination of Na^+-dependent membrane depolarization, as well as potential concomitant K^+ efflux (Shabala et al. 2006; Coskun et al. 2013a), enhanced H^+-ATPase activity, and shifts in external osmotic potential (Racusen et al. 1977; Shabala and Lew 2002), in the determination of Na^+-dependent membrane depolarization.

Materials and methods

Plant culture

Experiments were conducted in 3-week-old seedlings of rice [*O. sativa* L., cv. 'Pokkali', a salt-tolerant cultivar; International Rice Research Institute (IRRI)]. Seeds were surface-sterilized in 1% NaClO for 15 min, then rinsed in dH_2O every 0.5 h for 3 h, and germinated in aerated dH_2O for 48 h in a growth chamber equipped with fluorescent lights (450-µmol photons m^{-2} s^{-1}; Philips Silhouette High Output F54T5/850HO; Philips Electronics Ltd, Markham, ON, Canada; lights were on for 12 h day^{-1}) and maintained at 70% RH with a day/night temperature cycle of 30°C/20°C. Germinated seeds were transferred to aerated, hydroponic growth tanks (14 l), which initially contained only a modified Johnson's solution ("high-nutrient", HN, solution) composed of: 2-mM $MgSO_4$, 0.5-mM K_2SO_4, 0.5-mM $(NH_4)_2SO_4$, 0.3-mM KH_2PO_4, 0.3-mM $CaCl_2$, 0.1-mM Fe EDTA, 20-µM H_3BO_3, 9-µM $MnCl_2$, 1.5-µM $CuSO_4$, 1.5-µM $ZnSO_4$, and 0.5-µM Na_2MoO_4, with a pH of 6.30–6.50 (adjusted with 1 M KOH). 72 h prior to experiments, a subset of the plants was switched to a simple "low-nutrient" (LN) solution, containing only 0.2-mM $CaSO_4$, pH 6.1–6.15. To ensure steady-state growth conditions, solutions were replaced on days 9, 13, 16, 18, and 20, with experiments starting on day 21.

Electrophysiology

Plasma-membrane electrical potentials were measured using protocols similar to those described by Glass et al. (1992). Sharp-electrical recordings were conducted in a grounded Faraday cage that housed an inverted Leica DME microscope (Leica Microsystems Inc., Concord, Ontario, Canada), mounted with a narrow Plexiglas measuring chamber (volume = 125 ml), and an electrode micromanipulator (SD instruments; MX310R, Siskiyou Corporation, Grants Pass, Oregon, USA). Roots of 21–24-day-old intact rice seedlings were positioned over platinum pins in the measuring chamber, which was filled with growth solution (HN or LN). Single-barrel micro-electrodes were prepared from borosilicate glass capillary tubes using a vertical micropipette puller (Sutter Instruments Co., model P-30, Novato, CA), back-filled with 3 M KCl, and inserted into an electrode holder connected to an electrometer (World Precision Instruments Inc., Duo773) and an oscilloscope (Tektronix Inc., Tektronix TDS2002B, Beaverton, OR, USA). Micro-electrodes were used to impale cortical and epidermal root cells, 1–2 cm from the root tip, and voltage readings were taken manually from the oscilloscope, with the electrometer providing confirmation for these values. Initial impalement resulted in membrane electrical potential differences ($\Delta\Psi$) that fell into two distinct populations (see Maathuis and Sanders 1993; Nieves-Cordones et al. 2008); the less negative $\Delta\Psi$ values agreed with literature values reported across the tonoplast, while the more negative values agreed with those across the plasma membrane (Walker et al. 1996; Carden et al. 2003; Nieves-Cordones et al. 2008). Only impalements that yielded steady initial $\Delta\Psi$ values, consistent with measurements across the plasma membrane, were used. In addition, we ensured that tip resistances remained within a reasonable range (15–25 MΩ) and that the impalements were without leakage.

The chamber was connected to a perfusion system with an inflow and outflow rates set to 7.5 ml min^{-1}. Once an initial stable membrane potential was achieved, treatment solutions were perfused into the chamber, delivering a range of compounds (NaCl, Na_2SO_4, and Na_2HPO_4, as well as sorbitol and *N*-methyl-D-glucamine ($NMDG^+$, chloride salt), two osmolytes with low membrane permeation rates; Kaiser et al. 1981; Shabala and Lew 2002; Essah et al. 2003), at concentrations ranging from 0.5 to 200 mM. Concentrations were increased in a stepwise fashion, with each resulting change in membrane potential difference (i.e., depolarization, $\Delta\Delta\Psi$) recorded 1–2 min following impalement; this brief period was sufficient to allow for stabilization of the new potential (for examples, see Suppl. Figs. S1 and S2). Subtraction procedures in Figs. 5, 6, and 7 were carried out using $\Delta\Delta\Psi$ values generated by osmolytes at concentrations iso-osmolar to NaCl; osmolarities were confirmed using a vapor-pressure osmometer (VAPRO 5520, Wescor Inc). In the case of sorbitol-dependent depolarization, subtraction was not made at the lowest concentration of 2 mM, as this value of $\Delta\Delta\Psi$ was likely due less to osmotic effects than to high-affinity H^+/sorbitol symport (with $K_m \approx 1$ mM; Marquat et al. 1997; Gao 2003; Watari et al. 2004). For experiments involving the metabolic inhibitor diethylstilbestrol (DES), 50-µM DES was washed in after the initial $\Delta\psi$ was measured, and once a new baseline membrane potential was recorded, a NaCl treatment solution (also containing DES) was introduced as mentioned above. $\Delta\Delta\psi$ was calculated relative to the baseline membrane potential found after DES addition, and not to the initial resting membrane potential.

$^{24}Na^+$ influx

For $^{24}Na^+$ radiotracer-influx experiments, each individual replicate consisted of three 21-day-old intact plants bundled together at the mesocotyl. Roots of these bundles were immersed for 2 min in a radioactive load solution, identical to growth solution except that it also contained $^{24}Na^+$

(half-life = 14.96 h), received as $^{23/24}$NaCl from the McMaster University Nuclear Reactor (Hamilton, ON, Canada). Load solutions contained different concentrations (1, 10, 50, and 100 mM) of Na^+, which allowed us to generate influx isotherms (e.g., Fig. 2). Labelled plants were transferred to non-radioactive growth solutions otherwise chemically identical to the load, first for a 2-min desorption, then a 3-min desorption in a second solution. All solutions were aerated. Immediately after desorption, shoots and basal shoots were separated from the roots, and roots spun in a low-speed centrifuge for 30 s to remove surface-adhering solution before weighing. Gamma-radioactivity was counted in root, basal shoot, and shoot tissue, using a γ-counter that automatically corrected for isotopic decay (Packard Instrument Quantum Cobra Series II, model 5003).

