



Review article

Sodium efflux in plant roots: What do we really know?



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ARTICLE INFO

Article history:

Received 23 June 2015

Received in revised form 3 August 2015

Accepted 3 August 2015

Available online 15 August 2015

Dedicated to the memory of André Läuchli.

Keywords:

Sodium

Efflux

Ion transport

Energetics

Compartmental analysis

Toxicity

ABSTRACT

The efflux of sodium (Na^+) ions across the plasma membrane of plant root cells into the external medium is surprisingly poorly understood. Nevertheless, Na^+ efflux is widely regarded as a major mechanism by which plants restrain the rise of Na^+ concentrations in the cytosolic compartments of root cells and, thus, achieve a degree of tolerance to saline environments. In this review, several key ideas and bodies of evidence concerning root Na^+ efflux are summarized with a critical eye. Findings from decades past are brought to bear on current thinking, and pivotal studies are discussed, both “purely physiological”, and also with regard to the SOS1 protein, the only major Na^+ efflux transporter that has, to date, been genetically characterized. We find that the current model of rapid transmembrane sodium cycling (RTSC), across the plasma membrane of root cells, is not adequately supported by evidence from the majority of efflux studies. An alternative hypothesis cannot be ruled out, that most Na^+ tracer efflux from the root in the salinity range does not proceed across the plasma membrane, but through the apoplast. Support for this idea comes from studies showing that Na^+ efflux, when measured with tracers, is rarely affected by the presence of inhibitors or the ionic composition in saline rooting media. We conclude that the actual efflux of Na^+ across the plasma membrane of root cells may be much more modest than what is often reported in studies using tracers, and may predominantly occur in the root tips, where SOS1 expression has been localized.

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1. Introduction

It is estimated that soil salinity occurs over 6–10% of the earth's land surface, or as much as 900 million hectares, including 20–50% of all irrigated farmlands, which provide nearly half the food used by humans (Shabala, 2013; Pessaraki and Szabolcs, 2011).

Substantial declines in the productivity of agricultural plants in many regions are caused by excessive concentrations of sodium ions (Na^+) in soil solution, which exert deleterious effects on plant water status, nutrient acquisition, and metabolic pathways (Munns and Tester, 2008; Kronzucker and Britto, 2011). The sensitivities and tolerances of higher plants to Na^+ have been linked both to the transport of this ion into plant tissues, cells, and sub-cellular compartments, and also to its exit, or efflux, from the plant back to the external environment. Accordingly, the activities of transport systems (e.g., membrane-bound, vascular) moving

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Na⁺ into, within, and from the plant have long been the subject of intense theoretical and applied study by plant physiologists. Given this effort, however, it is surprising that Na⁺ transport under saline conditions remains poorly understood (Kronzucker and Britto, 2011; Cheeseman, 2013). In the modern context, it is revealing for instance that no plasma-membrane transporters responsible for the primary influx of Na⁺ into root cells have as yet been definitively identified at the genetic level. For Na⁺ transport in the opposite (efflux) direction, only one transporter, SOS1 ("Salt Overly-Sensitive 1"), has been genetically characterized, but, despite a very large number of published studies on SOS1, its precise functional character is not well understood, nor is the general physiology or ecological significance of Na⁺ efflux from the root.

Despite our limited understanding, a general model of Na⁺ transport in plants under salinity has become accepted, based mainly on physiological observations of unidirectional and net Na⁺ fluxes and their thermodynamics (e.g., Higinbotham et al., 1967; Pitman and Saddler, 1967; Cheeseman, 1982; Cheeseman et al., 1985; Lazof and Cheeseman, 1986, 1988a,b; Schubert and Lauchli, 1988; Essah et al., 2003; Tester and Davenport, 2003; Davenport et al., 2005; Kronzucker et al., 2006; Malagoli et al., 2008; Wang et al., 2009). In this model, it is proposed that Na⁺ passively enters root cells at rates which can be extremely high (often one or two orders of magnitude higher than nutritionally relevant fluxes of ions such as K⁺; see Britto and Kronzucker, 2009), via non-selective cation channels, and driven by the twin forces of an inwardly negative membrane potential across the plasma membrane (typically 80–200 mV), and a cytosolic Na⁺ concentration ($[Na^+]_{cyt}$) of possibly no greater than ~40 mM (Carden et al., 2003; Kader and Lindberg, 2005; Munns and Tester, 2008; Britto and Kronzucker, 2009), i.e., significantly less than external concentrations of Na⁺ ($[Na^+]_{ext}$) under saline conditions. Further to the model, sodium ions subsequently exit the cell at rates nearly as high as influx rates, via the SOS1 protein, which catalyzes secondarily active Na⁺ transport (i.e., Na⁺/H⁺ antiport) at the plasma membrane (Fig. 1; Britto and Kronzucker, 2006; Munns and Tester, 2008; Kronzucker and Britto, 2011 cf. Garciadeblás et al., 2007). The net result is that only a small fraction of Na⁺ entering the symplasm remains there, the rest being shuttled back to the root medium. In this model, the efflux component of Na⁺ transport is considered to be of fundamental importance to plant salinity tolerance, as it limits the cellular accumulation of Na⁺ in the root, which could otherwise lead to the poisoning of enzymatic and transport processes, and to the increased translocation of Na⁺ to the shoot, where it can inhibit photosynthesis (Munns, 2002; Tester and Davenport, 2003; Munns and Tester, 2008; Kronzucker et al., 2013).

While this model of rapid transmembrane sodium cycling (RTSC) appears, at least superficially, to offer a plausible explanation for the common observation that Na⁺ rapidly cycles in and out of plant roots (Malagoli et al., 2008 and references therein), and, moreover, suggest a means by which plants may engage membrane transport systems to achieve salt tolerance, it is beset with a number of problems that may limit its utility and acceptability. These include, perhaps most significantly, the very large energy expenditure that would be required to maintain the active-transport (efflux) phase of the rapid cycle (Malagoli et al., 2008; Britto and Kronzucker, 2009). For instance, in a tracer-efflux study in rice roots, we have calculated, using an established flux-energetics model (Kurimoto et al., 2004), that the rate of oxygen use required by respiring roots to drive the apparent Na⁺ efflux (at a rate of 107 $\mu\text{mol g}^{-1}(\text{FW}) \text{h}^{-1}$) would be double that of the actual, measured, oxygen consumption of the entire root, were the RTSC model to be correct (Malagoli et al., 2008). We have catalogued similarly excessive rates of calculated oxygen demand elsewhere (Britto and Kronzucker, 2009). Another consequence of the pronounced efflux often reported for Na⁺ under salinity conditions is that it

