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Assessing the role of root plasma membrane and tonoplast Na^+/H^+ exchangers in salinity tolerance in wheat: *in planta* quantification methods

TRACEY A. CUIN[1]*, JAYAKUMAR BOSE[1], GIOVANNI STEFANO[2], DEEPA JHA[3], MARK TESTER[3], STEFANO MANCUSO[2] & SERGEY SHABALA[1]

[1]*School of Agricultural Science, University of Tasmania, Private Bag 54, Hobart, Tasmania 7001, Australia,* [2]*LINV – DOFI, University of Florence, 50019 Sesto Fiorentino, Italy and* [3]*Australian Centre for Plant Functional Genomics, University of Adelaide, Glen Osmond, South Australia, 5064, Australia*

ABSTRACT

This work investigates the role of cytosolic Na^+ exclusion in roots as a means of salinity tolerance in wheat, and offers *in planta* methods for the functional assessment of major transporters contributing to this trait. An electrophysiological protocol was developed to quantify the activity of plasma membrane Na^+ efflux systems in roots, using the microelectrode ion flux estimation (MIFE) technique. We show that active efflux of Na^+ from wheat root epidermal cells is mediated by a SOS1-like homolog, energized by the plasma membrane H^+-ATPase. SOS1-like efflux activity was highest in Kharchia 65, a salt-tolerant bread wheat cultivar. Kharchia 65 also had an enhanced ability to sequester large quantities of Na^+ into the vacuoles of root cells, as revealed by confocal microscopy using Sodium Green. These findings were consistent with the highest level of expression of both SOS1 and NHX1 transcripts in plant roots in this variety. In the sensitive wheat varieties, a greater proportion of Na^+ was located in the root cell cytosol. Overall, our findings suggest a critical role of cytosolic Na^+ exclusion for salinity tolerance in wheat and offer convenient protocols to quantify the contribution of the major transporters conferring this trait, to screen plants for salinity tolerance.

Abbreviations: NHX, tonoplast Na^+/H^+ exchanger; SOS1, plasma membrane Na^+/H^+ exchanger.

Key-words: cytosol; salinity tolerance; sodium; vacuolar sequestration.

Correspondence: S. Shabala. Fax: +61 3 6226 2642; e-mail: sergey.shabala@utas.edu.au

*Present address: Biochimie et Physiologie Moléculaire des Plantes, CNRS UMR 5004, Institut National de la Recherche Agronomique (INRA) U386, Montpellier SupAgro, Université Montpellier II, PlaceViala, F34060 Montpellier Cedex, France.

INTRODUCTION

The ability of a plant to exclude Na^+ from the shoot is considered to be a crucial feature of salinity tolerance in glycophytes such as wheat (Gorham, Wyn Jones & Bristol 1990; Munns & James 2003; Tester & Davenport 2003; Colmer, Munns & Flowers 2005; Munns & Tester 2008). Indeed, the greater salinity tolerance of bread wheat is attributed to its superior ability, compared with durum wheat, to restrict Na^+ transport to the shoot (Joshi, Sharma & Dhari 1982; Francois *et al.* 1986; Gorham *et al.* 1987; Colmer *et al.* 2005). This helps prevent a high Na^+/K^+ ratio within the metabolically active cytosol (Gorham *et al.* 1990), a ratio that is considered critical to plant salinity tolerance (Maathuis & Amtmann 1999; Shabala & Cuin 2008).

Glycophytes have three main mechanisms for minimizing the accumulation of Na^+ in the shoot: minimizing the initial entry of Na^+ into the roots, maximizing the efflux of Na^+ back out of the root and restricting the transfer of Na^+ to the shoot. However, the unidirectional influx of Na^+ in most glycophytes is thermodynamically passive and appears to be quite poorly controlled (Tester & Davenport 2003). Furthermore, in wheat genotypes that differ in their Na^+ accumulation, tracer experiments using $^{22}Na^+$ have shown that there is little difference in unidirectional Na^+ uptake (Davenport, Reid & Smith 1997). Thus, the active extrusion of Na^+ back into the rhizosphere and the restriction of Na^+ loading into the xylem are likely to be more central to the control of the variation in Na^+ accumulation in wheat.

Thermodynamically, Na^+ extrusion from the cytosol to the external medium under saline conditions is an active, energy-consuming process. While Na^+ extrusion in animals and microorganisms also occurs through Na^+-ATPases, energized directly by the hydrolysis of ATP, such Na^+ pumps are absent in higher plants (Garciadeblás, Benito & Rodríguez-Navarro 2001). Instead, plasma membrane Na^+/H^+ antiporters are thought to drive the active transport of Na^+ out of plant cells (Apse & Blumwald 2007), and this electroneutral exchange is the only mode of transport that has been measured for Na^+ export under physiological conditions (Zhu 2003; Apse & Blumwald 2007). Such

energy-dependent Na^+ transport is coupled to the H^+ electrochemical potential difference established by H^+ translocating pumps (Blumwald, Aharon & Apse 2000; Hasegawa *et al*. 2000; Shi *et al*. 2000; Gaxiola, Fink & Hirschi 2002). A Na^+/H^+ antiporter function has been proposed for the *Arabidopsis* SOS1 transporter (Shi *et al*. 2000, 2003; Qiu *et al*. 2003), although exchange activity resulting in Na^+ extrusion has not been reported *in vivo*. Nonetheless, SOS1 promoter–GUS fusions show expression in the epidermis at the root tip (Shi *et al*. 2000), *sos1* mutant plants accumulate more Na^+ (Wu, Ding & Zhu 1996; Shi *et al*. 2000) and overexpression of SOS1 has been found to reduce Na^+ accumulation and improve salinity tolerance in transgenic *Arabidopsis* (Shi *et al*. 2003).

Despite the critical importance of active efflux systems for salinity tolerance (see Munns & Tester 2008 for review), such systems have not been properly characterized in wheat. Early studies did show Na^+/H^+ antiporter activity in plasma membrane vesicles from wheat genotypes (Allen, Wyn Jones & Leigh 1995), and more recently, a possible SOS1 homolog has been identified from sequence similarity in bread wheat (Mullan, Colmer & Francki 2007), although this gene has yet to be characterized. A putative bread wheat plasma membrane Na^+/H^+ antiporter (TaSOS1) has been shown to enhance Na^+/H^+ activity and Na^+ extrusion when expressed in yeast cells (Xu *et al*. 2008), and the level of its expression *in planta* is up-regulated by salinity. This suggests that SOS1-type antiporters could contribute to plasma membrane Na^+/H^+ exchange in wheat and may play a role in the salinity tolerance of this crop plant. However, the functional activity of these SOS1-type antiporters *in planta* remains to be demonstrated. Hence, the possible contribution made by endogenous Na^+/H^+ antiporters to the export of Na^+ from wheat roots, and in turn to salinity tolerance, remains unresolved.

In Australia alone, it is projected that by 2050, 24% of the wheat belt will be significantly affected by salinity (see http://audit.ea.gov.au). However, attempts to quantify the activity of active Na^+ efflux systems in wheat and to correlate this activity with differential salinity tolerance among contrasting varieties is limited (e.g. Davenport *et al*. 2005). Moreover, no methods exist that would provide a rapid and convenient assessment of the activity of such Na^+ efflux systems in any plant species.

As well as extrusion out of the root, another important mechanism that prevents the accumulation of Na^+ in the shoot is the control of Na^+ loading into the xylem (Møller *et al*. 2009). Therefore, in order to maintain an optimal cytosolic K^+/Na^+ ratio, while restricting xylem loading, Na^+ export from the root must be combined with an efficient mechanism of sequestration of the retained Na^+ into the vacuoles of root cells. Such sequestration can be achieved by the activity of tonoplast Na^+/H^+ exchangers (Apse *et al*. 1999; Blumwald *et al*. 2000). However, although tonoplast Na^+/H^+ exchangers have been described and characterized in bread wheat (Wang *et al*. 2002; Xue *et al*. 2004; Brini *et al*. 2005, 2007; Saqib *et al*. 2005; Yu *et al*. 2007), their contribution to the differential tolerance observed among wheat cultivars remains unexplored, there being no convenient method for the rapid assessment of this trait.

The aims of this study were threefold: (1) using genotypes contrasting in their salinity tolerance, to evaluate the role of cytosolic Na^+ exclusion in this trait in wheat; (2) to reveal the role and relative contribution of SOS and NHX transporters towards the above trait; and (3) to provide a reliable and relatively straightforward method for the rapid assessment of the ability of plants to actively exclude Na^+ from the root cytosol either by export out of the plant and/or through vacuolar sequestration in root cells.