$^{42}K^+$ efflux

Roots of replicate bundles, each consisting of six 21-day-old intact plants, were immersed for 1 h in an aerated radioactive load solution identical to growth solution, except that it also contained $^{42}K^+$ (half-life = 12.36 h; provided as K_2CO_3 by McMaster University Nuclear Reactor). For plants grown under LN conditions, the load solution contained 0.1-mM $^{39/42}K^+$. After loading, the seedlings were transferred to glass efflux funnels, and their roots were eluted of radioactivity into a series of glass vials containing 13-ml aliquots of non-radioactive growth solution (Coskun et al. 2014). Eluates were collected at 1-min intervals for the first 10 min, and then at 2-min intervals for the next 44 min, for a total of 54 min of elution, or (in the case of 100-mM NaCl runs) for the next 20 min, for a total of 30 min of elution. For $^{42}K^+$ efflux runs that included NaCl step-ups, NaCl was introduced first with 1-mM NaCl at 16 min, followed by 10-mM NaCl at 26 min, 50 mM at 36 min, and 100 mM at 46 min. For $^{42}K^+$ efflux runs involving only 100-mM NaCl, the salt was introduced at 16 min. Radioactivity from the elution vials was measured and corrected for decay using a γ-counter (Packard Instrument Co., Meriden, CT, USA).

Statistics

Each data point represents average influx, efflux, or $\Delta\Delta\psi$, and statistics are shown as mean ±SE. Propagation of error from data sets that were subtracted from each other (e.g., depolarization due to NaCl, and that due to sorbitol) was determined using the square root of the sum of the squares of errors from each data set. Graphs and nonlinear (Michaelis–Menten) regressions were generated using GraphPad Prism (v. 5.01).

Results

$[Na^+]_{ext}$-dependent depolarization and $^{24}Na^+$ influx under HN and LN conditions

Plants grown under the complete, "high-nutrient" (HN) regime, or switched from HN to a simple $CaSO_4$ ("low-nutrient", or LN) solution, 3 days before experimentation, had resting root cell plasma-membrane potentials ($\Delta\Psi$) of − 101.18 (± 3.06) and − 143.22 (± 8.32) mV, respectively. The introduction of Na^+, at concentrations that were increased incrementally between 1 and 100 mM, resulted in net plasma-membrane depolarizations ($\Delta\Delta\Psi$) in roots of both LN and HN plants (Fig. 1). The depolarizations were short-lived, with new stable potentials reached within 1–2 min following a given change in $[Na^+]_{ext}$ (Suppl. Fig. S1). As $[Na^+]_{ext}$ was increased, $\Delta\Delta\Psi$ asymptotically approached an upper limit in both HN and LN plants; i.e., it exhibited saturating patterns, which conformed well to the Michaelis–Menten model of enzyme kinetics (R^2 = 0.995 and 0.983 for HN and LN plants, respectively; Fig. 1). Depolarization was much greater in LN plants, particularly at the lower $[Na^+]_{ext}$ values of 1 and 10 mM, where $\Delta\Delta\Psi$ was, respectively, about 5 and 4 times higher than in HN plants. This distinction was reflected in a first approximation of Michaelis–Menten kinetic parameters for depolarization, found using nonlinear regression (Table 1): LN conditions substantially increased V_{max}, and lowered K_m, of $[Na^+]_{ext}$-dependent membrane depolarization. Preliminary results in another cultivar of rice, the salt-sensitive IR29, showed very similar trends in terms

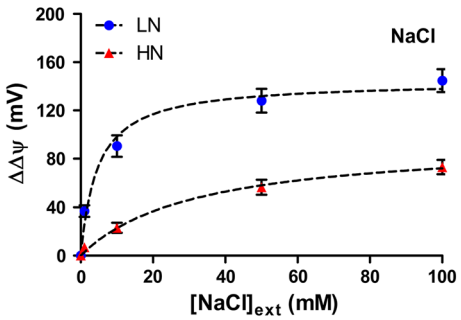

Fig. 1 Sodium-dependent membrane depolarization ($\Delta\Delta\Psi$) in root cortical and epidermal cells of intact rice seedlings, cv. Pokkali. Plants were grown (with only trace Na^+) under high-nutrient (HN) growth solution or switched to low-nutrient (LN) solution for 72 h, then subjected to stepwise increases in external concentration of NaCl; error bars generated via error propagation. Michaelis–Menten regression analysis yielded K_m values of 32.1 and 4.7 mM in HN and LN plants, respectively. V_{max} values were 95.6 and 144.2 mV in HN and LN plants, respectively. Error bars represent ±SE (LN NaCl: n = 8–10; HN NaCl: n = 12–16)

Table 1 Estimates of K_m values for Na^+ uptake, as obtained using a variety of plant systems and techniques

Species	K_m (mM)		Comments	References
Oryza sativa (cv. Pokkali)	HN	LN		Present study
	32.1	4.7	Before subtraction	
	22.1	1.4	After sorbitol subtraction	
	16.1	1.2	After NMDG-Cl subtraction	
Oryza sativa (cv. Nipponbare; TosWT2; 1-1)	0.66		Short-term $^{22}Na^+$ influx analysis (LN conditions)	Horie et al. (2007)
Oryza sativa (cv. Nipponbare; TosWT2; 1-2)	0.48			
Oryza sativa (cv. Nipponbare; *oshkt2; 1-1*)	15.7			
Oryza sativa (cv. Nipponbare; *oshkt2; 1-2*)	11.5			
Oryza sativa (cv. Nipponbare),	0.15		HKT2;1 expressed in *Xenopus* oocytes	Jabnoune et al. (2009)
Dionaea muscipula (Venus flytrap)	21		Non-stimulated gland cells	Böhm et al. (2016)
	7		Prey-stimulated gland cells	
Hordeum vulgare (cv. Golden Promise)	2.2		Trans-root potential (LN conditions)	Mian et al. (2011)
Triticum aestivum (cv. Hunter)	1.2		Planar lipid bilayer technique	Davenport and Tester (2000)

In the present study, V_{max} values were (before subtraction, after sorbitol subtraction, and after NMDG-Cl subtraction, respectively) 95.6, 44.8, and 48.6 mV in HN plants, and 144.2, 87.8, and 55.2 mV in LN plants

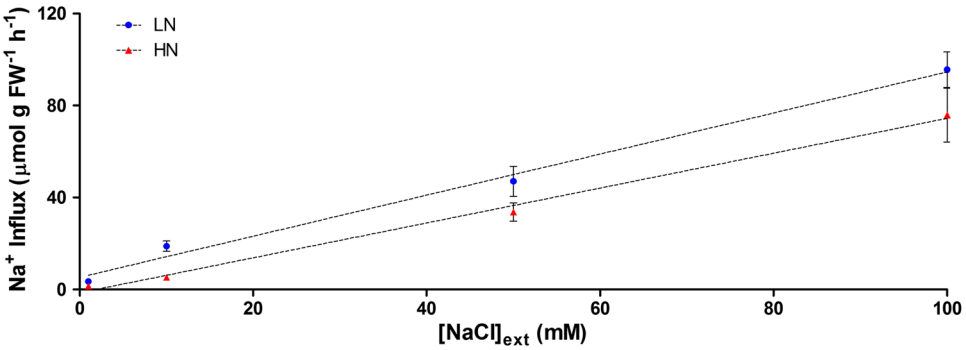

Fig. 2 Unidirectional influx of Na^+ in roots of intact rice seedlings, cv. Pokkali, as measured using $^{24}Na^+$ radiotracer. Plants were grown, as shown in Fig. 1, then roots were exposed to various concentrations of labelled NaCl for 2 min, followed by two non-radioactive washes of 2 and 3 min. Error bars represent ±SE (LN: $n = 6$; HN: $n = 4$–8)

of depolarization under LN and HN conditions (Suppl. Fig. S2).