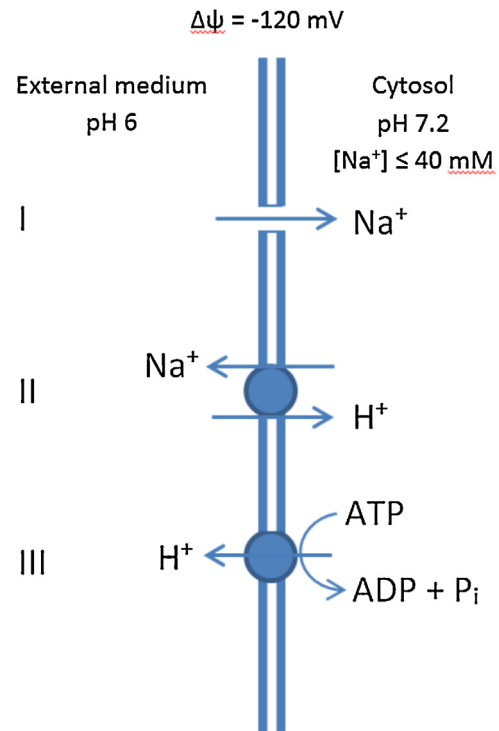


Fig. 1. Depiction of the current model of rapid transmembrane sodium cycling (RTSC) occurring across the plasma membrane of root cells under saline conditions. Numerals I, II, and III refer to the three major transport components of the cycle: I, Na⁺ influx via cation channels (probably non-selective); II, Na⁺ efflux via Na⁺/H⁺ antiport (SOS1); III, regeneration of the proton gradient, which provides a thermodynamic driving force for steps I and II. $\Delta\Psi$ refers to the electrical potential difference across the membrane.

is linked to the measurement of very large cytosolic Na⁺ pools, under the assumption that these are the pools from which efflux traces originate (see Britto and Kronzucker, 2001). The cytosolic Na⁺ concentrations often found using compartmental analysis by tracer efflux (CATE) are typically much higher than those found using more direct (and more widely accepted) methods such as ion-selective intracellular electrodes (see comparison of estimates in Kronzucker and Britto, 2011), a discrepancy that requires explanation.

Other problems with the RTSC model include the lack of rigorously analyzed efflux traces in physiological studies, in terms of identification of compartments from which these traces originate (see Sections 3 and 7), the consequences of the RTSC model for flux analyses, and the lack of information to corroborate physiological observations with findings from genetics and molecular biology. We have presented aspects of this critique elsewhere (Malagoli et al., 2008; Britto and Kronzucker, 2009; Kronzucker and Britto, 2011; Kronzucker et al., 2013), but have not until now focused specifically on the efflux component of the current Na⁺ transport model. In this review, we therefore aim to critically assess the evidence for the involvement of a powerful Na⁺ efflux mechanism (or mechanisms), at the plasma membrane of root cells, in the context of salt tolerance by plants. It is hoped that an analysis of this nature will help guide the way through the current impasse in the understanding of Na⁺ transport mechanisms in plants.

2. A hypothesis

Our discussion will be framed in part by a hypothesis that is rarely considered in discussions of Na⁺ efflux (cf. Pitman, 1963; Ritchie and Larkum, 1984), but which may be key to the resolution of problems surrounding this phenomenon. In brief, we propose

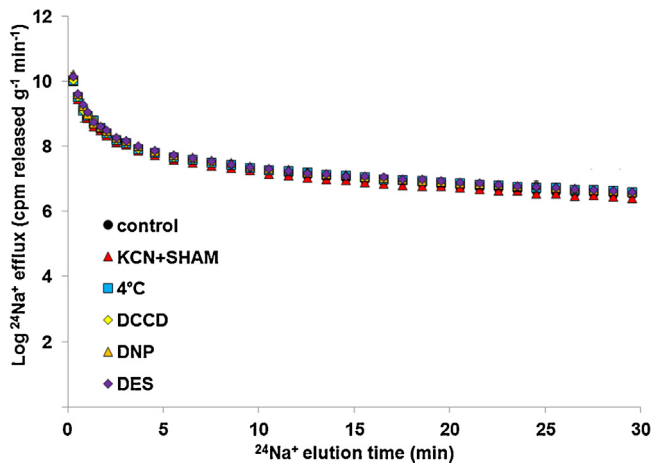


Fig. 2. Representative plots of Na^+ efflux traces from roots of intact barley seedlings labelled with $^{24}\text{Na}^+$ at 150 mM external $[\text{Na}^+]$. Inhibitors were present for 70 min prior to, and also during, tracing. Dashed line indicates regression of slow phase to obtain initial efflux from this phase. Redrawn from Schulze (2013).

that unidirectional sodium-efflux traces (and time-courses of Na^+ influx; see Section 3) made using Na^+ tracers (e.g., $^{22}\text{Na}^+$, $^{24}\text{Na}^+$), which provide the empirical foundation of the current model of rapid, transmembrane sodium cycling, may have been incorrectly concluded to represent the energy-linked movement of Na^+ across cellular plasma membranes in the root system. An alternative possibility, which cannot be dismissed at present, is that, under salinity conditions, a significant, or dominant, portion of tracer released from roots in efflux (“washout”) experiments does not represent a transmembrane flux but rather the cycling of Na^+ through the root via extracellular (apoplastic) regions of the root. If such an apoplastic hypothesis is borne out, the RTSC model will need to be discarded or redefined.

3. Methodological summary

It is worthwhile first to summarize the range of methods used to investigate the efflux of ions in general from plant roots, with special consideration to sodium efflux. At the outset, we emphasize that, strictly speaking, efflux in the RTSC model refers to a *unidirectional* flux (as distinct from a net flux), which, unfortunately, greatly limits the range of methods available for its study. Indeed, the use of tracers has appeared to provide the only means available for measurement of unidirectional efflux under steady-state conditions (i.e., not in response to perturbations in ion supply; Greenshpan and Kessler, 1970; Lazof and Cheeseman, 1986; Fraile-Escanciano et al., 2010), and therefore will be considered first.