An efficient protocol was developed involving use of the non-invasive microelectrode ion flux estimation (MIFE) technique to quantify the activity of the plasma membrane Na^+ efflux system(s) in plant roots. *In planta* evidence is provided that demonstrates that active Na^+ efflux from wheat root epidermal cells is mediated by a SOS1-like homolog, powered by the plasma membrane H^+-ATPase. SOS1-like activity is shown to be highest in Kharchia 65, a bread wheat cultivar often referred to as a 'standard' for salinity tolerance (Hollington 2000). It is also shown that Sodium Green dye confocal imaging can be used to efficiently evaluate the ability of a particular genotype to sequester Na^+ into the root cell vacuoles. This could reveal correlations between Na^+ compartmentation and salinity tolerance in wheat. Taken together, our findings suggest a critical role for cytosolic Na^+ exclusion for salinity tolerance in wheat and offer convenient protocols to quantify the contribution of the major transporters conferring this trait. When combined with marker-assisted selection, these techniques may be applied to locate and clone the genes underlying salinity tolerance.

MATERIALS AND METHODS

Plant materials and growth conditions

Four bread (*Triticum aestivum*) and four durum (*Triticum turgidum* L. ssp. *durum*) wheat cultivars were obtained from the Tamworth Agricultural Institute, NSW Department of Primary Industries, Australia. Hydroponically grown seedlings were used for all electrophysiology, $^{22}Na^+$ flux measurements and quantitative RT-PCR. Sterilized seeds were grown in an aerated solution (0.5 mM KCl and 0.1 mM $CaCl_2$, pH 5.7) in a dark growth cabinet at 23–24 °C with a relative humidity 65–70%, except where noted. The main reason for using dark-grown seedlings was to maximize the genotypic difference in ion flux responses from plant roots (Jingyi Zhang and Sergey Shabala, unpublished results).

Arabidopsis thaliana (wild-type Columbia and *sos1* mutant) seeds were obtained from NASC (Nottingham, England). Seeds were surface sterilized with commercial bleach and sown on the surface of 90 mm Petri dishes containing 0.35% phytagel, half-strength Murashige and Skoog media (Sigma-Aldrich, Castle Hill, NSW, Australia) and 1% (w/v) sucrose at pH 5.7. Plates were sealed with parafilm and placed in an upright position, so roots grew down the phytagel surface without penetrating it. Seeds were

grown for 6 d under constant white fluorescent light at room temperature.

Non-invasive ion flux measuring (the MIFE) technique

Net ion fluxes were measured non-invasively using ion-selective microelectrodes (the MIFE technique, UTas Innovation, Hobart, Australia), as described previously (Newman 2001; Shabala, Shabala & Van Volkenburgh 2003). Briefly, microelectrodes were pulled and salinized with tributylchlorosilane, and tips backfilled with commercially available ion-selective cocktails (K^+, 60031; Na^+, 77176; Ca^{2+}, 21048; all from Sigma-Aldrich). Electrodes were mounted with tips positioned close together, on a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan), 40 μM above the root surface. During measurement, a computer-controlled stepper motor moved the electrode between two positions (40 and 80 μM, respectively) from the root surface in a 10 s square-wave manner. The CHART software (see Newman 2001) recorded the potential difference between the two positions and converted them into electrochemical potential differences using the calibrated Nernst slope of the electrode. Net ion fluxes were calculated using the MIFEFLUX software for cylindrical diffusion geometry (Newman 2001).

MIFE measurements on Na^+ uptake kinetics

Six-day-old seedlings were used for ion flux measurements. The root length was between 10 and 12 cm and did not differ significantly between genotypes (Supporting Information Fig. S1). In the majority of experiments, ion fluxes were measured from the mature root zone, 10 mm from the root tip. Some measurements were conducted further along the root axis, 40 mm from the root tip. No apparent difference in root morphology was apparent at any of these positions (Supporting Information Fig. S2); root hairs were present in both regions (Supporting Information Fig. S3), and no evidence of lateral roots were found. Seedlings were placed in a 10 mL Perspex measuring chamber containing 5 mL of the basic salt medium (BSM; in mM: 0.5 KCl, 0.1 $CaCl_2$, pH 5.7 unbuffered), 1 h prior to measurement. Net ion fluxes were measured in BSM solution for 5 to 10 min to ensure steady initial values. A double stock of NaCl-containing solution was applied to reach the final NaCl concentration required (50 mM, except where noted), and transient Na^+ fluxes were measured for up to 60 min. The time required for stock addition and the establishment of diffusion gradients is reported to be about 40 s (Shabala & Hariadi 2005). Accordingly, the first 60 s after the solution change was discarded from the analysis.

Measuring net Na^+ efflux in 'recovery' experiments

In a typical protocol, roots of six-day-old wheat or *Arabidopsis* plants were exposed to 150 mM NaCl for 24 h. For wheat, the bathing medium also contained 0.1 mM $CaCl_2$ and 0.5 mM KCl (BSM solution). For *Arabidopsis*, K^+ was omitted from the bathing medium because removal of NaCl resulted in a large influx of K^+ into *Arabidopsis* roots (data not shown), which, due to the poor discrimination of the Na^+ liquid ion exchangers (LIX) between K^+ and Na^+, substantially masked the resultant Na^+ efflux. No such confounding effects were present in wheat roots; only a very small K^+ uptake was measured in response to Na^+ removal (Fig. 3).

One hour prior to measurement, a seedling was transferred to a 10 mL Perspex measuring chamber containing the bathing medium, still in the presence of 150 mM NaCl. After 1 h, this solution was poured off and the root was quickly rinsed three times in 10 mM $CaCl_2$ to remove surface NaCl. The root was then transferred to a clean Perspex chamber containing the bathing medium, minus NaCl. The time taken for this procedure was a maximum of 1.5 min. Thus, when recordings were made, the first few minutes were discarded. Washing with 10 mM $CaCl_2$ did not significantly affect the calcium activity of the bathing medium if the root was transferred to a clean chamber, as measured by a Ca^{2+}-selective electrode (data not shown). In wheat, fluxes were recorded on the mature zone, 10 mm from the root tip (unless specified otherwise). In the case of *Arabidopsis*, measurements were made 100 μm from the tip, an area from which expression levels in root epidermal cells of SOS1 is highest (Shi *et al*. 2002) and where the highest SOS1 activity in roots has previously been found (Shabala *et al*. 2005).

Abrupt removal of 150 mM NaCl might potentially cause a hypo-osmotic shock and associated non-specific leak of solutes, including Na^+. To address this issue, additional methodological experiments were conducted using either lower (50 mM) NaCl treatment, or in the presence of an isotonic, non-ionic osmoticum in a bath upon the removal of 150 mM NaCl so as to avoid shock.

For pharmacology experiments, seedlings were pre-treated with the pharmacological agent, dissolved in the bathing medium for 1 h prior to the removal of NaCl, except where noted.

$^{22}Na^+$ Influx Measurements

Na^+ influx was measured using $^{22}Na^+$ radiotracer essentially as described by Essah, Davenport & Tester (2003). Seedlings were grown hydroponically in 0.1 mM $CaCl_2$ and 0.5 mM KCl in the light. Ten- to 12-day-old plants were used in these experiments and plants were subjected to a sudden-shock treatment at 150 mM NaCl. Whole seedlings were suspended with their roots in 10 mL $^{22}Na^+$-labelled solution (approximately 40 kBq mL^{-1} of $^{22}Na^+$) containing 150 mM NaCl plus 0.5 mM KCl and 0.1 mM $CaCl_2$, on a gently rotating shaker. At the end of the influx period (5 or 60 min), the seedling was separated into root and shoot. The root was briefly rinsed in solution containing 150 mM NaCl and 10 mM $CaCl_2$, followed by successive rinses of 2 and 3 min in 500 mL of ice-cold 150 mM NaCl plus 10 mM $CaCl_2$ to

displace any apoplastic $^{22}Na^+$ or $^{22}Na^+$ adhered to the root surface. All solutions were stirred on gently moving shakers at 45 rpm. The roots were blotted gently, and both the shoot and root were weighed and transferred to a plastic vial containing 2.5 mL scintillation cocktail (Optiphase HiSafe, Fisher Chemicals, Loughborough, UK). Samples were counted with a liquid scintillation counter (Beckman Coulter LS6500, Fullerton, CA, USA).

Confocal laser scanning microscopy

Wheat seedlings were grown for 6 d and treated with 150 mM NaCl as described above. Root segments, between 8 and 10 mm long, were cut from the mature zone, 10 to 15 mm from the root apex. Eight to 10 segments were incubated in Eppendorf tubes in 500 μL of the 10 μM Sodium Green solution (S-6901, Molecular Probes, Eugene, OR, USA). After 1 h of incubation, the samples were examined using confocal microscopy. Images were recorded with a Leica TCS SP5 confocal microscope (Leica Microsystems CMS GmbH, Germany), equipped with an acusto-optical beam splitter (AOBS), and an upright microscope stand (DMI6000). A 775×775 μm area (512×512 pixel) using a 20× objective (HCX PL APO CS 20.0×0.70 DRY UV) or a 246×246 μm area (512×512 pixel) using a 63× objective (HCX PL APO lambda blue 63.0×1.40 OIL UV) was imaged, with an image spatial calibration of 1.5 or 0.5 μm per pixel, respectively. The pinhole was set to one 'Airy unit'. The microscope setting for detecting Sodium Green was $\lambda_{exc} = 488$ nm and λ_{em} over the 520–560 nm spectral band. A series of confocal optical XY images through the thickness of the samples (total scanning volume was ~100 μm, with a slice thickness of 5 μm) were acquired in XYZ scanning mode, using the Leica LAS AF software package. Comparison of different levels of fluorescence between cells was carried out by visualizing cells with the identical imaging settings of the confocal microscope (i.e. laser intensity, pinhole diameter and settings of the imaging detectors).