Unidirectional $[Na^+]_{ext}$-dependent Na^+ influx into the roots was also measured in both HN and LN plants, using the radiotracer $^{24}Na^+$ (Fig. 2). In contrast to the saturating patterns seen with depolarization, a linearly proportional increase of Na^+ influx with increasing $[Na^+]_{ext}$ was found in both cases ($R^2 > 0.99$). However, as with depolarization, a greater flux was observed in LN plants throughout the concentration range.

H^+-ATPase activity and K^+ efflux

The role of the H^+-ATPase in ΔΔΨ patterns was assessed electrophysiologically by washing the ATPase inhibitor DES into the measuring chamber, prior to the introduction of the NaCl gradient. We found that, in both HN and LN plants, DES made the resting potential less negative prior to NaCl addition, and also reduced ΔΔΨ, but did not prevent ΔΔΨ from saturating in response to NaCl additions (Fig. 3a, b).

K^+ efflux was measured by monitoring the release rates of $^{42}K^+$ radiotracer from roots pre-loaded with $^{42}K^+$, while gradually stepping up $[Na^+]_{ext}$ from 1 to 100 mM (as in electrophysiology experiments), and also by suddenly introducing 100-mM NaCl (Britto et al. 2010; Coskun et al. 2013a). Between 1- and 50-mM $[Na^+]_{ext}$, a concentration range over which saturation of ΔΔψ was already pronounced, we found no indication of Na^+-stimulated $^{42}K^+$ efflux in either HN or LN plants (Fig. 4a, b). On the other hand, we did find a small degree of $^{42}K^+$ efflux stimulation at 100-mM NaCl (applied either suddenly or as a step-up) under both nutritional conditions, but most notably in LN plants (Fig. 4a, b).

Influence of osmotic strength and Cl^- transport on ΔΔψ

To gauge the contribution of changes in osmotic strength to changes in membrane potential in our system, we measured the electrical response of root cells to concentrations of sorbitol that were iso-osmotic to the Na^+ and Cl^- concentrations used in the NaCl experiments of Fig. 1. Sorbitol elicited

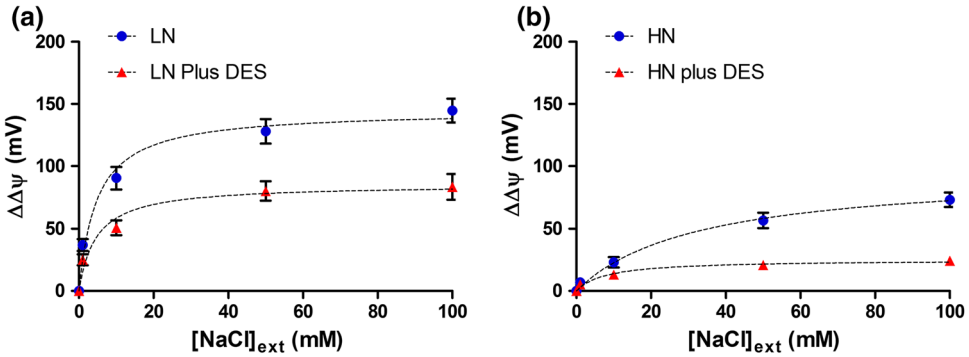

Fig. 3 Sodium-dependent membrane depolarization (ΔΔψ) in root cortical and epidermal cells of intact rice seedings, cv. Pokkali, with and without the addition of 50-µM DES. Plants were grown, as shown in Fig. 1 [LN conditions (**a**) and HN conditions (**b**)], then subjected to the metabolic inhibitor DES or no DES followed by a stepwise increases in external concentration of NaCl. Error bars represent ±SE (LN NaCl: $n = 8–10$; HN NaCl: $n = 12–16$; LN NaCl plus DES: $n = 4$; HN NaCl plus DES: $n = 4$)