Using CATE, direct efflux experiments with tracers such as $^{22}\text{Na}^+$ and $^{24}\text{Na}^+$ involve the immersion of intact or excised roots in a radioactive solution during a “labelling” period, typically ranging from 1 to 24 h, and followed by a “washout” period during which roots are eluted of radioactivity into a timed series of non-radioactive aliquots (Fig. 2; Walker and Pitman, 1976; Britto and Kronzucker, 2012; Coskun et al., 2014). The specific activity of the released tracer is estimated using the kinetics of tracer release, which under steady-state conditions are typically exponential and assumed to be similar to the kinetics with which the compartments that release tracer become labelled (Walker and Pitman, 1976; Britto and Kronzucker, 2001). In general, the accuracy of this form of analysis depends critically on the correct identification (“phase identification”—e.g., Kronzucker et al., 1995) of the releasing compartments, although in practice this identification is rarely performed (cf. Pitman, 1963; Lazof and Cheeseman, 1986; Ritchie

and Larkum, 1984; Kronzucker et al., 1995; Britto and Kronzucker, 2003; Coskun et al., 2010; see Section 7).

An important means by which compartment identification can be approached is through the application of physical and chemical treatments, or genetic constructs, that have the potential to modify tracer-release kinetics (e.g., changes in temperature, presence of competing substrates, provision of metabolic or channel/transporter inhibitors, altered expression of transporter genes). For any given experimental system, it is critical that such treatments be known to work properly, and thus eliminate confounding possibilities such as the lack of penetration of a chemical inhibitor through the layered tissues of the root cross-section, or to justify the use of an inhibitor known to be effective in animal systems but not in plants (e.g., ouabain; see Section 6).

Following such an approach, we have previously presented an in-depth analysis of potassium ($^{42}\text{K}^+$) efflux from labeled roots of barley seedlings (Coskun et al., 2010), and found that it could indeed be inhibited by a wide array of treatments, but only when K^+ was provided at external concentrations ($[\text{K}^+]_{\text{ext}}$) less than 1 mM. In stark contrast, efflux traces obtained above 1 mM $[\text{K}^+]_{\text{ext}}$ were completely resistant to the same treatments (Coskun et al., 2010). Interestingly, this threshold is close to that at which the thermodynamics of K^+ import into root cells switches from an energetically active state to a passive one (and at which the influx mechanism switches from a high-affinity, proton-coupled mechanism, to a low-affinity, channel-mediated one (Epstein et al., 1963; Szczerba et al., 2009)). It was shown that, under active-influx conditions, a passive, channel-mediated, mechanism, transporting K^+ down its electrochemical potential gradient (in this case, via Shaker-type efflux channels), was responsible for K^+ efflux below 1 mM $[\text{K}^+]_{\text{ext}}$, while no evidence in support of active efflux at higher $[\text{K}^+]_{\text{ext}}$, could be found. We concluded that the recalcitrant efflux traces still observed under these conditions represented K^+ movement not across the plasma membrane, but rather through relatively slowly exchanging apoplastic (extracellular) fractions of the root (Coskun et al., 2010). Importantly, the efficacy of inhibitors at low $[\text{K}^+]_{\text{ext}}$ confirmed that the lack of inhibitory effects at high $[\text{K}^+]_{\text{ext}}$ was not due to their exclusion from sites of action, e.g., due to barriers imposed by root geometry (see above). This study illustrates some of the unexpected findings and pitfalls that may be encountered while engaged in efflux analysis, and underscores the requirement that efflux traces be rigorously examined (also see Walker and Pitman, 1976; Zierler, 1981; Lazof and Cheeseman, 1986, 1988a,b; Jacquez, 1996). We shall return to this crucial issue in our discussion of published reports of Na^+ tracer efflux under saline conditions, and in the context of the apoplastic hypothesis outlined in Section 2.

Several indirect tracer-based methods of measuring efflux involve comparisons between the unidirectional influx of a traced substance and its net accumulation in plant tissues (also with tracer; Davenport et al., 1997; Chen et al., 2007). Similarly, efflux has been characterized in experiments in which time-courses of tracer influx are analyzed (e.g., using $^{22}\text{Na}^+$ or $^{24}\text{Na}^+$; Lazof and Cheeseman, 1986, 1988a,b; Essah et al., 2003; Malagoli et al., 2008). The rationale behind this latter approach is that, the longer a plant root system is exposed to tracer in an influx time-course, the smaller the overall rate of tracer accumulation will be (and the more a net flux will be approximated), because over time a greater portion of incoming tracer will be lost through efflux. The intensity of this decline will be proportional to the intensity of efflux relative to influx, and to the turnover rate of the pool from which the efflux trace originates (e.g., the cytosol; see Britto and Kronzucker, 2001). However, this procedure can produce problematic results if administered under non-steady-state conditions, particularly if a “shock treatment” is involved (Munns, 2002; Cheeseman, 2013). For example, in a widely cited study by Essah et al. (2003), a non-steady-state

design was used to show changes in unidirectional uptake of $^{22}\text{Na}^+$ by excised roots of *Arabidopsis*, using a shock treatment of 200 mM NaCl. Within 2 or 3 min, the net influx of Na^+ ceased altogether in these roots. While the authors attributed this to a very rapid and efficient efflux mechanism, it is problematic that a net Na^+ flux should become zero so quickly, in a species known to accumulate Na^+ over much longer periods of time (e.g., Guo et al., 2008). It should be emphasized that compartment identification is as essential in studies using this type of method as it is with direct efflux measurements.

A major non-tracer method of measuring efflux, which has proven to be of wide utility in recent years, involves ion-specific, extracellular, microelectrode techniques (e.g., Shabala et al., 2005a,b; Guo et al., 2008; Cuin et al., 2011). While this method allows for the real-time, in-vivo, non-invasive tracking of the transport of a wide range of ions (including Na^+ , Ca^{2+} , K^+ , and H^+), however, it cannot be used to measure unidirectional fluxes under steady-state conditions (and thus cannot provide evidence for or against the RTSC model), but can only track net fluxes of ions. For this reason, virtually all reports of Na^+ efflux from roots using this type of microelectrode system are characterized by sudden, often extreme, changes in the external concentration of Na^+ ($[\text{Na}^+]_{\text{ext}}$, typically from several hundred millimolar, to 1 mM or less). While this methodology has provided considerable insight into Na^+ transport in plants, it is also deeply problematic in that the concentration shifts entail a radical alteration in the thermodynamics of transport, favoring a passive outward flow of Na^+ from root cells, via systems that may not, under normal conditions, mediate Na^+ efflux (Fraile-Escanciano et al., 2010). In addition, the Na^+ -specific microelectrode cocktails in these procedures are still relatively new, and can pose problems including insufficient discrimination between Na^+ and similar ions such as K^+ (Shabala et al., 2005a,b; Cuin et al., 2011; Jayakannan et al., 2011; Lu et al., 2013).