Quantitative real-time PCR analysis

Six-day-old hydroponically grown wheat seedlings, treated with 150 mM for 24 h were harvested, snap frozen in liquid nitrogen and the root RNA extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the protocol described by Chomczynski (1993). Genomic DNA contamination was removed using Ambion's DNA-*free* (Promega, Madison, WI, USA) and 200 ng of total RNA was used to synthesis cDNA using Superscript III (Invitrogen). Quantitative real-time PCR (Q-PCR) was performed on the cDNA for the transporters *TaSOS1* (*Locus:AY326952*) and *TaNHX1* (*Locus:AY040245*) using a RG6000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, Sydney, Australia). Primer sequences are shown in Supporting Information Table S1.

A two round normalization of Q-PCR data was carried out by geometric averaging of multiple control genes as described by Vandesompele, De Paepe & Speleman (2002). *T. aestivum* glyceraldehyde 3-phosphate dehydrogenase, elongation factor alpha, cyclophilin and actin were used as control genes for normalization of cDNA from the bread wheat lines Kharchia 65 and Baart 46. For the normalization of cDNA obtained from Wollaroi and Tamaroi, the durum lines, *Triticum turgidium* ssp. *durum* glyceraldehyde 3-phosphate dehydrogenase, elongation factor alpha, heat shock protein 70 and tubulin were used. The results are presented as mean ± SE ($n = 3$–6 batches).

RESULTS

Sodium uptake kinetics do not differ in wheat cultivars with contrasting salinity tolerance

The four durum and four bread wheat cultivars used in this study differed significantly in their salinity tolerance (Cuin *et al*. 2008, 2009; Cuin, Parsons & Shabala 2010). Nonetheless, net Na^+ influx, measured by the MIFE technique, was not significantly different (at $P < 0.05$) between the cultivars. A dramatic but short-lived (several minutes) net Na^+ uptake of several thousand nmol m^{-2} s^{-1} was measured in all cultivars immediately upon an increase in external Na^+ (Fig. 1a), but 5 min later, the net Na^+ flux was not significantly ($P < 0.05$) different from zero. Any net Na^+ uptake that did occur after that period could not be detected due to methodological limitations, such as poor Na^+ LIX selectivity (Chen *et al*. 2005) and/or low signal-to-noise ratio (discussed below).

In an attempt to overcome such limitations, radiotracer experiments were carried out to measure unidirectional $^{22}Na^+$ uptake in response to sudden salt-shock treatment (150 mM NaCl) (Fig. 1b,c). It appears that the confounding effect of $^{22}Na^+$ efflux becomes evident 5 min after NaCl application, due to non-linearities in influx after that time, so measurements prior to this time point represent the unidirectional influx (Davenport *et al*. 2005). As is evident from Fig. 1b, no clear differences were revealed between cultivars, despite their contrasting salinity tolerance (e.g. Cuin *et al*. 2008, 2010). Indeed, no statistically significant difference (at $P < 0.05$) in Na^+ uptake was found between Westonia and Baart 46, two bread wheat cultivars of contrasting salinity tolerance (Cuin *et al*. 2009) (Fig. 1b). Similarly, unidirectional $^{22}Na^+$ fluxes in Odin and Towner, two contrasting durum wheat cultivars (tolerant and sensitive, respectively; Cuin *et al*. 2009), were not statistically different. In addition, no major difference in unidirectional $^{22}Na^+$ uptake was detected 60 min after salt stress was applied (Fig. 1c). Thus, the results presented corroborate previous reports that the contrasting Na^+ accumulation and salinity tolerance in wheat is not conferred by differences in the unidirectional uptake of Na^+ (Davenport *et al*. 1997).

Evaluating root Na^+ efflux ability in 'recovery' experiments

It is not the unidirectional Na^+ uptake but the *net* Na^+ flux (i.e. the difference between Na^+ uptake and its extrusion)

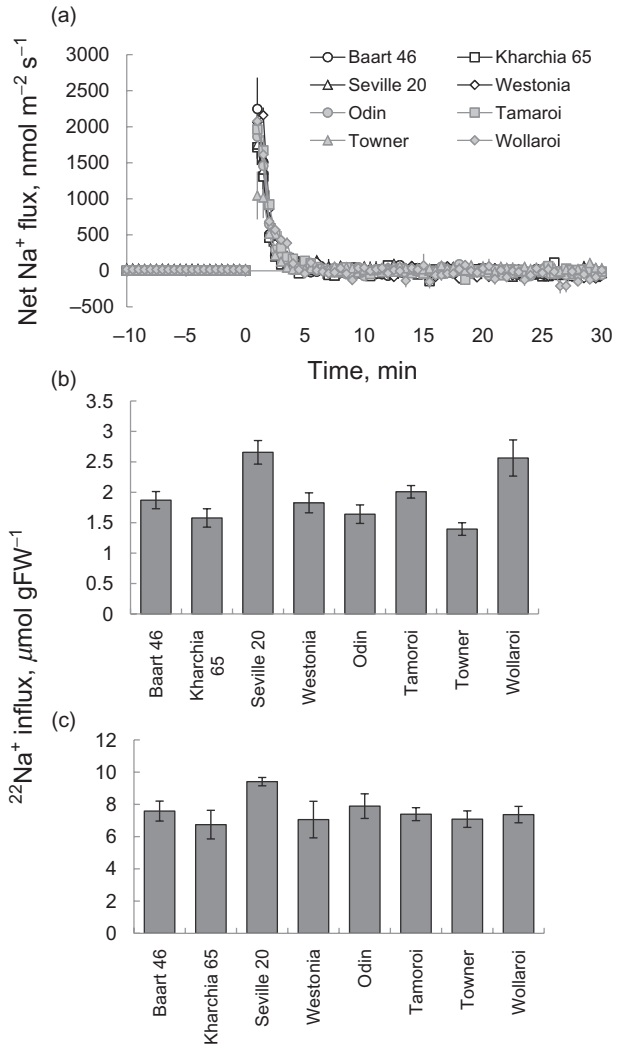

Figure 1. (a) net Na^+ flux kinetics measured in 6-day-old wheat seedlings following 50 mM NaCl treatment. Fluxes were measured in the mature zone, about 10 mm from the root tip. Means ± SE ($n = 6$ seedlings). In all microelectrode ion flux estimation measurements, the sign convention is efflux negative. (b,c) Unidirectional $^{22}Na^+$ uptake into the root of 10- to 12-day-old wheat roots after 5 and 60 min of 150 mM NaCl treatment, respectively. Mean ± SE ($n = 12$ seedlings).

that is critical to salinity tolerance. Nonetheless, the detection of potentially small but critical differences between influx and efflux is impeded by the poor signal-to-noise ratio that occurs in the presence of high Na^+ concentrations in the bathing medium. As shown in Fig. 1a, even at the relatively low concentration of NaCl used in the present study (50 mM), resolution of the Na^+ flux is still problematic.

This problem was mitigated by adapting an approach used previously to study the effects of extreme pH treatment on the activity of H^+ transporters: a so-called 'recovery' protocol (Fuglsang *et al*. 2007). In this method, the net efflux of Na^+ is recorded immediately following the cessation of salinity treatment. Accordingly, 6-day-old wheat roots were treated with 150 mM NaCl for 24 h, then NaCl was quickly removed and Na^+ flux responses were measured.

A rapid and massive efflux of Na^+ from roots was recorded in all cultivars immediately after the removal of NaCl from the bathing medium (Fig. 2). In seven out of the eight cultivars examined, the net efflux rapidly subsided within 5 min. It could be argued that this initial efflux from the roots might originate from the removal of loosely bound Na^+ from the root surface as well as from the apoplastically bound Na^+ (Wang *et al*. 2006). For this reason, a time point of 20 min after NaCl removal was chosen for comparative purposes. By this time, net Na^+ fluxes reached their steady levels, and no confounding effects of either K^+ or Ca^{2+} were present (discussed below). At this time point, a small but significant ($P < 0.05$) net Na^+ efflux was still detected in all cultivars (Fig. 2b). Notably, Kharchia 65 (a genotype which is often considered to be a standard for salinity tolerance; Hollington 2000), showed a considerably greater export of Na^+ compared with all other genotypes measured (Fig. 2b).