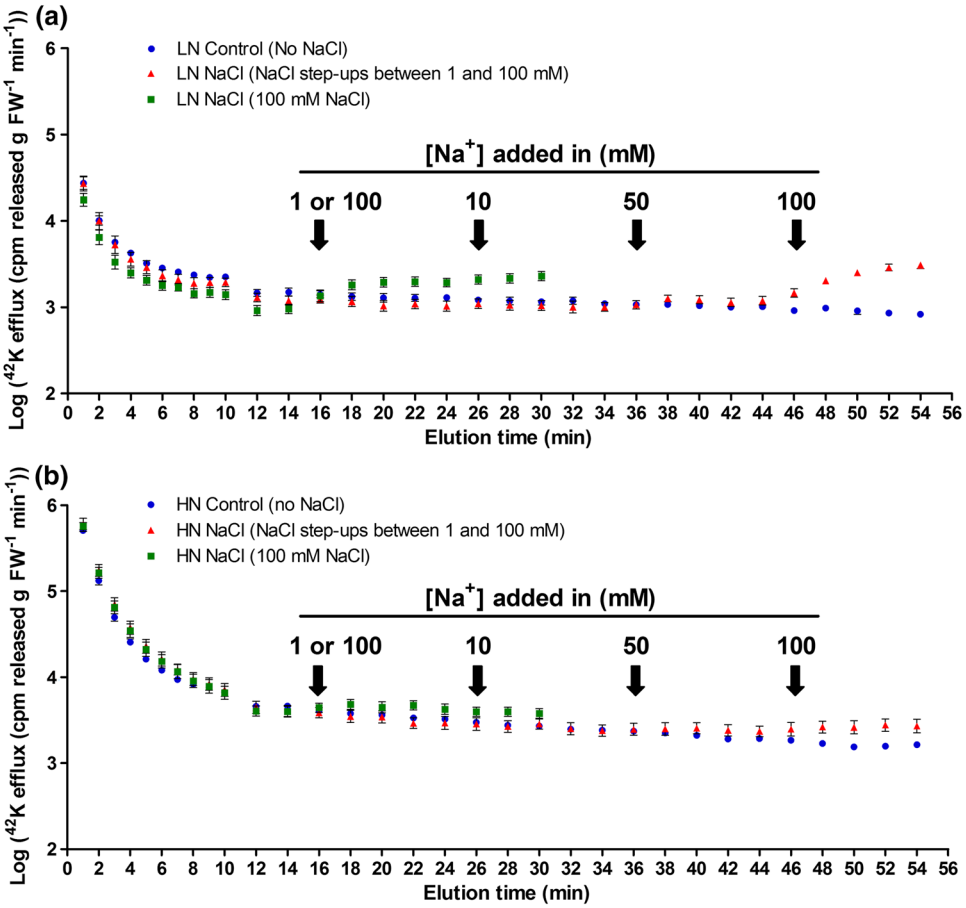

Fig. 4 Sodium-stimulated $^{42}K^+$ efflux from roots of intact rice seedlings. Plants were grown, as shown in Fig. 1, then labelled with 100 µM (LN) or 1.3-mM K^+ (HN), spiked with $^{42}K^+$. Radioactivity was eluted from roots using growth solution with non-radioactive K^+ for the first 14 min, followed by the same solution supplemented with various concentrations of Na^+, as indicated. Error bars represent ±SE (LN control: $n = 7$; LN NaCl step-ups: $n = 6$; LN 100 mM NaCl: $n = 6$; HN control: $n = 4$; HN NaCl step-ups: $n = 6$; HN 100 mM NaCl: $n = 6$)

substantial, concentration-dependent depolarization in roots of HN and LN plants, although the extent was not markedly different between the two growth conditions (Fig. 5a).

To assess the influence of Cl^- transport on ΔΔψ, we measured ΔΔψ in solutions containing varying concentrations of the chloride salt of the membrane-impermeable cation $NMDG^+$ (Fig. 6a). We found that, unlike with sorbitol, NMDG-Cl-dependent membrane depolarization was much more pronounced in LN plants.

Figures 5b and 6b show the arithmetic differences resulting from the subtraction of the data in Figs. 5a and 6a, respectively, from the data in Fig. 1 (see "Materials and methods" and "Discussion").

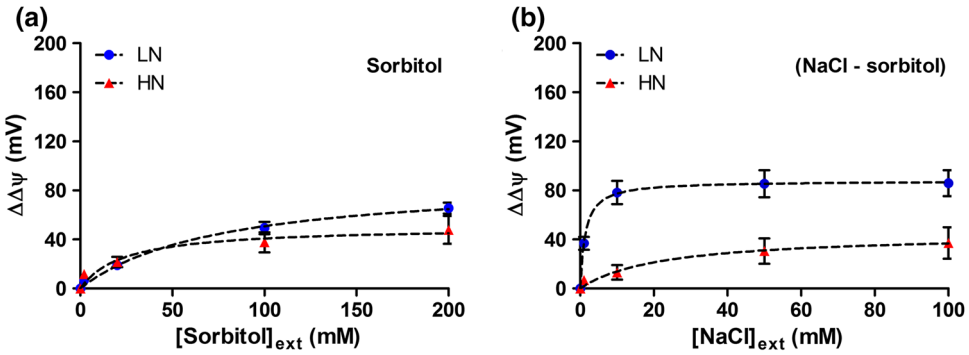

Fig. 5 Sorbitol-dependent membrane depolarization (ΔΔψ) in root cortical and epidermal cells of intact rice seedlings, cv. Pokkali. Plants were grown, as shown in Fig. 1, then subjected to stepwise increases in external concentration of sorbitol (**a**). **b** The difference between Fig. 1 and b in Fig. 2; error bars generated via error propagation. Michaelis–Menten regression analysis yielded K_m values in **c** of 22.1 and 1.4 mM in HN and LN plants, respectively. V_{max} values were 44.8 and 87.8 mV in HN and LN plants, respectively. Error bars represent ± SE (LN NaCl–NMDG-Cl: n = 8–10; HN NaCl–NMDG-Cl: n = 12–16; LN sorbitol: n = 9–13; HN sorbitol: n = 6–14)

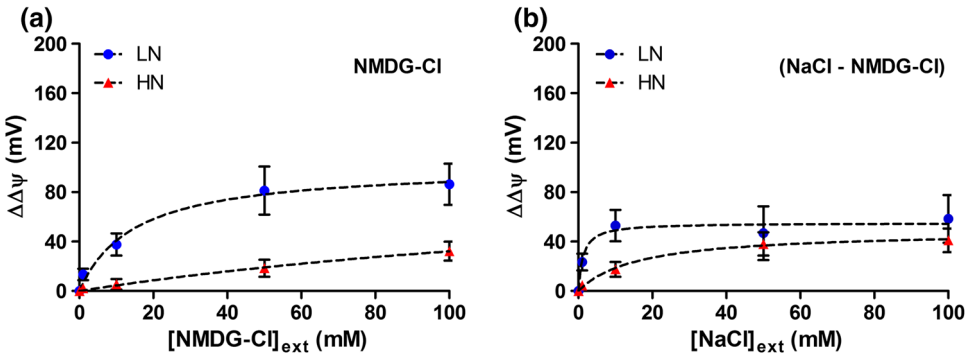

Fig. 6 NMDG-Cl-dependent membrane depolarization (ΔΔψ) in root cortical and epidermal cells of intact rice seedlings, cv. Pokkali. Plants were grown, as shown in Fig. 1, then subjected to stepwise increases in external concentration of NMDG-Cl (**a**). **b** The difference between Fig. 1 and b of Fig. 2; error bars generated via error propagation. Michaelis–Menten regression analysis yielded K_m values in **c** of 16.1 and 1.2 mM in HN and LN plants, respectively. V_{max} values were 48.6 and 55.2 mV in HN and LN plants, respectively. Error bars represent ±SE (LN NaCl–NMDG-Cl: n = 8–10; HN NaCl–NMDG-Cl: n = 12–16; LN NMDG-Cl: n = 4–7; HN NMDG-Cl: n = 4)

Counterion effects on ΔΔΨ

Another set of experiments was conducted, to explore the influence of anions on Na^+-dependent plasma-membrane depolarization, using sulfate (SO_4^{2-}) and hydrogen phosphate (HPO_4^{2-}) as counterions to replace Cl^-. We found that, under HN conditions, all sodium salts depolarized root cells to about the same extent (Fig. 7a). By contrast, LN conditions brought out differences among them, particularly after subtraction of corresponding osmotic components (Fig. 7b). While all salts showed an increased ΔΔΨ in LN plants, they did so in the distinct order NaCl > Na_2SO_4 > Na_2HPO_4 (when compared on a Na^+-equivalent basis; Fig. 7b).