Sodium efflux from roots has also been examined by measuring the accrual of Na^+ in an initially Na^+ -free solution bathing the roots of plants previously exposed to Na^+ (e.g., Mengel and Pflüger, 1972; Nassery, 1972; Takahashi et al., 2007). This approach is beset with a problem similar to that described above for microelectrodes, i.e., a dramatic change in the thermodynamic gradient for Na^+ transport, and a movement away from steady-state conditions. This problem is also encountered in experiments where efflux is estimated by measuring the loss of Na^+ from plant tissues after transfer to Na^+ -free solution (e.g., Oh et al., 2009; Fraile-Escanciano et al., 2010). New developments in Na^+ -specific fluorescent dyes (Halperin and Lynch, 2003; Kader and Lindberg, 2005; Anil et al., 2007) and triple-barrelled Na^+ -selective microelectrodes (Carden et al., 2003), however, offer the prospect of finer resolution of changes in symplastic $[\text{Na}^+]$ in the cytosol and vacuole, which might be attributable to changes in Na^+ efflux. However, few reports exploiting these new possibilities have been published so far (cf. Anil et al., 2007).

Yet another method estimates Na^+ efflux by monitoring increases in net Na^+ uptake brought about by metabolic inhibition of the putatively active efflux mechanism(s) (e.g., Mennen et al., 1990). Two final methods, both rather specialized, should be briefly mentioned here. The first is the use of nuclear magnetic resonance (NMR), by which active Na^+ efflux from root tips of maize and *Spartina anglica* was inferred from ^{23}Na -NMR observations of vanadate-stimulated net Na^+ uptake (Spickett et al., 1993). The second is the use of plasma-membrane-derived vesicles in conjunction with pH-sensitive dyes such as quinacrine (Hassidim et al., 1990; Allen et al., 1995; Wilson and Shannon, 1995; Qiu et al., 2002; Rubio et al., 2011). By this method, it has been shown, for instance, that the SOS1 protein operates via a Na^+/H^+ antiport mechanism (Qiu et al., 2002).

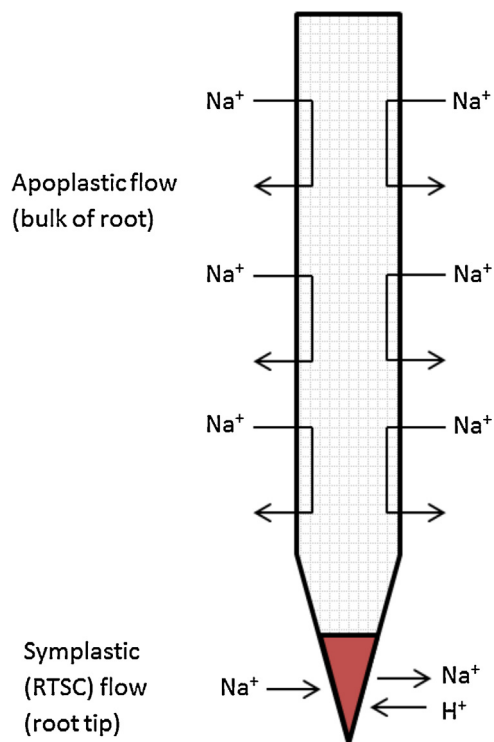


Fig. 3. Revision of RTSC model (Fig. 1) in an idealized section of root, showing the greater part of Na^+ efflux to be apoplastic, with a small amount of rapid transmembrane Na^+ cycling occurring via SOS1 at the root tip.

4. The phenomenon of ionic efflux from plant roots

For comparative purposes, a few remarks on the general nature of ionic efflux from plant root cells will be useful. While hundreds of studies demonstrating this phenomenon can be found in the primary literature (see, e.g., Britto and Kronzucker, 2006; for a brief review), the underlying mechanisms and physiological utility of efflux processes remain poorly understood, with a few exceptions. These exceptions include the efflux of ions such as H^+ and Ca^{2+} via specialized ATPases (Sze et al., 1999, 2000; Mäser et al., 2001), and the efflux of potassium ions (K^+) from root cells. The latter is known to occur via outwardly rectifying K^+ channels, and provides a means by which electrical homeostasis may be rapidly achieved in response to stimuli that cause membrane depolarization (Maathuis and Sanders, 1996; Shabala et al., 2006). Efflux also functions in the removal of harmful substances from within the cell, as we have proposed in the case of excessive ammonium (NH_4^+) supply to plant roots (Britto et al., 2001; Coskun et al., 2013), and which is widely regarded to be essential to the present case, that of excessive Na^+ supply. In this respect, efflux of toxic materials from root cells resembles the efflux of heavy metals and antibiotics from bacterial cells, an important pathway of drug resistance in medical microbiology (Nikaido, 1994; Nies, 2003). In the case of Na^+ , the NHX1 transporter located in the tonoplast membrane of the vacuole can provide a function similar to efflux across the plasma membrane, by removal of Na^+ from the cytosolic compartment (Apse et al., 1999). A third function of efflux transporters in plant roots might be to provide additional control mechanisms for nutrient uptake processes; for example, in cases where the influx of a nutrient exceeds plant assimilation and sequestration capacities, efflux systems could act as “overflow valves”. In the case of Na^+ at low external concentrations, considerable work has been done to describe the physiological character of K^+ -stimulated Na^+ efflux from roots (see Jeschke, 1983; for review), which may be a mechanism by which plants achieve selectivity for the preferred nutrient

cation (K^+) over the less preferred one (Na^+ ; see Kronzucker et al., 2013; for review of Na^+ as a plant nutrient).

Nevertheless, many fundamental questions pertaining to efflux from plant root cells remain open. One paradoxical (though readily observable) issue can be seen in the case of several nutritionally important ions, such as K^+ , NH_4^+ , and nitrate (NO_3^-), which display substantial steady-state effluxes from roots, even when their external supply is low, indeed limiting to plant growth (e.g., Memon et al., 1985; Kronzucker et al., 1997; Kurimoto et al., 2004; Britto and Kronzucker, 2006; Coskun et al., 2010). There is no satisfactory explanation for this aspect of efflux behavior, which is made still more intriguing by the possibility that, in some experimental systems, efflux across the plasma membrane of root cells might only operate under relatively low substrate provision (Coskun et al., 2010; see discussion of K^+ efflux in Section 3).

At higher substrate concentrations, i.e., in the millimolar range (i.e., 1–100 mM or higher, often termed the “low-affinity” range), it is notable that a linear increase in the unidirectional influx of that substrate is typically seen (Kronzucker and Britto, 2011), in contrast to the saturating influx usually seen in the lower (“high-affinity”) concentration range. In the case of Na^+ and other ions, it is also commonly observed that unidirectional efflux increases even more steeply than influx does as substrate concentrations increase, resulting in an increasingly rapid cycle, and a ratio of efflux to influx that approaches unity (Britto and Kronzucker, 2006). While this issue may be mainly of academic importance for most ions, which rarely reach such high concentrations in soils, it is of potentially great practical significance in the case of sodium.