The fluxes of K^+ and Ca^{2+} were also measured on the basis that these are potentially interfering ions that may confound Na^+ flux measurements (see Chen *et al*. 2005 for

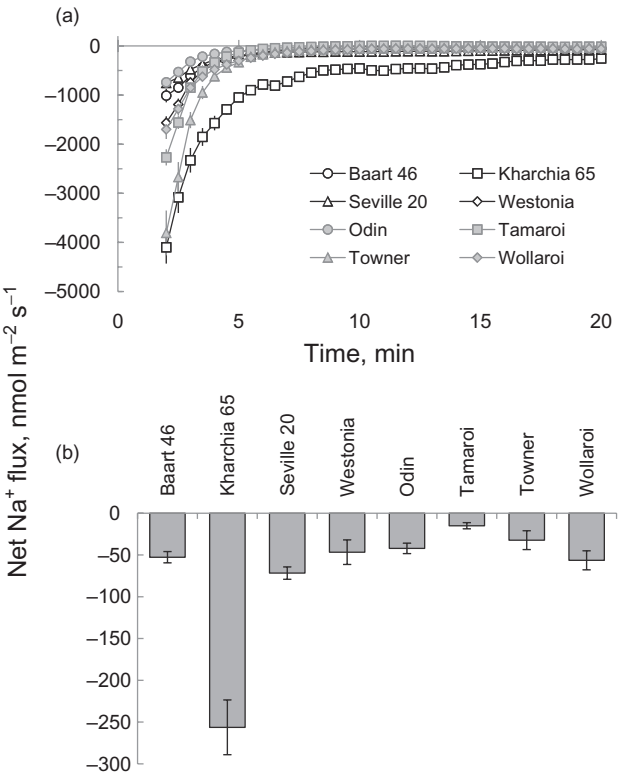

Figure 2. Sodium efflux from wheat roots after the removal of 150 mM NaCl. Six-day-old wheat seedlings were treated with 150 mM NaCl for 24 h before its removal, and the resultant net Na^+ fluxes measured, *c*. 10 mm from the root tip. (a) transient net Na^+ efflux immediately after NaCl removal and (b) steady-state net Na^+ efflux, 20 min after NaCl removal. Mean ± SE ($n = 6$ seedlings).

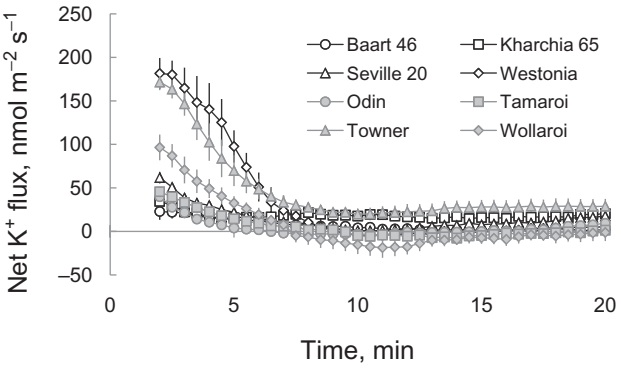

Figure 3. Transient net K^+ flux responses from wheat roots after the removal of 150 mM NaCl. Six-day-old wheat seedlings were treated with 150 mM NaCl for 24 h before its removal, and the resultant net K^+ fluxes measured, *c.* 10 mm from the root tip. Mean ± SE ($n = 6$ seedlings).

details). In the majority of cultivars, the net Ca^{2+} flux 20 min after Na^+ removal from the bath solution was not significantly different from zero (at $P < 0.01$, data not shown). For K^+, a rapid and significant initial uptake was measured immediately after Na^+ removal in all cultivars. However, this ceased within 8 to 10 min, and was followed by a relatively small and stable net K^+ influx (Fig. 3). Thus, no confounding effects from any of potentially interfering ions are present 20 min after NaCl removal, justifying this as a suitable time for screening protocols.

The genotypic difference in root Na^+ efflux ability is independent of position along the root axis and is not associated with hypo-osmotic shock

In the above experiments, net Na^+ efflux was measured in mature root zone (in the midst of root hairs), 10 mm from the root tip. However, the extent to which other root regions may contribute to overall Na^+ exclusion in wheat roots is unknown. Accordingly, measurements of Na^+ efflux were performed in four contrasting bread wheat cultivars 40 mm from the root tip. As shown in Fig. 4, the results were qualitatively very similar to those reported for the 10 mm region (shown in Fig. 2). A rapid and massive efflux of Na^+ from roots was recorded in all cultivars immediately after the removal of NaCl from the bathing medium (Fig. 4a), and the fluxes stabilized 15 to 20 min later. At this time, Kharchia 65 showed the highest net Na^+ efflux, three- to fourfold higher than three other varieties (Fig. 4b). Thus, it appears that active Na^+ efflux systems are present in much of the wheat root epidermis.

As mentioned in the Materials and Methods section, abrupt removal of 150 mM NaCl might potentially cause a hypo-osmotic shock and associated non-specific leak of solutes, including Na^+. To address this issue, additional methodological experiments were conducted. Two contrasting bread wheat varieties, Kharchia 65 and Baart 46, were used. Plants were treated with 150 mM NaCl for 24 h, and fluxes were measured as described above for Fig. 2, except that the bath solution upon removal of NaCl contained non-ionic (sorbitol) solution, isotonic to 150 mM NaCl, to avoid the osmotic shock. As shown in Fig. 5a, the results were very similar to those reported for the 'standard recovery protocol', both qualitatively and quantitatively. Hence, it appears that the genotypic difference in root Na^+ efflux ability is not associated with plant osmotic sensitivity. The genotypic difference in Na^+ efflux ability was also present when plants were incubated at lower (50 mM) NaCl concentrations (Fig. 5b). However, although statistically significant (at $P < 0.05$), the difference between genotypes was not as pronounced as for the 150 mM treatment. Hence, 24 h incubation in 150 mM NaCl may be recommended for screening protocols.

Pharmacology of transporters mediating sodium efflux in wheat roots suggests the role of SOS1-like proteins

In order to elucidate the transporter(s) responsible for mediating the recorded Na^+ efflux, a series of pharmacological experiments were carried out on Kharchia 65. The presence in the bathing medium of 20 mM tetraethylammonium (TEA) (a known blocker of K^+ selective channels, Hille 2001) did not affect the magnitude of the Na^+ efflux after the removal of 150 mM NaCl from the bathing medium

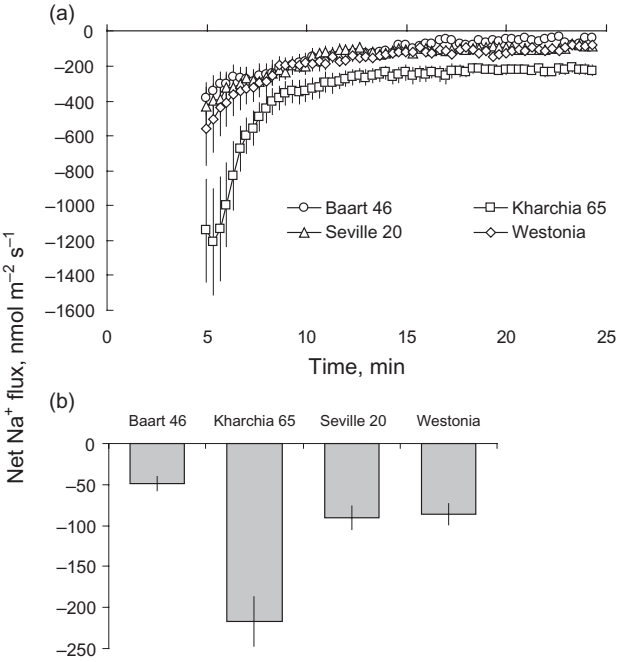

Figure 4. Sodium efflux from wheat roots after the removal of 150 mM NaCl measured at 40 mm from the root tip. Six-day-old seedlings were treated with 150 mM NaCl for 24 h before its removal. (a) Transient net Na^+ efflux immediately after NaCl removal and (b) steady-state net Na^+ efflux, 20 min after NaCl removal. Mean ± SE ($n = 7$ to 9 seedlings).