Discussion

Data interpretation and system tests

We interpret the depolarizing effect of $[Na^+]_{ext}$ on the plasma membrane of root cells (Fig. 1; Suppl. Figs. S1, S2) as being indicative of, and proportional to, the net movement of positive charge into the cell, in accordance with many precedents in the literature, for Na^+ and other ions (Glass et al. 1992; Wang et al. 1994; Maathuis et al. 1996; Walker et al. 1996; Jabnoune et al. 2009; Mian et al. 2011; Kavitha et al. 2012; Böhm et al. 2016; Coskun et al. 2016). As $[Na^+]_{ext}$ was increased, diminishing increments of depolarization yielded the saturating curves, as shown in Fig. 1. Saturating patterns

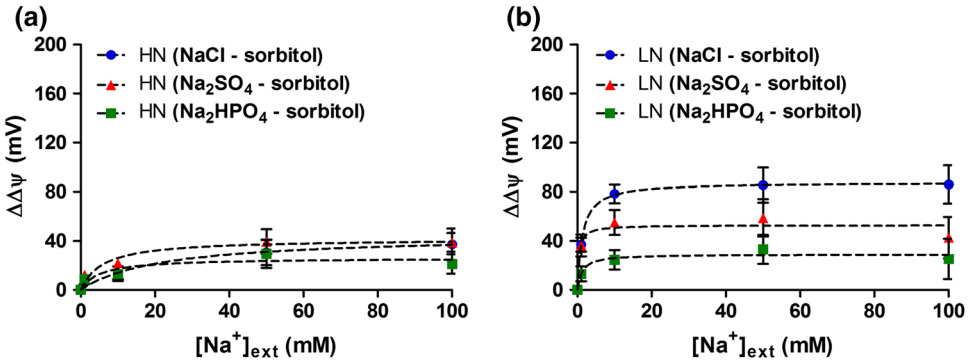

Fig. 7 Counterion effects on membrane depolarization ($\Delta\Delta\psi$) in root cortical and epidermal cells of intact rice seedlings, cv. Pokkali. Plants were grown, as shown in Fig. 1, then subjected to stepwise increases in external concentration of either NaCl, Na_2SO_4, or Na_2HPO_4. Osmotic effects (i.e., $\Delta\Delta\Psi$ due to sorbitol; see Fig. 1) were subtracted from these plots. Error bars represent ±SE (LN NaCl: n = 8–10; HN NaCl: n = 12–16; LN Na_2SO_4: n = 4; HN Na_2SO_4: n = 4; LN Na_2HPO_4: n = 4; HN Na_2HPO_4: n = 3–5)

are typically found using this electrophysiological approach, even if Michaelis–Menten parameters are not explicitly calculated (e.g, Kavitha et al. 2012; Flam-Shepherd et al. 2018). Here, we suggest that the depolarization plateau is observed, because Na^+ influxes across the root plasma membrane also reach an upper limit over this concentration range. However, this suggestion runs counter to the linearly rising low-affinity Na^+ influx isotherms that derive from radiotracer studies (Davenport 1998; Davenport and Tester 2000; Kronzucker and Britto 2011; Essah et al. 2003; Coskun et al. 2016), and lie at the foundation of the RTSC model (see above). We highlighted this apparent contradiction here, by reconfirming the linearity of radiotracer isotherms, using the $^{24}Na^+$ isotope in LN and HN plants (Fig. 2). In a previous study, a similar disparity was noted, though not explained, between linear $^{22}Na^+$ radiotracer uptake by wheat roots, and saturating electrophysiological traces in membrane lipid bilayers derived from these roots (Davenport and Tester 2000).

Because the tracer-based concept of a linear, low-affinity Na^+ influx system has become a widely assumed tenet of plant–sodium relations, it was important to test our interpretation of the apparently contradictory depolarization data. Therefore, we considered factors that could alternatively explain saturating depolarization, other than the saturation of Na^+ transport itself. In particular, we hypothesized that hyperpolarizing ion fluxes across the plasma membrane could balance depolarization by Na^+, and result in the apparent saturation of $\Delta\Delta\psi$, if they were to be stimulated under saline, or hyperosmotic, conditions, or in response to depolarization. Two major candidates for such fluxes, which might mask a putatively linear Na^+ influx into the cell, were ATPase-driven proton (H^+) efflux (Kochian et al. 1989; McClure et al. 1990; Maathuis and Sanders 1993; Shabala and Newman 1998; Shabala and Mackay 2011; Bose et al. 2015) and channel-mediated K^+ efflux (Nassery 1979; Kochian et al. 1989; McClure et al. 1990; Maathuis and Sanders 1993; Shabala and Newman 1998; Shabala et al. 2006; Britto et al. 2010; Shabala and Mackay 2011; Coskun et al. 2013a; Bose et al. 2015).

The possibility of H^+-ATPase involvement was tested by applying the ATPase inhibitor diethylstilbestrol (DES) to roots, prior to imposing the sodium gradient (Fig. 3). Although DES substantially depolarized the resting membrane potential prior to Na^+ addition, and decreased subsequent Na^+-dependent depolarizations, $\Delta\Delta\psi$ still conformed strongly ($R^2 = 0.86$) to a saturating, Michaelis–Menten model in both LN and HN roots. This finding suggests that saturation should not be attributed to increasing H^+-ATPase activity at the plasma membrane, at least over the time scale of our experiments (see also Roy et al. 2005, who showed that steady-state H^+ ATPase activity in Pokkali rice was not increased, but greatly reduced by salinity).

To test the involvement of K^+ efflux, we measured release rates of radioactive potassium ($^{42}K^+$) from $^{42}K^+$-loaded roots of plants exposed to the $[Na^+]_{ext}$ step gradient (Fig. 4). We found no stimulation of $^{42}K^+$ efflux at sodium concentrations below 100 mM, which was consistent with previously published work conducted in IR72, another cultivar of rice (Suppl. Fig. S1 in Coskun et al. 2013a). In that study, Na^+-stimulated K^+ efflux only became prominent at 160-mM NaCl, where it was nevertheless much smaller in Pokkali rice than in the cultivars IR72 or IR29. On the other hand, in the present study, we did find a small degree of Na^+-stimulated $^{42}K^+$ efflux at 100-mM NaCl (applied either in a step gradient, or as a single dose) under both nutritional conditions, but most notably in LN plants (Fig. 4a, b). Importantly, however, saturation of $\Delta\Delta\psi$ was already pronounced at $[Na^+]_{ext}$ values well below this threshold concentration, particularly in LN plants (Fig. 1). Indeed, in LN plants, K_m for $\Delta\Delta\psi$ was found to be within the first 5% of the 1–100-mM NaCl concentration range tested (Table 1; see below). It should also be noted that, while we examined

unidirectional K^+ efflux in the experiments of Fig. 4, it is the net flux of K^+ that will determine changes in $\Delta\Psi$ due to movement of this ion; however, the net flux of K^+ is by definition less than its unidirectional efflux (hence even less significant in the present case).

Moreover, it is possible that some, or all, of the enhanced K^+ efflux observed at 100-mM $[Na^+]_{ext}$ could be attributable to loss of plasma-membrane integrity (resulting from osmotic shock) and not necessarily to electrogenic, channel-mediated K^+ transport, as has been discussed in detail elsewhere (Nassery 1975, 1979; Cramer et al. 1985; Britto et al. 2010; Coskun et al. 2013a). Here, loss of membrane integrity was suggested in our electrophysiological experiments by the occasional loss of a previously well-sealed impalement when $[Na^+]_{ext}$ was raised to 100 mM (not shown). Indeed, the greater stimulation of $^{42}K^+$ efflux in LN plants might be indicative of their reduced osmotic stress tolerance, due to nutrient starvation in general, and reduced tissue K^+ in particular (Drew and Saker 1984; Walker et al. 1996; Coskun et al. 2013a).