5. How important is Na^+ efflux to Na^+ tolerance?

Central to the model depicted in Fig. 1 is the idea that Na^+ efflux from plant root cells is essential in protecting the plant from the deleterious effects of sodium over-accumulation in the cytosol (Munns, 2002; Munns and Tester, 2008; Kronzucker et al., 2013). This requirement appears to be particularly important given the widespread observation that roots of many species exhibit extremely high rates of Na^+ uptake under saline conditions (often well over $100 \mu\text{mol g}^{-1}(\text{FW}) \text{h}^{-1}$; reviewed in Britto and Kronzucker, 2006 also, see Section 6). If such rates are genuine, even a small decrease in Na^+ efflux could therefore result in a substantial net accumulation of Na^+ within the cell. Therefore, the idea has been put forward that the maximization of efflux is a desirable trait among salt-tolerant plants (Tester and Davenport, 2003; Cuin et al., 2011). However, it should be noted that many species of halophytic plants, which thrive under saline conditions, exclude Na^+ from their tissues to a lesser extent than glycophytes, probably to maintain sufficiently negative water potential; moreover, even in some glycophytes, the extent of sodium accumulation is not clearly linked to salt sensitivity (e.g., barley, wheat, rice, and *Arabidopsis thaliana*; Yeo et al., 1990; Rajendran et al., 2009; Jha et al., 2010; Mian et al., 2011; see Kronzucker et al., 2013, for discussion), while some authors have suggested that the sodium sensitivity of corn is linked to its tendency to exclude Na^+ (e.g., Schubert and Lauchli, 1986). It should also be noted that, in cases where sodium accumulation is beneficial, Na^+ ions nevertheless still appear to be transported out of the cytosolic compartment, though instead of moving across the plasma membrane to the external medium, they are moved across the tonoplast membrane and sequestered in the vacuole via NHX1 (this constitutes what is known as “tissue tolerance”; Munns and Tester, 2008; Kronzucker et al., 2013; see above). Given the multiplicity of strategies by which plants respond to high soil Na^+ concentrations, the difficulty of drawing broad conclusions about mechanisms of sodium tolerance and toxicity amongst the vast diversity of plants on earth should be kept in mind.

Moreover, there is substantial evidence in the literature contradicting the idea that an increased rate of Na^+ efflux is characteristic of Na^+ -tolerant plants. For example, studies comparing closely related species or cultivars differing in Na^+ tolerance do not always indicate higher efflux in the tolerant plants. These include studies comparing *Citrus* species (Greenspan and Kessler, 1970), *A. thaliana* and *Thellungiella salsuginea* (Wang et al., 2006; the lower efflux reported here for the halophyte *T. salsuginea* is perhaps surprising, given that Oh et al., 2009; found there to be higher SOS1 transcript abundance in *T. salsuginea* under all conditions tested), *Triticum aestivum* and *Puccinellia tenuiflora* (Wang et al., 2009), and between two cultivars of *Zea mays* (Schubert and Lauchli, 1990). In two of these studies (Greenspan and Kessler, 1970; Wang et al., 2009), it was suggested that salt sensitivity in the species exhibiting higher efflux may have been due to the energy costs of active Na^+ efflux (see Section 1, and Lessani and Marschner, 1978). In another, rather striking, example, Ding and Zhu (1997) concluded there to be no difference in $^{22}\text{Na}^+$ efflux between wild-type *A. thaliana* and a mutant lacking the putative efflux transporter SOS1. Even more peculiarly, there was much less sodium accumulation in the *sos1* plants, across the entire range of tested $[Na^+]_{\text{ext}}$ (5–75 mM), despite the characterization of these plants as 20 times more sensitive to Na^+ than the wild type. More recently, Jacobs et al. (2011) showed that expression in rice plants of PpENA1, a sodium-pumping ATPase from the moss *Physcomitrella patens*, conferred greater biomass production under salt stress in the transgenic plants; however, the enhanced growth could not be attributed to improved exclusion of Na^+ , nor to lower Na^+ accumulation in root or leaf.

One final example deserves mention, albeit with a caveat. In a study involving seven plant species varying in Na^+ tolerance (a total of nine cultivars), Lessani and Marschner (1978) showed strong inverse correlations between Na^+ tolerance and Na^+ efflux from roots of plants growing at 25, 50, and 100 mM $[Na^+]_{\text{ext}}$. The authors also suggested that the energy required for active efflux may have been an underlying reason for this correlation (see Section 1). It should be noted, however, that in this study $^{22}\text{Na}^+$ was fed to the leaves of the experimental plants, so the variations in $^{22}\text{Na}^+$ efflux from the roots may have reflected processes upstream from the actual efflux step, such as the loading of $^{22}\text{Na}^+$ into the phloem.

On the other hand, Elphick et al. (2001) were able to show reduced $^{22}\text{Na}^+$ efflux in *sos3* mutants of *A. thaliana* (*SOS3* is a calcium-binding upstream regulator of *SOS1*, which acts via the kinase protein, *SOS2*; Qiu et al., 2002). Moreover, a comparison between salt-tolerant and -sensitive accessions of reed (*Phragmites australis*) showed five-fold higher efflux in the tolerant accession (Takahashi et al., 2007). Cuin et al. (2011), using extracellular ion-selective electrodes, also showed higher efflux in cv. Kharchia 65, known as the “standard” of salt-tolerance in wheat, and which simultaneously showed the highest expression of *SOS1*. In the case of four barley cultivars examined by Chen et al. (2007), strong differences in Na^+ influx were not seen, but net flux was significantly lower in the tolerant cultivars; this was attributed to a higher efflux of Na^+ in these cultivars, although efflux was not directly measured. In general, enhanced expression of *SOS1* in many plant species is associated with their improved growth under saline conditions (Kronzucker and Britto, 2011), but unidirectional efflux of Na^+ has almost never been measured in roots of these transgenic organisms (see Section 7).

6. Other contrasting findings

The conflicting findings about the importance of Na^+ efflux reflect a substantial body of conflicting results in the broader realm of Na^+ efflux research. Many of these results are contrary to the RTSC model in Fig. 1, and their variability may preclude at present

a more general model of this phenomenon. Some of these inconsistencies may be due to differences in methods (see Section 3), levels of Na^+ supply, or to physiological variability among plant species. For example, Nassery (1972) showed that Na^+ loss (under non-steady-state conditions) from barley roots was reduced by low temperature and by the powerful ionophore dinitrophenol (DNP), whereas in bean roots, these same treatments stimulated Na^+ loss.