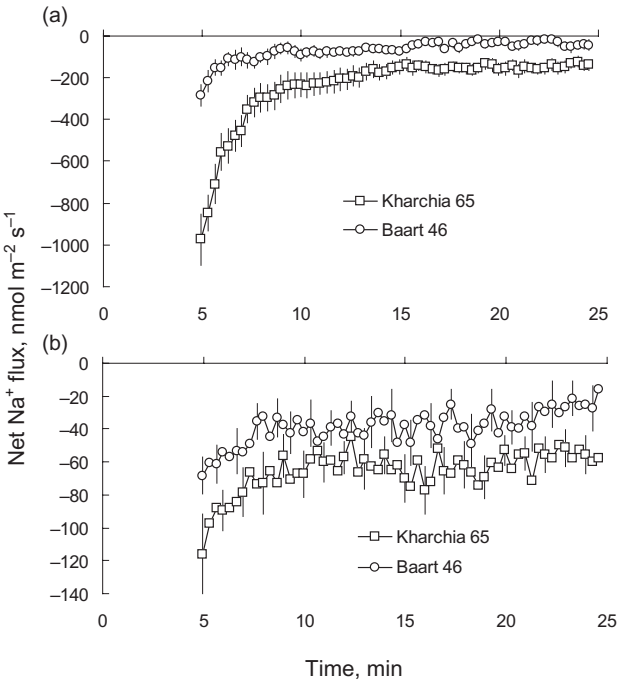

Figure 5. (a) Transient net sodium efflux from wheat roots measured immediately after the removal of 150 mM NaCl, but in an iso-equivalent amount (280 mM) of sorbitol. Six-day-old seedlings were treated with 150 mM NaCl for 24 h before its recovery in 280 mM sorbitol, and the resultant net Na^+ fluxes measured 10 mm from the root tip. Mean ± SE ($n = 9$ seedlings). (b) Transient net sodium efflux from wheat roots measured immediately after the removal of 50 mM NaCl. Six-day-old seedlings were treated with 50 mM NaCl for 24 h before its removal, and the resultant net Na^+ fluxes measured 10 mm from the root tip. Mean ± SE ($n = 6$ seedlings).

(Fig. 6). In contrast, root pre-treatment for 30 min with either 50 μM carbonyl cyanide 3-cholorophenylhydrazone (CCCP) (a protonophore) or 0.5 mM vanadate (an inhibitor of P-type H^+-ATPase) significantly ($P < 0.01$) reduced the Na^+ efflux (Fig. 6). While it is acknowledged that this may also affect the transport of other ions across the plasma membrane, it does suggest that the maintenance of an electrochemical potential difference for H^+ is critical to the removal of Na^+ from the root. Furthermore, the addition of 0.1 mM NAA (an activator of the plasma membrane H^+ pump, Hager 2003) also significantly ($P < 0.01$) stimulated Na^+ efflux.

These results indicate that the pumping of H^+ plays a significant role in Na^+ removal from salinized wheat roots, most likely by powering a Na^+/H^+ antiport system. To investigate this possibility, measurements were made of Na^+ fluxes in the presence of amiloride, an inhibitor of Na^+/H^+ exchange activity in both animal and plant systems (Blumwald & Poole 1985; Kleyman & Cragoe 1988). Amiloride was found to significantly reduce the magnitude of Na^+ efflux from Kharchia 65 roots in a dose-dependent manner (Fig. 7b). The inhibitory effects of amiloride were highly

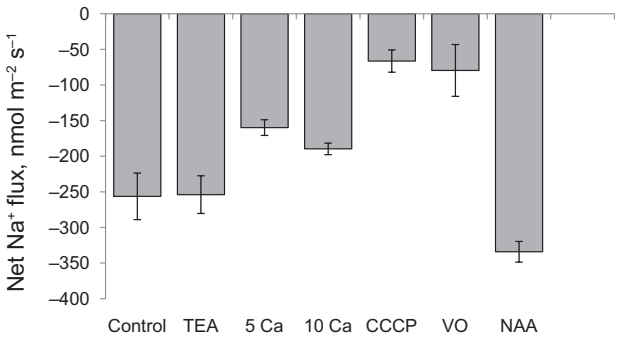

Figure 6. Pharmacology of Na^+ efflux from Kharchia 65 roots after removal of NaCl, treated with a range of channel blockers (a) and transport inhibitors (b). Six-day-old Kharchia 65 was exposed to 150 mM NaCl (as described previously) that was then removed and the resultant Na^+ fluxes measured in the mature zone (10 mm from the tip) of roots supplied with known channel blockers. Steady-state net Na^+ efflux, 20 min after 150 mM NaCl removal. Mean ± SE ($n = 6$ seedlings).

significant ($P < 0.001$) at an applied concentration as low as 25 μM (Fig. 7b), saturation being observed between 125 and 150 μM, with a measured reduction of 87% compared with the control at 100 μM (Fig. 7a). This indicates strongly that

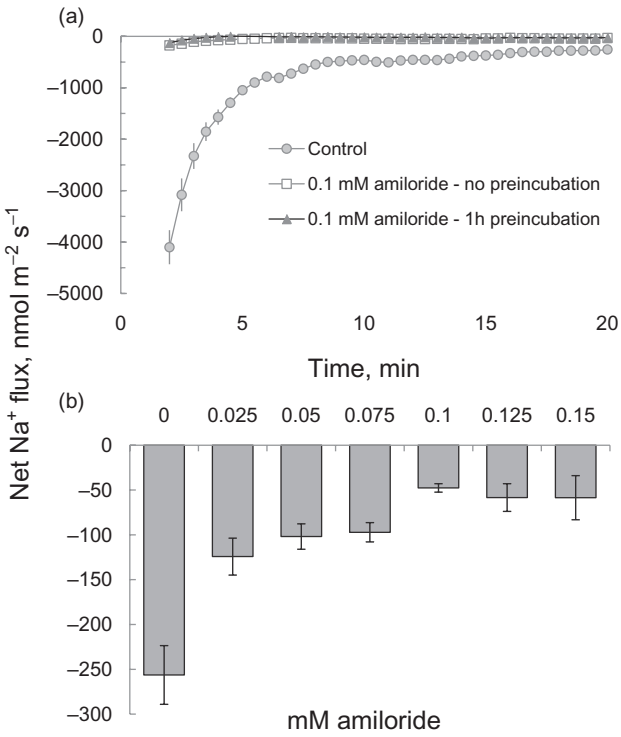

Figure 7. Sodium efflux from the mature zone of Kharchia 65 roots after removal of NaCl, treated with amiloride, either with 1 h pre-incubation or added when NaCl is removed. Other experimental details are as for Fig. 4. (a) Transient net Na^+ efflux measured upon 150 mM NaCl removal; (b) dose dependency of amiloride on Na^+ efflux measured after 20 min of 150 mM NaCl removal. Mean ± SE ($n = 6$ seedlings).

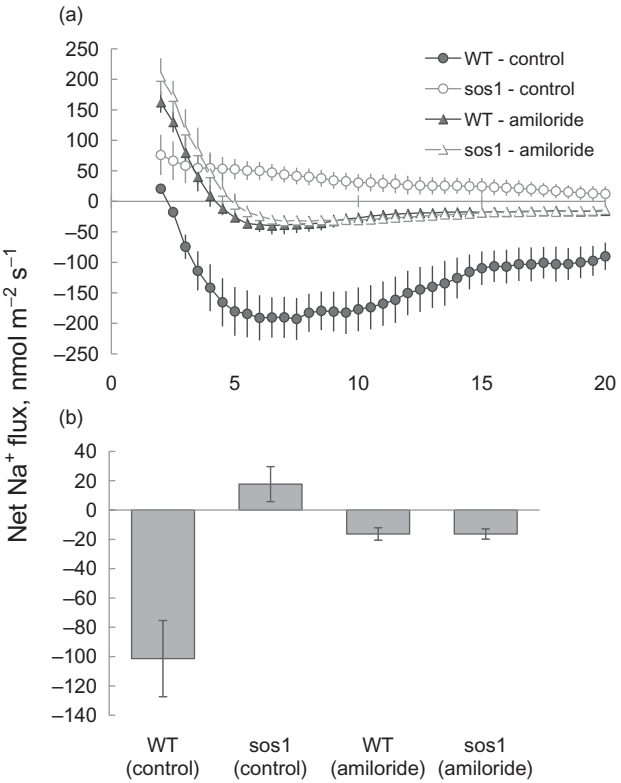

Figure 8. Sodium efflux from *Arabidopsis* wild -ype and *sos1* root apex after the removal of 150 mM NaCl, with and without 0.1 mM amiloride. Six-day-old *Arabidopsis* seedlings were subjected to 150 mM NaCl for 24 h before its removal, and the resultant Na^+ fluxes measured in the root apex, 100 μM from the root tip. (a) transient net Na^+ efflux immediately after NaCl removal; (b) steady-state net Na^+ efflux measured 20 min after NaCl removal. Mean ± SE ($n = 6$ seedlings).

a SOS1-like Na^+/H^+ antiporter is responsible for the active transport of Na^+ out of the root cells of the salt-tolerant Kharchia 65 bread wheat cultivar, and substantial amounts of Na^+ are removed in this way.