These system tests indicate that the saturating depolarization patterns observed are not likely to be caused by the stimulation of K^+ or H^+ efflux by increases in $[Na^+]_{ext}$, strengthening our interpretation the patterns signify a leveling off of Na^+ transport across the plasma membrane. However, it is not completely clear what underlies this leveling off. It could be due to kinetic limitations on ion-channel activity (Hedrich and Schroeder 1989; Maathuis et al. 1996). Alternatively, it could be attributed to a diminishing electrochemical potential gradient driving Na^+ influx, which would result from both a steadily depolarizing membrane over the course of the experiment, and an increasing cytosolic sodium concentration. These kinetic and thermodynamic limitations are not mutually exclusive, and it is possible that the saturation of $\Delta\Delta\psi$ is brought about by a combination of factors.

Characterization of Na^+-dependent membrane depolarization

Nutrient withdrawal

Because withdrawal of nutrients, particularly K^+, is known to stimulate Na^+ uptake in rice and many other plants (Rains and Epstein 1967a, b; cf. Davenport and Tester 2000; Subbarao et al. 2003; Horie et al. 2007; Schulze et al. 2012; Kronzucker et al. 2013), plants were grown either under a HN regime, or switched from HN to a simple LN solution 3 days before experimentation. Since LN root cells had significantly more polarized resting membrane potentials than HN plants, LN cells were expected to have a "primed" electrical potential gradient, more favorable to Na^+ influx than HN cells (Kavitha et al. 2012). In addition to being electrically primed, LN roots were expected to be enzymatically primed for Na^+ uptake, particularly in the form of enhanced expression and activity of OsHKT2;1, a Na^+-influx transporter previously shown to be strongly upregulated during nutrient (K^+) starvation in rice (Garciadeblás et al. 2003; Horie et al. 2007; Jabnoune et al. 2009; Kavitha et al. 2012; Miyamoto et al. 2015; Reddy et al. 2017). As predicted, the doubly primed condition of LN plants resulted in substantially greater Na^+-dependent depolarization than found in HN plants (Fig. 1). Interestingly, root Na^+ influx, measured using the radiotracer $^{24}Na^+$, was also greater in LN plants than HN (Fig. 2); in this instance, agreement was found between methods.

Michaelis–Menten patterns

Saturating depolarization patterns in other work have allowed investigators to estimate Michaelis–Menten kinetic parameters (V_{max} and K_m) for the membrane transport of Na^+, K^+, and other ions, in intact plants (Glass et al. 1992; Maathuis et al. 1996; Mian et al. 2011; Böhm et al. 2016; cf. McClure et al. 1990). Following such precedents, we derived a first approximation of kinetic parameters for Na^+ influx from the data in Fig. 1 (Table 1). These values were similar to those found electrophysiologically by Böhm et al. (2016; Table 1) for low-affinity Na^+ transport into digestive-gland cells of the Venus flytrap (*Dionaea muscipula*). In this interesting study, a shift in V_{max} and K_m was observed with changing environmental conditions; however, this was not due to nutrient deficiency, as it was in the present study, but to the prey-induced expression of DmHKT1, a channel-like Na^+-transport protein in the flytrap's digestive glands (Böhm et al. 2016; see Table 1). Our values are also similar to those found by Mian et al. (2011), who examined NaCl-dependent depolarization of the transroot electrical potential in barley (Table 1). However, as shown in Table 1, our initial ("unsubtracted"; see below) results of 4.7–32.1 mM are still much higher than several other reported K_m values corresponding to root Na^+ influx, including those from a radiotracer study of OsHKT2;1-mediated Na^+ influx in K^+-starved roots of *japonica* rice under low (non-saline) $[Na^+]_{ext}$ conditions (Horie et al. 2007), and two electrophysiological studies, one of channel-mediated plasma-membrane conductance in wheat (Davenport and Tester 2000), and one of rice OsHKT2;1 activity expressed in *Xenopus* oocytes (Jabnoune et al. 2009; we note that heterologous expression systems might not, however, faithfully reflect root kinetic characteristics; Garciadeblás et al. 2003). Interestingly, however, the study by Horie et al. (2007) showed that insertional disruption of *oskht2;1* in several mutant lines of rice led to pronounced increases in the K_m value for root Na^+ uptake, to a degree similar to what we found in HN, relative to LN, plants, in the present study (Table 1).

Osmotic effects on ΔΔψ

Because elevated external osmotic strength can by itself cause membrane depolarization over the time scale of our measurements (Racusen et al. 1977), we examined the effects of osmolytes on membrane potential (Figs. 5, 6). From the pronounced depolarization elicited by additions of sorbitol (Fig. 5a), a solute with low membrane permeation rates (Kaiser et al. 1981; Shabala and Lew 2002; Essah et al. 2003), it was reasonable to assume that NaCl-dependent depolarization also includes an osmotically responsive component, in addition to a component attributable to ion transport. To isolate the ion-transport component, we subtracted the values of ΔΔΨ generated independently by sorbitol additions (iso-osmotic to NaCl additions, with a small additional correction to account for known low rates of high-affinity sorbitol influx; see "Materials and methods") from the total depolarization due to NaCl. Subtraction of this osmotic component accentuated the differences between LN and HN plants, with LN plants showing an even stronger conformation to the Michaelis–Menten model ($R^2 = 0.9997$), and a more pronounced inflection point that reflected a greatly reduced K_m (1.4 mM; Fig. 5b; Table 1). Interestingly, this second K_m approximation yielded a value (1.4 mM) very close to that found previously for Na^+ conductance via NSCCs in wheat lipid bilayer experiments (1.2 mM; Davenport and Tester 2000), and also closer to the very low K_m values found for rice OsHKT2;1-mediated Na^+ transport, using radiotracer (Horie et al. 2007) and electrophysiological (Jabnoune et al. 2009) methods (Table 1). The surprisingly strong osmotic effects on membrane depolarization are as yet unexplained; however, this finding underscores the need to include osmotic controls when tracing electrical activities in plant cells under saline (and even sub-saline) conditions.

Influence of Cl^-

When the chloride salt of $NMDG^+$ was used in place of sorbitol, substantially greater depolarization was observed in the case of LN plants (Fig. 6a). This increase was attributed to an increased uptake of chloride in LN plants (via nH^+/Cl^- symport, a depolarizing flux with $n > 1$ H^+ per 1 Cl^-;Teakle and Tyerman 2010), and is consistent with the known enhancement of Cl^- influx under nutrient deprivation, similar to that of Na^+ influx (White and Broadley 2001).