In another non-steady-state efflux study, in corn, low temperature and anaerobiosis were also found to have no effect on Na^+ efflux from roots, while, by contrast, producing a significant stimulation of K^+ efflux (Mengel and Pflüger, 1972). On the other hand, a steady-state radiotracer study in the same species showed that low temperature stimulated Na^+ efflux slightly (Schubert and Lauchli, 1988). In this study, it was remarkable that two powerful inhibitors of ion transport and cellular metabolism, *N*-ethylmaleimide (NEM) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), produced no changes in the $^{22}\text{Na}^+$ efflux trace, even though they both had strong depolarizing effects on the membrane potentials of root cells in the same study (see Section 7). Moreover, the lack of an NEM effect suggests that efflux did not occur via vesicular transport (see Uemura et al., 2004), a possibility previously questioned on the basis of the unrealistically high membrane turnover rates that might be required to achieve high rates of vesicle-mediated efflux (Lazof and Cheeseman, 1988a). In another tracer study, comparing unidirectional Na^+ fluxes in corn and barley root tips, Jacoby and Rudich (1985) also indicated no effects of CCCP on sodium efflux, even though this agent reduced tissue ATP supplies in both test species by 80%, within five minutes of its application. The authors concluded that a sodium efflux pump in corn or barley, were it to exist, would have to be powered by an energy source other than the ATP-driven proton-motive force, in contradiction to the RTSC model. A similar conclusion was reached by Cheeseman (1982), who observed no effect of DCCD (*N,N'*-dicyclohexylcarbodiimide), another potent inhibitor of transport and metabolism, on efflux traces from labeled corn roots. Thirty years later, however, an alternative energy source to power Na^+ efflux in root cells has yet to be identified.

It is worth highlighting that corn stands out from other model species (e.g., barley; Ratner and Jacoby, 1976) in that most studies have not supported the idea that Na^+/H^+ antiport operates at the plasma membrane of corn root cells (e.g., Cheeseman, 1982; Jacoby and Rudich, 1985; Schubert and Lauchli, 1988; Mennen et al., 1990). Moreover, although thermodynamic considerations suggest that Na^+ efflux under saline conditions is an energy-demanding, active-transport process (Cheeseman, 1982; Schubert and Lauchli, 1988), there is substantial evidence indicating that this flux is resistant to treatments that powerfully suppress metabolism (NEM, CCCP, DCCD, anaerobiosis, low temperature; see above). Thus, in at least one major agricultural species, we find substantial evidence against the universality of the RTSC model (Fig. 1). Curiously, this species also does not seem to exhibit the extremely high “toxic” unidirectional influxes of Na^+ (Davenport and Tester, 2000) that have been reported in many other species, and comprise a significant part of the model. Compare, for instance, the unidirectional influx of $5.74 \mu\text{mol g}^{-1}(\text{FW}) \text{h}^{-1}$ found by Schubert and Lauchli (1988) in corn, with one as high as $154 \mu\text{mol g}^{-1}(\text{FW}) \text{h}^{-1}$ found in rice at the same $[\text{Na}^+]_{\text{ext}}$ (25 mM; Malagoli et al., 2008), or influxes as high as $600 \mu\text{mol g}^{-1}(\text{FW}) \text{h}^{-1}$ estimated in *Spergularia marina* at a higher concentration (90 mM; Lazof and Cheeseman, 1986). In addition, efflux in corn at 25 mM $[\text{Na}^+]_{\text{ext}}$ was found to be $3.31 \mu\text{mol g}^{-1}(\text{FW}) \text{h}^{-1}$, or only 58% of influx (Schubert and Lauchli, 1988), a far less intense cycling of Na^+ than is typically measured (e.g., efflux was 86% of influx in Malagoli et al., 2008 at the same $[\text{Na}^+]_{\text{ext}}$). The lack of conformity of corn physiology to the RTSC model, however, may

not be isolated; in a survey of 16 higher plant species, Mennen et al. (1990) found unequivocal evidence for plasma-membrane Na^+/H^+ antiport in only four.

Nevertheless, a few studies have suggested that Na^+/H^+ antiport may in fact occur in corn, as indicated by sensitivities to ouabain (Davis and Jaworski, 1979 see below) and vanadate (Spickett et al., 1993). Moreover, recent work has shown that the Na^+/H^+ antiporter SOS1 is indeed expressed in corn, as is the CBL-CIPK signaling network which controls expression of the SOS pathway (Zhao et al., 2009; Estrada et al., 2013). Sodium-transport studies on corn genotypes altered in SOS1 are eagerly awaited.

Amiloride, an inhibitor of Na^+/H^+ antiporters, has been shown to have a pronounced ability to reduce Na^+ efflux in several plant species, including poplar (Sun et al., 2009), wheat (Cuin et al., 2011), *A. thaliana* (Cuin et al., 2011), cotton (Kong et al., 2012), and several species of mangrove (Lu et al., 2012; Lang et al., 2014 Lang et al., 2014). It is worth noting that in these studies, the flux was measured with non-invasive ion-selective microelectrodes (see Section 3), under conditions in which roots were preloaded at high $[\text{Na}^+]$, then monitored in solutions of much lower $[\text{Na}^+]$ (see Section 3). It was noted by Guo et al. (2009), however, that SOS1 has no amiloride-binding domain (Zhu, 2000), and these authors proposed that the inhibitor acts by altering proton fluxes at the plasma membrane, thus decreasing the proton-motive force required by SOS1. The measurement of efflux at a low measuring concentration of Na^+ may have allowed the amiloride effect to be unmasked; by contrast, it was not seen in a tracer experiment involving measurement at high $[\text{Na}^+]_{\text{ext}}$ (150 mM), in *A. thaliana* (Essah, 2002). In addition, amiloride was found to have no effect on Na^+/H^+ transport in plasma-membrane vesicles derived from two tomato species (Wilson and Shannon, 1995), and an NMR study on corn root tips showed no effect of amiloride on Na^+ fluxes (Spickett et al., 1993), possibly due to incomplete inhibition *in vivo*.

Lastly, it should be mentioned that ouabain, a well described inhibitor of ATPase activity in animal systems, has also produced equivocal effects on Na^+ efflux from plant roots. This was discussed by Davis and Jaworski (1979), who have been the only authors to show ouabain-inhibited Na^+ efflux under saline conditions ($[\text{Na}^+]_{\text{ext}}$ ranged from 1 to 30 mM). Interestingly, this study was in corn, and remains one of the few demonstrations of a possible Na^+/H^+ antiport in this species (see above). The authors suggested that insufficient incubation time, and/or poor quality of ouabain stocks, may have contributed to the negative findings regarding this inhibitor in the majority of other studies. However, their own incubation time of several hours was questioned for its excessive length by Schubert and Lauchli (1986), while Cheeseman (1982) criticized their use of excised roots. Moreover, while the mode of action of ouabain on the sodium-potassium ATPase in animal systems is well known (Ogawa et al., 2009), this type of pump is not known to be present in plants, nor have any mechanisms of ouabain inhibition in plant systems been characterized.