Experiments with Arabidopsis *sos1* mutants are consistent with a role for the plasma membrane Na^+/H^+ exchanger in Na^+ efflux from wheat roots

To further investigate the role for a plasma membrane Na^+/H^+ antiport system in wheat roots cells, the Na^+ efflux from the roots of wild-type *Arabidopsis* and *sos1* knockout mutant plants was measured (Zhu 2002, 2003). The root apex is the region where levels of SOS1 expression have been shown to be highest (Shi *et al*. 2002) and a large net Na^+ efflux was recorded in this region in wild-type plants after the removal of 150 mM NaCl from the bathing medium. This efflux was totally abolished in the *sos1* mutant (Fig. 8). Furthermore, and as in wheat, it was found that the presence of 100 μM amiloride significantly ($P < 0.01$) reduced the extent of Na^+ efflux in wild-type *Arabidopsis* plants. This is despite the fact that the predicted sequence analysis of *SOS1* contains no amiloride-binding domain (Zhu 2000), although recent work has shown that SOS1 activity is affected by amiloride (Guo, Babourina & Rengel 2009). No significant difference in Na^+ flux was found between wild-type and *sos1* knockout plants when subjected to this inhibitor.

Salinity tolerance in wheat is also associated with better Na^+ sequestration in root vacuoles

In addition to the export of Na^+ out of the root, the ability to compartmentalize Na^+ in the cell vacuole provides an effective mechanism to avert the toxic effects of Na^+ in the cytoplasm (Apse & Blumwald 2007). In order to evaluate the potentially differing abilities among wheat varieties to compartmentalize Na^+, confocal laser scanning microscopy was used to observe the intracellular distribution of Na^+ in salinized wheat roots loaded with Sodium Green. After 24 h of 150 mM NaCl treatment, most of the Na^+ accumulated by Kharchia 65 epidermal root cells was found to be located in the vacuole (Fig. 9), indicating efficient compartmentalization of accumulated Na^+. By contrast, in the three other varieties used (Baart 46, Tamaroi and Wollaroi), the strongest signal originated from the cytosol, with only a few cells showing efficient Na^+ compartmentalization in the vacuole (Fig. 9).

The intracellular spatial distribution of Na^+ within the root epidermal cells was further quantified using Leica

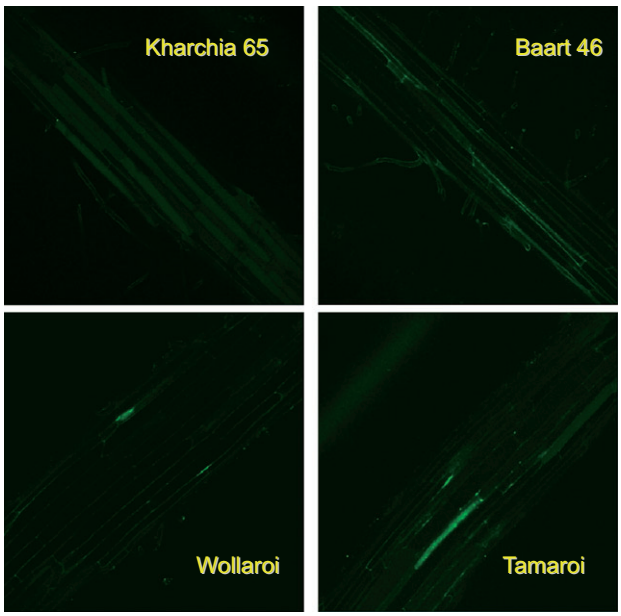

Figure 9. Sodium compartmentation in epidermal root cells of four wheat cultivars. Six-day-old wheat roots were treated with 150 mM NaCl for 24 h and then stained with 10 μM Sodium Green dye for 1 h before the confocal images were taken. One (of six) typical plant for each genotype is shown. Measurements were made in the mature zone, between 10 and 20 mm from the root apex.

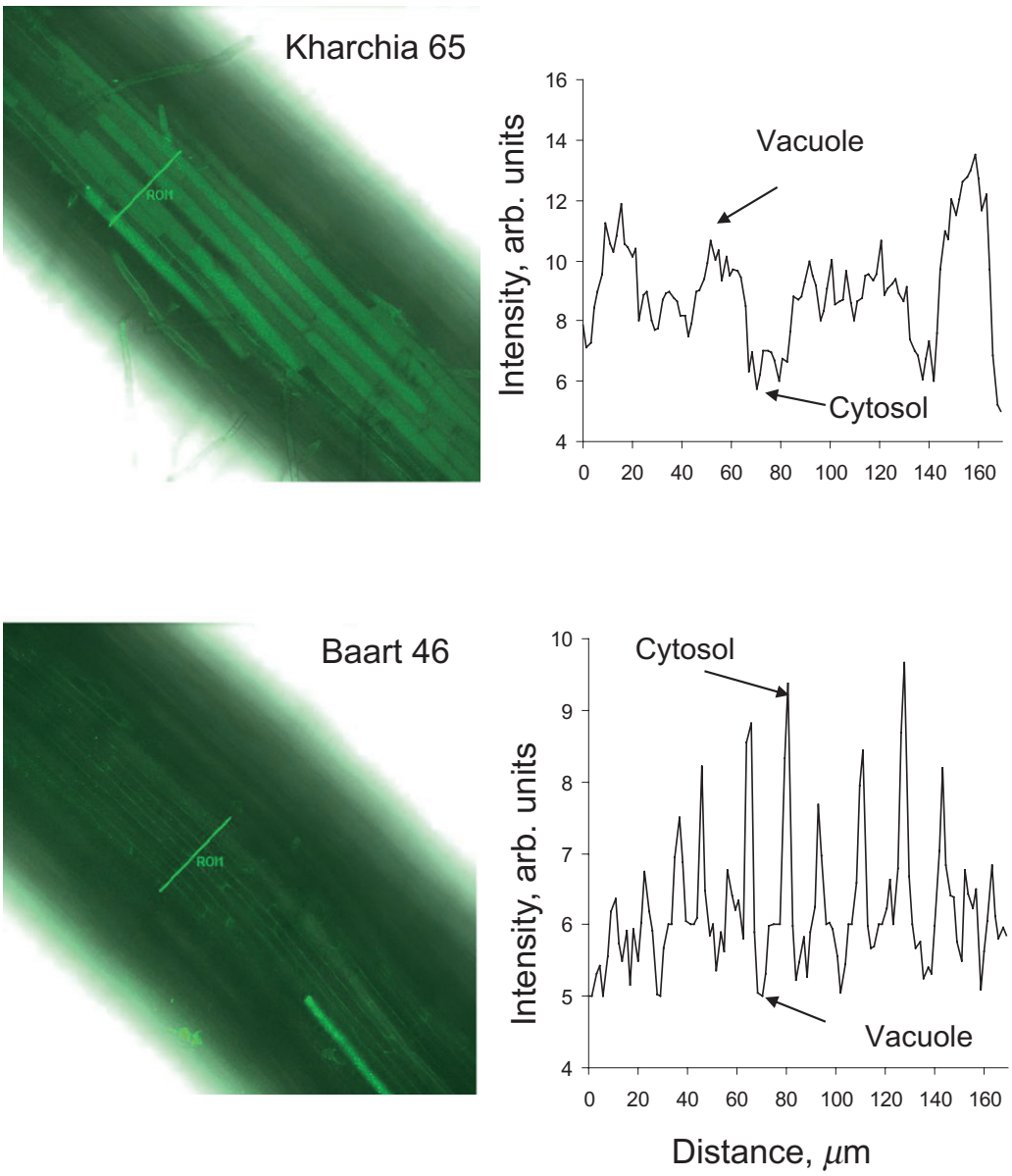

Figure 10. Quantification of the cytosolic to vacuolar Na^+ content ratio in epidermal root cells of two bread wheat genotypes contrasting in their salt tolerance. Kharchia 65 – tolerant; Baart 46 – sensitive. The Na^+ content in each cell compartment is proportional to the intensity of Sodium Green fluorescence (showed in arbitrary units). One (of 8 to 12) typical examples is shown for each cultivar.

Application Suite software. As illustrated in Fig. 10, the cytosolic Na^+ content in the salt-tolerant Kharchia 65 cultivar was consistently (by *c.* 40%) lower than that of the vacuole. Conversely, in the other cultivars examined, the cytosolic Na^+ content was found to be approximately 50% higher than that in the respective vacuole (Fig. 10). As a result, the cytosolic-to-vacuolar Na^+ content ratio ranged from approximately 0.7 in the salt-tolerant Kharchia 65 cultivar to 1.4–1.6 in the other (less tolerant) varieties (Fig. 11), indicating a correlation between vacuolar Na^+ sequestration and salinity tolerance (data not shown). Thus, it would appear that the salt-tolerant Kharchia 65 genotype not only has an intrinsically higher activity of a SOS1-like plasma membrane Na^+/H^+ exchanger, but that this cultivar also has a superior ability to efficiently sequester Na^+ into root cell vacuoles.

In this work, arbitrary but not absolute values for intracellular Na^+ concentrations were used. Although the probe can be calibrated relatively easy, so overcoming this issue, we felt that for comparative purposes of screening, this semi-quantitative method would be perfectly valid and, in fact, is the preferred one. Indeed, if one wants to rank, for example, hundreds of genotypes according to their vacuolar Na^+ sequestration ability, this can be achieved much faster by using arbitrary units, assuming the instrument settings are kept constant.