Subtraction of NMDG-Cl-dependent ΔΔψ from NaCl traces allowed us to simultaneously correct for both the estimated osmotic contribution to ΔΔψ (as with subtracted sorbitol traces, above) and that attributable to nH^+/Cl^- symport (Fig. 6b; we assume here and above that Na^+, Cl^-, $NMDG^+$, and sorbitol have similar osmotic effects on membrane potential). Thus, a third iteration of kinetic parameters was undertaken, which further reduced the K_m values for ΔΔΨ in both LN and HN plants (Table 1). For LN plants, the resulting values coincided exactly with the K_m of 1.2 mM found for Na^+ conductance via an NSCC by Davenport and Tester (2000; but see below).

Counterion effects

The substitution of Cl^- by SO_4^{2-} or HPO_4^{2-} did not affect ΔΔψ in HN plants, but had a pronounced effect in LN plants (Fig. 7), further suggesting that the LN condition involves a stimulation of anion transport. The differing effects of NaCl and Na_2SO_4 are consistent with the pioneering work of Rains and Epstein (1967a, b), who observed that low-affinity Na^+ fluxes in barley were substantially greater when Cl^-, rather than SO_4^{2-}, was the counterion. Similarly, Kochian et al. (1985) showed greater low-affinity K^+ transport with Cl^- than with SO_4^{2-} or HPO_4^{2-} as the accompanying anion. This effect has been attributed to the generally more rapid uptake of Cl^- than of SO_4^{2-} (Kochian et al. 1985; Franklin and Zwiazek 2004; Paz et al. 2008), and the even lower ΔΔΨ seen when HPO_4^{2-} is the counterion may be similarly attributable to the lesser uptake of this ion, relative to that of SO_4^{2-}, particularly under nutrient deprivation (Clarkson et al. 1992; *cf.* Kochian et al. 1985).

Identity of the low-affinity Na^+-uptake system in LN plants

Particularly, once corrections are made for osmotic and chloride effects (Fig. 6b), it appears that the saturating curve describing depolarization by Na^+ under LN conditions is dominated by a single Na^+ influx system of medium affinity (K_m of 1.2 mM). Given the similarity in K_m values, this system is functionally, if not phylogenetically, similar to the non-specific cation channel identified in wheat by Davenport and Tester (2000). However, these convergent K_m values may be purely coincidental: Kavitha et al. (2012) concluded that low-affinity Na^+ transport in roots of Pokkali rice occurred predominantly via pathways other than NSCCs, such as HKT-type transporters. Moreover, Horie et al. (2007) and Jabnoune et al. (2009) found that OsHKT2;1 dominated Na^+ transport and electrical conductance (respectively) in *japonica* rice, under low-K^+ conditions. While the K_m values in these two studies were rather lower than in ours (Table 1), the starvation-induced character of transport, conductance, and depolarization in the three studies suggests that OsHKT2;1 might be the dominant contributor to the depolarization behavior of Pokkali here.

"Translating" ΔΔψ into chemical flux values

From a broader perspective, it is worth considering how one might mathematically "translate" a polarization shift

($\Delta\Delta\psi$, in mV), into a chemical tracer flux [expressed as μmol g (FW) $^{-1}$ h^{-1}, for example, a unit of influx found in the present study and many others]. The ratio between these two terms ("*D*:*I*", or depolarization:influx) is theoretically linked to the utilisation of the electrochemical energy gradient across the cell membrane to drive ion fluxes, and the expenditure of this gradient is particularly important from the perspective of distinguishing active, high-affinity transport from passive, low-affinity transport across the plasma membrane (Rodriguez-Navarro et al. 1986; Maathuis and Sanders 1993).

It is useful to compare our *D*:*I* values to values calculated from the relatively well-established literature on K^+-dependent depolarization and K^+ influx (Fig. 8, Suppl. Table S1). We find that, in almost all cases, the *D*:*I* ratio derived from K^+-transport studies is substantially higher than *D*:*I* in our present study, which suggests that more energy is consumed in the transport of a given quantity of K^+ than Na^+ into the cell. This is consistent with the additional energy barrier presented to K^+ influx in the form of a high cytosolic K^+ activity (~100 mM), and also with the engagement of high-affinity active transporters for K^+ (Britto and Kronzucker 2008). Some *D*:*I* values in the high-affinity K^+-transport range were exceptionally large, such as the extreme values of 136–214 mV [μmol g (FW)$^{-1}$ h^{-1}]$^{-1}$ derived from an important study in *Arabidopsis thaliana* (Hirsch et al. 1998). These values are inexplicably large, not only because they are much higher than all other tabulated values (Suppl. Table S1), but also because they are thought to correspond to passive fluxes mediated by K^+ channels, rather than active fluxes mediated by high-affinity transporters (these had been suppressed in the study by the presence of NH_4^+). The outlying *D*:*I* values appear to be linked to the extremely low $^{86}Rb^+$ fluxes reported in this study (e.g., only 0.28 μmol g (FW)$^{-1}$ h^{-1} at 1 mM external [K^+]).

At the other end of the spectrum, the lowest *D*:*I* values we calculated were those found for Na^+ transport in our study, at high $[Na^+]_{ext}$ (50, and, especially, 100 mM). This is partially because few other studies have reported both depolarization and influx at such high substrate concentrations. The gradual decline of our *D*:*I* values to their minima of 1.0 and 1.5 mV [μmol g (FW)$^{-1}$ h^{-1}]$^{-1}$ (in HS and LS plants, respectively) is consistent with our hypothesis that large, linearly increasing Na^+-radiotracer fluxes measured at high $[Na^+]_{ext}$ result mainly from apoplastic transport (Britto and Kronzucker 2015; Coskun et al. 2016; Flam-Shepherd et al. 2018), and do not occur across the plasma membrane, as indicated by the lack of continued plasma-membrane depolarization at high $[Na^+]_{ext}$. One might be tempted to predict that *D*:*I* would continue to decline and approach zero with ever-increasing $[Na^+]_{ext}$, but above 100 mM, root cells and membranes would start to become damaged, and electrode impalements to become non-viable.

As an addendum, we can suggest a first step towards a mathematical modeling of the twin phenomena of depolarization and influx, albeit by use of a simplifying concept, in which we frame *D*:*I* as a Michaelis–Menten function (for $\Delta\Delta\psi$) divided by a simple linear equation (for linearly increasing tracer influx; intercept at 0,0). This can be shown to reduce to

$$D:I = \frac{D_{\max}}{a(K_M + [S])},$$

where D_{max} is the maximum depolarization (or "velocity", as in V_{max}; Maathuis and Sanders 1993), a is the slope in the linear equation, K_M is the Michaelis (half-saturation) constant, and [S] is the substrate concentration. Inspection of this equation shows an asymptotic trend of *D*:*I* towards zero, as substrate concentrations become very large.