7. Is the rapid transmembrane sodium cycling model based on an artefact?

Because of the assumed importance of Na^+ efflux to salt tolerance in the RTSC model, and the reliance of this model on measurements of unidirectional Na^+ fluxes made using tracers, it is essential here to critically examine this body of evidence. In particular, the central question of flux malleability must be addressed; that is, the extent to which an ion flux can be altered by an external physical or chemical treatment, or by genetic modification (see Section 3). Without such information, it is not possible to deter-

mine the compartment of origin for the efflux trace in the complex tissue of intact plant roots, even if the compartments are as broadly distinct as “symplast” and “apoplast”.

Surprisingly, it is under benign or beneficial sodium conditions that the majority of detailed tracer-efflux studies have been conducted, rather than under more agriculturally relevant salinity conditions, where Na^+ cycling is pronounced. This includes most of the early work in this area, which focused on Na^+ transport at $\sim 1 \text{ mM } [\text{Na}^+]_{\text{ext}}$ or less. In this concentration range, pronounced stimulation and inhibition of Na^+ release from radiolabeled plant tissues have been frequently observed in reaction to many experimental treatments including ouabain (Davis and Jaworski, 1979), fusicoccin (Marrè, 1979), abscisic acid (Behl and Jeschke, 1981), changes in external pH (Behl and Raschke, 1986; Jacoby and Teomy, 1988; Mennen et al., 1990), changes in temperature (Nassery and Baker, 1972; Macklon, 1975), and changes in external K^+ supply (Jeschke, 1983; Schulze et al., 2012).

The situation is markedly different at higher concentrations of Na^+ , with very few studies showing the types of changes commonly seen at lower $[\text{Na}^+]_{\text{ext}}$. Table 1 shows 25 published tracer studies examining Na^+ efflux at $10 \text{ mM } [\text{Na}^+]_{\text{ext}}$ or higher. Remarkably, in only nine of these studies was there an attempt made to test the malleability of efflux. These deserve a closer look. Seven of the studies involved changing the chemical composition (or temperature) of the root solution. In a study on corn by Cheeseman (1982), the results of such tests were negative, with no effects on the flux brought about by DCCD (see Section 6) or by changing the external concentrations of K^+ and Na^+ (thus ruling out Na^+/K^+ or Na^+/Na^+ exchange). Two studies by Ratner and Jacoby (1976) and Jacoby and Rudich (1985), by contrast, showed that switching the dominant external salt from Na_2SO_4 to K_2SO_4 resulted in an acceleration of Na^+ efflux in barley (but not corn) root tips; moreover, a reversible stimulation of Na^+ efflux was produced in barley root tips by lowering the external solution pH from 6.2 to 3.7 (and back). In these studies, however, CCCP did not alter Na^+ efflux in either barley or corn. In excised corn roots, on the other hand, Davis and Jaworski (1979) saw an inhibition of Na^+ efflux by ouabain. In their study of Na^+ efflux from roots of intact corn seedlings, Schubert and Lauchli (1988) showed that moderate stimulations of efflux were brought about by added KCl, chelation of external Ca^{2+} by EGTA, and low temperature, but no effects were seen with CCCP or NEM. In addition to these papers, two doctoral theses document tests for the malleability of Na^+ efflux traces. In one, Essah (2002) was unable to show any changes in efflux from roots of *A. thaliana* due to alterations of external Ca^{2+} and pH; similarly, the addition of amiloride had no effect. In the other, Schulze (2013) showed a lack of response of Na^+ efflux traces in barley to a wide range of inhibitors (some shown in Fig. 2): amiloride, DCCD, DNP, NEM, diethylstilbestrol, and potassium cyanide (applied with salicylhydroxamic acid, or SHAM), in addition to other treatments (high pH and low temperature). To summarize, two of these studies showed moderate effects on Na^+ efflux in barley brought about by changing the ionic composition of the medium, while one showed similar effects in corn (in contrast to three other studies in this species). More significantly, only a single study provided evidence of efflux malleability resulting from the use of a powerful metabolic inhibitor (ouabain; Davis and Jaworski, 1979), a study which, as noted, has not been free from criticism (Cheeseman, 1982; Schubert and Lauchli, 1988; see Section 6).

Three studies in this group were conducted in mutants of *A. thaliana*, to examine the influence of genetic modifications on Na^+ efflux. In one, Ding and Zhu (1997) found that the newly discovered *sos1* mutant of *A. thaliana* displayed no difference in the $^{22}\text{Na}^+$ efflux rate relative to wild type, and, startlingly, showed lower Na^+ accumulation between 5 and 75 $\text{mM } [\text{Na}^+]_{\text{ext}}$, despite being vastly more salt-sensitive than the wild type (see Section 5). It should be noted

that in these experiments, the entire seedlings were exposed to the uptake and efflux solutions, making the interpretation of tracer efflux challenging (De Boer and Volkov, 2003). In addition, the study did not provide a steady-state baseline flux (it is not explained why plants were labeled in a 0.5-mM NaCl solution $^{22}\text{Na}^+$, and then eluted with a non-radioactive solution containing 20 mM NaCl). By contrast, Schulze (2013) conducted $^{24}\text{Na}^+$ -efflux experiments (at 25 $\text{mM } [\text{Na}^+]_{\text{ext}}$) in wild-type and *sos1* mutants of *Arabidopsis*, using standard, steady-state efflux protocols (e.g., Malagoli et al., 2008; Coskun et al., 2010), but nevertheless confirmed the lack of difference in efflux between genotypes (Schulze, 2013). The third study in this sub-category is that of Elphick et al. (2001), who compared Na^+ -efflux traces from roots of *sos3* mutants with those from wild-type roots (SOS3 is a regulator of SOS1; Qiu et al., 2002; see Section 5). Curiously, although Ding and Zhu (1997) and Schulze (2013) found no effect of the *sos1* mutation on Na^+ efflux, Elphick et al. (2001) found that *sos3* mutants had a substantially smaller efflux. These studies are thus difficult to reconcile, but, as pointed out by Fraile-Escanciano et al. (2010), the effect seen by Elphick et al. (2001) may be related to the complex signalling network into which SOS proteins are integrated. Disruption of this network by knocking down an upstream component (in this case, SOS3) could have systemic consequences, and result in pleiotropies that are likely to include changes in ion fluxes. This consequence is especially plausible given the broad range of functions that have been at least tentatively attributed to the proteins in the SOS pathway, including roles in the sensing of Na^+ , the regulation of intracellular K^+ , Ca^{2+} , and pH homeostases, elements of root development under salt stress (including auxin responses, cortical microtubule, and microfilament organization and gravitropism), protection of endocytosis, oxidative stress tolerance (including the scavenging of reactive oxygen species), light control of germination, and low-affinity K^+ uptake (Zhu, 2002; Katiyar-Agarwal et al., 2006; Batelli et al., 2007; Auge et al., 2009; Guo et al., 2009; Oh et al., 2009; Fraile-Escanciano et al., 2010; Zhao et al., 2011; Undurraga et al., 2012; Yadav et al., 2012; Bose et al., 2013; Ye et al., 2013; Feki et al., 2011). Indeed, Gong et al. (2001) found that in *sos3* mutants, at least six genes were expressed differently from wild type, illustrating the reality of pleiotropies.