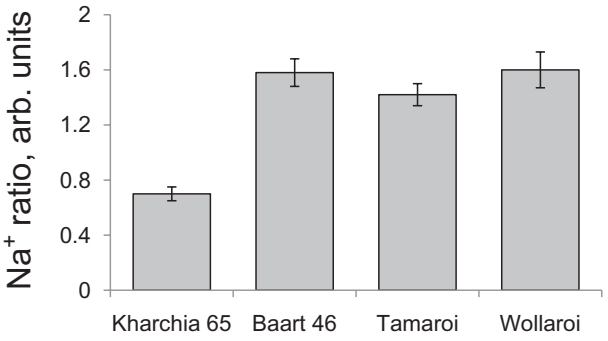

Figure 11. Cytosolic to vacuolar Na^+ ratio in root epidermal cells of wheat plants grown under saline (150 mM NaCl) conditions. The Na^+ content in each compartment was quantified as shown in Fig. 9. Mean ± SE ($n = 6$ to 9 cells).

SOS1 and NHX1 expression levels in roots of bread and durum wheat cultivars

The expression level of both SOS1 and NHX1 genes were investigated using real-time quantitative PCR on total RNA extracted from 150 mM NaCl-treated roots. Both types of transporters were present in all cultivars studied. The highest level of SOS1 transcript accumulation was found in the best Na^+ exporting variety, Kharchia 65, although the results were not statistically significant at $P < 0.05$ (Fig. 11). The two bread wheat varieties, Kharchia 65 and Baart 46, also had much higher level of NHX1 expression compared with the durum wheat varieties Tamaroi and Wollaroi (a twofold difference, significant at $P < 0.05$; Fig. 12).

It could be argued that durum and bread wheats have different size genomes and that the absence of a third genome in durum wheat would predict a lower level of total tissue expression of these two genes. However, while the results demonstrate higher SOS1 expression in Kharchia 65 over Baart 46 and the two durum lines, Tamaroi and Wollaroi, the values are not significant (Fig. 12), whereas NHX1 expression was significantly higher in the two bread wheat lines compared to durum. If the difference in the level of expression was related to the absence of the third genome in durum, it should reflect for both SOS1 and NHX1 and not just for NHX1 alone. Further experiments with chromosome substitution lines (e.g. deletions or additions of the relevant chromosomes) are needed to fully address this issue.

DISCUSSION

The MIFE microelectrode technique can be used for rapid screening of root plasma membrane Na^+ efflux activity

Microelectrode ion flux measurements have been proven to be an efficient tool in studying plant adaptive responses to the environment (Shabala 2006). The MIFE technique has been used to investigate many aspects of the ionic mechanisms of salt stress perception and signalling in various species including barley (Shabala *et al*. 2003, 2005; Chen *et al*. 2005, 2007a,b), wheat (Cuin *et al*. 2008), *Arabidopsis* (Shabala *et al*. 2005) and lucerne (Smethurst *et al*. 2008). In all these studies, the focus has been on NaCl-induced K^+ fluxes and the importance of K^+ retention in salinity tolerance (reviewed by Shabala & Cuin 2008). However, measurements of Na^+ fluxes under conditions of high salinity have been confounded by several methodological limitations.

First, all commercially available Na^+ LIX have a poor Na^+ selectivity as they are sensitive to K^+ and Ca^{2+} as well as Na^+ (Carden, Diamond & Miller 2001; Chen *et al*. 2005), having an almost ideal Nernst response for each of these ions (Chen *et al*. 2005). Because the imposition of salt stress causes a very rapid and massive efflux of both K^+ (a result of membrane depolarization; Shabala *et al*. 2005; Chen *et al*. 2007b) and Ca^{2+} (the result of Donnan exchange in the cell wall; Shabala & Newman 2000), the measured net Na^+ influx is substantially underestimated, and any measured efflux is likely to represent fluxes of K^+ and Ca^{2+}.

Also, a low signal-to-noise ratio in the presence of a high background of Na^+ in the bath solution substantially limits the resolution of Na^+ flux measurements. Patch-clamp studies on epidermal protoplasts isolated from Kharchia roots reported inward Na^+ currents of 20 to 30 mA m^{-2} range when measured at physiologically relevant voltages (Tyerman *et al*. 1997). Similar data were reported in

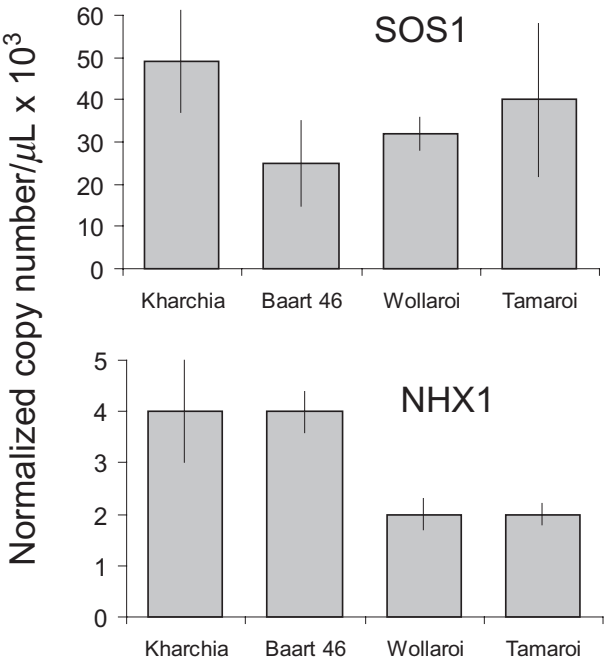

Figure 12. Real-time quantitative PCR measurements of SOS1 and NHX1 transcript levels in the roots of 6-day-old hydroponically grown bread and durum wheat cultivars after exposure to 150 mM NaCl for 24 h. Data points represent mean ± SE ($n = 3$ to 6 batches).

patch-clamp experiments on *Arabidopsis* root protoplasts (Demidchik & Tester 2002). This is equivalent of 200 to 300 nmol m^{-2} s^{-1} flux measured by the MIFE technique and is comparable with the noise level in net Na^+ fluxes measured under the similar experimental conditions (Supporting Information Fig. S4).

The above methodological limitations may be overcome by conducting Na^+ efflux measurements in a Na^+-free medium immediately after the removal of Na^+ from the bathing medium. Accordingly, a 'recovery' protocol was developed (see Materials and Methods), which allows quantification of the net Na^+ efflux from roots of wheat genotypes that have contrasting levels of salinity tolerance. Without background Na^+ in the bathing medium and without the confounding effects of either K^+ or Ca^{2+} fluxes (see below), any measured net Na^+ flux is likely to reflect the actual Na^+ movement across the plasma membrane of the root epidermal cells.

Immediately after the removal of 150 mM NaCl from the bathing medium, a significant Na^+ efflux was measured in all eight cultivars tested (Fig. 2). Twenty minutes after Na^+ removal, the potentially confounding effects of Donnan exchange in the cell wall on Ca^{2+} fluxes are completely eliminated and net K^+ fluxes were stable, relatively small and not significantly different between cultivars (Fig. 3). Even assuming that some long-lasting plasma membrane hyperpolarization may occur in response to Na^+ removal from the bath, the small uptake of K^+ measured for the wheat roots at this time (e.g. Fig. 3) can probably be attributed to the activation of inward-rectifying TEA-sensitive K^+ channels (0.5 mM K^+ as KCl is present in the bathing medium). However, 20 mM TEA^+ was found to have no significant effect on the measured Na^+ fluxes in wheat (Fig. 6), which also indicates that this small influx of K^+ had no confounding effect on the measurements of Na^+ efflux from wheat roots. In *Arabidopsis* however, a substantial net K^+ uptake was still evident 20 min after the removal of Na^+ (data not shown), so K^+ was omitted from the bath solution. This difference between two species warrants further discussion. As evident from our previous publications (Cuin *et al*. 2009), wheat species have a superior ability to retain K^+ in their roots upon NaCl application. This is in a stark contrast to *Arabidopsis* (Shabala *et al*. 2003, 2006) or barley (Chen *et al*. 2005, 2007a,b; Cuin & Shabala 2005, 2007). Moreover, over the longer term, the shoot sap K^+ in wheat actually increases in response to salinity treatment (Cuin *et al*. 2009), while in *Arabidopsis*, a substantial loss of the plant K^+ content over longer-term salinity treatment is observed. Therefore, when NaCl is removed, *Arabidopsis* does its best to replenish the lost K^+; this is not required for wheat species that are capable of better control of their K^+ homeostasis. However, to make the MIFE Na^+ flux measuring method universal and applicable to all species, it is recommended that plant screening be done in a K^+-free solution, with only Ca^{2+} present in the bath. Such measurements should then reflect the actual net Na^+ fluxes across the plasma membrane of root cells.