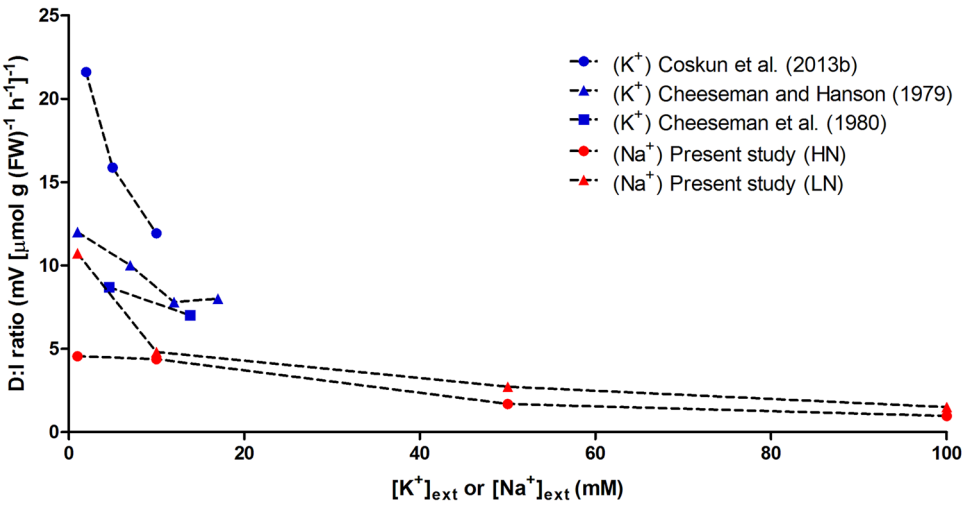

Fig. 8 Depolarization:influx (*D*:*I*) ratio as a function of external substrate concentration. Membrane depolarization (mV) and influx (µmol g FW^{-1} h^{-1}) values (when measured in the same study) were derived from the literature for K^+, and from the present study for Na^+ (see legend)

Conclusions

Our study contains a number of significant findings: (1) Na^+-dependent $\Delta\Delta\Psi$ saturates at moderate $[Na^+]_{ext}$ values; (2) saturation occurs with much lower K_m (and higher V_{max}) values in LN plants than in HN; (3) $\Delta\Delta\Psi$ due to Na^+ and Cl^- influx is increased under LN; (4) Na^+-stimulated K^+ efflux and enhanced H^+-ATPase activity cannot explain this saturation; (5) there is a major osmotic contribution to $\Delta\Delta\Psi$ that is independent of nutritional conditions; (6) counterion contributions to $\Delta\Delta\Psi$ are only evident under LN conditions; and (7) no saturation is observed in $^{24}Na^+$ (radiotracer) influx assays conducted over the same concentration range. While some of these phenomena have been previously reported by ourselves and other researchers in this area (as noted), they have not, to our knowledge, been brought together in this way, to show in particular the stark difference between what is reported by electrophysiological and tracer methods, and also to illustrate the high degree of Na^+ transport plasticity that can be made visible using sharp electrodes.

The electrophysiological results of our study do not support the current RTSC model, of a rapid transmembrane cycling of sodium occurring in plant roots and displaying linearly increasing kinetics when measured in short-term radiotracer uptake experiments, and in the saline range. The question remains as to what then is reported by such experiments if not genuine plasma-membrane transport of Na^+. As mentioned above, we have suggested that traces of this nature might contain a substantial apoplastic component, resulting in misleading patterns of linearity (Britto and Kronzucker 2015; Coskun et al. 2013a, 2016; Flam-Shepherd et al. 2018; Santa-María and Rubio 2018). Perhaps, most problematically, the Na^+-efflux component of the RTSC cycle, which can become very high under salinity (and nearly equal to influx), shows great resistance to transport inhibitors (Britto and Kronzucker 2015). It also shows similarities to apoplastic-tracer kinetics, as measured by the fluorescent extracellular dye 8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS; Yeo et al. 1987; Anil et al. 2005; Coskun et al. 2016). While these PTS studies provide a more direct demonstration that Na^+ fluxes in the RTSC model have an apoplastic character, the distinction between electrophysiological and radiotracer patterns presented here is consistent with, and provides lateral, if indirect, support for such an interpretation.

Very few studies have characterized electrophysiological responses to ion transport in a rapid-cycling context (Coskun et al. 2016; Flam-Shepherd et al. 2018). For this reason, it is not possible to estimate the extent to which the behavior found here in Pokkali rice is common among the angiosperms, or even among other genotypes of rice. Our preliminary results in salt-sensitive IR29 rice (Suppl. Fig. S2), however, indicate that $\Delta\Delta\Psi$ is saturable in this cultivar as well, and it is also stimulated by LN conditions. It would be worthwhile to investigate the electrophysiological characteristics of Na^+ transport in other cultivars of rice, as well as in barley, a species that shows a lower threshold for Na^+-stimulated $^{42}K^+$ efflux than rice (Britto et al. 2010).

We wish to emphasise that our critique does not pertain to most radiotracer-flux studies in plants. On the contrary, great progress has been made in the understanding of plant nutrition by means of radiotracer experiments. Moreover, some results found with radiotracers accord with those found using electrophysiology, including the elevated fluxes and depolarizations seen in LN plants in the present study (Figs. 1, 2), and the agreement between our anion study (Fig. 7) and that of Rains and Epstein (1967a; see above). Our critique refers specifically to the case of Na^+ transport under saline conditions, as described by the RTSC model.

There is a methodological outcome to our re-interpretation of this model: the distortions due to apoplastic-tracer contamination will be amplified when short-term labelling and desorption procedures are used ($\leq$5 min; Britto and Kronzucker 2012; Coskun et al. 2016), as is common practice today. Interestingly, a rare example of saturating low-affinity Na^+-radiotracer ($^{22}Na^+$) influx (measured between ~0.5 and 50 mM NaCl) was shown by Rains and Epstein (1967a) in barley roots. In their study, much longer labelling and desorption intervals (20 and 30 min, respectively) were used, which in our view could potentially produce more realistic results, and agreement with saturating $\Delta\Delta\Psi$ patterns.

While one hopes that the present study has made some key advances, further work will be required to satisfactorily answer this fundamental question in plant nutritional physiology: do Na^+ fluxes have saturable kinetics, or do they have an unlimited capacity (prior to membrane failure)? Such work may take the form of investigations under broader nutritional profiles, different cultivars of rice and other model and agricultural species, experimentation with transport mutants, and techniques allowing positive localization and visualization of apoplastic sodium cycling.

Author contribution statement AH conducted the experiments. DC assisted with the experiments. AH and DB co-wrote the paper. HK supervised the project. All authors contributed to the design and analysis of experiments, and to editing of the manuscript.

Acknowledgements We thank M. Butler and R. Pasuta at McMaster Nuclear Reactor (Hamilton, Ontario, Canada) for supplying $^{24}Na^+$ and $^{42}K^+$. We also thank Dr. D. Dubins at the University of Toronto Leslie Dan Faculty of Pharmacy for access to their osmometer. This work was supported by the Natural Sciences and Engineering Research Council

of Canada (NSERC), the Ontario Graduate Student Scholarship (OGS), and the University of Toronto.

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