A SOS1-like plasma membrane transporter is responsible for active Na^+ extrusion from wheat roots

Originally identified and characterized in *Arabidopsis* (Wu *et al*. 1996), the SOS1 Na^+/H^+ antiporter is the only Na^+ efflux protein characterized so far in plants (Hasegawa *et al*. 2000; Sanders 2000; Apse & Blumwald 2007). The AtSOS1 transporter localizes to the plasma membrane, including at the root tip (Shi *et al*. 2002). Overexpression of this transporter improves salt tolerance in transgenic *Arabidopsis* (Shi *et al*. 2003), and its removal in *sos1*-knockout mutants results in a greater accumulation of Na^+ under saline conditions (Wu *et al*. 1996). Here, *in planta* evidence is provided, suggesting that a SOS1-like homolog(s) operates at the root cell plasma membrane in wheat. First, both a protonophore (CCCP) and an inhibitor of H^+-ATPase (vanadate) were found to dramatically reduce the Na^+ efflux (Fig. 6), implying that the Na^+ efflux does rely on the generation of a H^+ gradient across the root plasma membrane. Secondly, NAA, a known activator of H^+ pumping, significantly increased the measured Na^+ efflux (Fig. 6). Thirdly, application of amiloride, a well-known inhibitor of the Na^+/H^+ exchanger in both animal and plant systems (Blumwald & Poole 1985; Kleyman & Cragoe 1988), had the effect of blocking 87% of the measured Na^+ efflux (Fig. 7). Fourthly, Na^+ efflux upon the removal of external NaCl is abolished in *sos1 Arabidopsis* mutant plants (Fig. 8). Finally, the high levels of SOS1 expression in Kharchia 65 (Fig. 12) indicate that this gene could be responsible for encoding a Na^+-exporting transport system. Taken together, these results indicate that a SOS1-like Na^+/H^+ homolog is responsible for the active Na^+ extrusion from wheat roots measured in this study. The absence of a statistically significant difference in the SOS1 expression level between cultivars (Fig. 12) suggests that the difference in Na^+ exclusion rate may be due to post-transcriptional and/or post-translational modification. The presence of another, yet unidentified type of Na^+/H^+ antiporter also cannot be completely ruled out.

Recently, a putative SOS1 homolog has been identified in bread wheat by sequence similarity (Mullan *et al*. 2007). Also, a TaSOS1 protein has been identified in bread wheat (Xu *et al*. 2008). This Na^+-extruding protein is closely related to AtSOS1 and the SOS1 homolog in rice (OsSOS1; Martínez-Atíenza *et al*. 2007), and its expression in heterologous yeast systems has shown that it mediates Na^+/H^+ exchange (Xu *et al*. 2008). We have indications of a high level of expression of a *TaSOS1* in wheat roots of both durum and bread wheat, with notably high levels in the highest Na^+-exporting Kharchia 65 (Fig. 12). Thus, the results presented in this study provide *in planta* evidence of a role for a SOS1-like Na^+/H^+ antiporter system in wheat salinity tolerance, the identification of which requires further study.

SOS1 activity and salinity tolerance in wheat

Among the genotypes examined in the present study, the salt-tolerant variety Kharchia 65 showed the highest Na^+

efflux in MIFE experiments (Fig. 2). Although the higher salinity tolerance in Kharchia 65 cannot be attributed exclusively to higher SOS1 activity at root plasma membrane, even a small increase in Na^+ export would have a large effect on the overall accumulation of Na^+ in a plant over time (Tester & Davenport 2003). Consequently, even relatively small differences in the activity of a SOS1-type Na^+/H^+ exchanger could lead to large differences in Na^+ accumulation in the shoot. It is arguable that the activity of a SOS1-type transporter in the roots of wheat may be a critical factor in the ability of certain cultivars to tolerate saline environments. The fact that such SOS1 activity in Kharchia 65 was much higher compared to the seven other varieties studied may therefore explain why this cultivar is often used as a salinity tolerance 'standard' for wheat (Hollington 2000). Interestingly, Westonia, which is another salt-tolerant wheat bread wheat cultivar (Cuin *et al*. 2009), did not possess this higher Na^+/H^+ antiporter activity, further confirmation that salinity tolerance in wheat cannot be attributed to one specific mechanism alone.

The Na^+ extruded from a root is likely to build up in the rhizosphere. Thus, continued active extrusion would be against an ever-increasing gradient and would become energetically expensive and, thermodynamically, more difficult. Certainly, the cost of this 'futile cycling' of Na^+ could be a considerable burden to the plant and may be a significant contributor to saline toxicity (Yeo 1998; Tester & Davenport 2003; Britto & Kronzucker 2006; Kronzucker *et al*. 2006). Interestingly, when under saline conditions and in contrast to *Arabidopsis*, the halophyte *Thellungiella halophila*, appears to restrict unidirectional influx of Na^+ rather than increasing efflux (Wang *et al*. 2006), and a similar response is seen in the halophyte monocot *Puccinellia tenuiflora* compared with wheat (Wang *et al*. 2009). If influx is restricted, the energetic cost of Na^+ export is reduced. Sodium extrusion would also result in increased osmotic stress to the plant as salt levels build up in the root zone. Thus, total exclusion of Na^+ from the plant may not be of benefit to the plant in the longer term. If Na^+ could be efficiently sequestered into the cell vacuole, it would act as a cellular osmoticum and, at the same time, the accumulation of Na^+ in the rhizosphere would be reduced. The overall effect would be to decrease the water potential in the plant and reduce the need for the synthesis of energetically expensive compatible solutes.

Evaluating vacuolar Na^+ sequestration ability using Sodium Green[1]

Of the four cultivars studied, confocal imaging using Sodium Green (Figs 9 & 10) clearly demonstrated that only Kharchia 65 plants were capable of efficiently sequestering Na^+ into the root cell vacuoles, while a greater proportion of Na^+ was located in the root cell cytosol in the more sensitive varieties (Fig. 11). This is despite a similar level of NHX1 expression in Baart 46 and Kharchia 65 (Fig. 12). This implies either a post-translational or post-transcriptional modification of functional activity that results in the up-regulation of activity in Kharchia 65, or alternatively, the involvement of other NHX-type transporters in vacuolar Na^+ sequestration in wheat. Although the ability of Kharchia 65 to extrude Na^+ from the roots may contribute to salinity tolerance, this strategy may only be 'buying time' for a controlled transfer of Na^+ to the shoots and its subsequent sequestration into the cell vacuole of both roots and shoots, where the Na^+ can serve as an osmoticum. Experiments with Sodium Green or other intracellular Na^+ measuring techniques in leaves are needed to fully address this issue.

The reported difference in vacuolar Na^+ sequestration shown in Figs 9–11 is most pronounced in the epidermal cells. It could be argued that the epidermis occupies only a small portion of the root tissue volume and that cortex cells and the stele behave differently. It may be envisaged that the cortical cells primarily fulfil a passage role, and so do not play a major role in Na^+ sequestration. More likely, however, their role becomes critical after much longer NaCl exposure. This issue is clearly beyond the scope of this paper and has to be addressed in a separate study.

Prospects and conclusions

This work provides the first functional assessment of the activity of two major transport systems involved in cytosolic Na^+ exclusion in wheat roots. *In planta* protocols were developed that target the important physiological trait of Na^+ exclusion from the cytosol by quantifying the relative contribution of SOS1 and NHX Na^+/H^+ exchangers. This approach may be used in the 'pyramiding' of physiological traits behind salinity tolerance, so contributing to the ultimate aim of developing salt-tolerant crops. Also, when combined with marker-assisted selection, these techniques may be of application in locating (and, eventually, cloning) the gene(s) that underpin salinity tolerance.

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[1]While this paper was being prepared, a study by Oh *et al*. (Plant Physiology (2009) 151: 210–222) was published reporting the use of another sodium-specific fluorophore, Corona Na-Green, to quantify Na^+ distribution in root tissues of *Thellungiella*, a salt-tolerant relative of *Arabidopsis*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Roots of 6-day-old wheat seedlings grown hydroponically in BSM solution (0.5 mM KCl + 0.1 mM $CaCl_2$). (a) Baart 46, (b) Kharchia 65, (c) Seville 20, (d) Westonia, (e) Odin, (f) Tamaroi, (g) Towner and (h) Wollaroi.

Figure S2. Root cross sections taken at 10 mm (top row) and 40 mm (bottom row) from the root tip of four 6-day-old bread wheat genotypes. (a) Baart 46, (b) Kharchia 65, (c) Seville 20 and (d) Westonia. The horizontal yellow bar is equivalent to 200 μm. Roots were stained with 0.5% w/v aqueous solution of Safranin O.

Figure S3. Distance to the first root hair from the root tip of 6-day-old wheat seedlings grown hydroponically in BSM (0.5 mM KCl + 0.1 mM $CaCl_2$). Mean ± SE (n = 7 to 8 seedlings).

Figure S4. A close up look at transient net Na^+ flux responses from Kharchia 65 roots after 100 mM NaCl treatment. Mean ± SE (n = 6).

Table S1. Primers used for quantitative real-time PCR

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