It is rather surprising, given that SOS1 is the only known Na^+ -efflux protein at the plant plasma membrane (Cuin et al., 2011; Guo et al., 2012; Zhang and Shi, 2013), and has been the subject of hundreds of published studies, that only two (Ding and Zhu, 1997; Schulze, 2013) should have directly examined Na^+ efflux in genotypes altered in SOS1 activity, and only one in a genotype altered in the SOS1-regulator, SOS3 (Elphick et al., 2001); to our knowledge, no studies on SOS2 of this type exist. Moreover, the SOS1 study by Ding and Zhu (1997) appears to be not without serious procedural flaws (see above). Although the salt tolerance conferred upon plants overexpressing SOS1 has often been associated with lower tissue Na^+ content (e.g., Shi et al., 2003), it is also important to remember that *sos1* mutants have shown reduced accumulation of Na^+ , within the $[\text{Na}^+]_{\text{ext}}$ range of 5–75 mM (Ding and Zhu, 1997). However, at 100 $\text{mM } [\text{Na}^+]_{\text{ext}}$, Shi et al. (2002) found that *sos1* mutants did accumulate more Na^+ than wild type. Nevertheless, changes in accumulation do not necessarily indicate that the Na^+ -transport function of SOS1 is directly responsible for them; for instance, in an *A. thaliana* study, mutants lacking the ability to synthesize spermine and thermospermine were shown to have higher Na^+ accumulation (Alet et al., 2012). Similarly, *Arabidopsis* plants enhanced in suberin synthesis also showed increased Na^+ accumulation (Baxter et al., 2009). Another relevant example of the hazards involved in assigning functions to gene products is that of the CHX21 transporter, which was initially thought to be a transporter regulating Na^+ content in the xylem and leaf tissue of *Arabidopsis*, but later determined

Table 1
Survey of tracer studies examining Na⁺ efflux at 10 mM [Na⁺]_{ext} or higher, with summary of malleability tests.

Author(s)	Species	[Na ⁺] _{ext} (mM)	Was malleability of the efflux trace tested?
Binzel et al. (1988)	Tobacco	428	No
Blom-Zandstra et al. (1998)	<i>Capsicum annuum</i> L.	15	No
Cheeseman (1982)	Corn	20	Yes. No effects of DCCD, K ⁺ , Na ⁺
Davenport et al. (2005)	Durum wheat	25	No
Davis and Jaworski (1979)	Corn	1–30	Yes. Moderate effects of ouabain
Ding and Zhu (1997)	<i>A. thaliana</i>	0.5 (load), 20 (elution)	Yes, via <i>sos1</i> mutation; no effect of mutation
Elphick et al. (2001)	<i>A. thaliana</i>	50	Yes, via <i>sos3</i> mutation; lower efflux in mutant
Essah (2002)	<i>A. thaliana</i>	100	Yes. No effect of changing pH, Ca ²⁺ , or amiloride
Essah et al. (2003) ^a	<i>A. thaliana</i>	200	No
Hajibagheri et al. (1989)	Corn	50	No
Jacoby and Rudich (1985)	Corn, barley	20	Yes. Replacing Na ⁺ with K ⁺ , and decreasing pH, changed slope in barley but not corn; no effect of CCCP in either species
Kronzucker et al. (2006)	Barley	1–100	No
Lazof and Cheeseman (1986) ^a	<i>Spergularia marina</i>	90	No
Lazof and Cheeseman (1988a) ^a	<i>Spergularia marina</i>	25	No
Lazof and Cheeseman (1988b) ^a	Lettuce	10	No
Mills et al. (1985)	Oat, <i>Atriplex</i>	3, 50	No
Ratner and Jacoby (1976)	Barley	20	Yes. pH shifts show clear and reversible changes in slope of efflux trace
Santa-María and Epstein (2001)	Wheat, amphiploid cross: wheat X <i>Lophopyrum elongatum</i>	100	No
Schubert and Lauchli (1988)	Corn	25	Yes. Only increases were seen, caused by KCl, cold, EGTA. No effect of NEM, CCCP
Schubert and Lauchli (1990)	Corn	25–50	No
Schulze (2013)	Barley, <i>A. thaliana</i>	150 (barley), 25 (<i>A. thaliana</i>)	Yes. No effect of amiloride, DCCD, DNP, NEM, diethylstilbestrol, and cyanide in barley; no effect of <i>sos1</i> mutation in <i>A. thaliana</i>
Wang et al. (2006)	<i>A. thaliana</i> , <i>Thellungiella salsuginea</i>	100	No
Wang et al. (2009)	<i>Puccinellia tenuiflora</i> , wheat	25, 100, 150	No
Yeo (1981)	<i>Suaeda maritima</i>	340	No
Yue et al. (2012) JPP	Tobacco	50–360	No

^a Indicates studies in which efflux was estimated indirectly, using a timecourse of influx.

to be involved in K⁺ homeostasis in the female gametophyte (Evans et al., 2012).

In summary, an extensive survey of efflux experiments conducted in the salinity range does not support the idea that large quantities of sodium rapidly cycle across root cell membranes under salinity, suggesting that Na⁺ efflux does not depend on significant expenditures of cellular energy. Nor is there compelling evidence that observed efflux traces are attributable to the activity of the SOS1 transporter. Naturally, these outcomes raise many questions. Firstly, what is the nature of the efflux traces, if they do not result from Na⁺ transport across the root plasma membrane? Much more detailed physiological work will be required to adequately resolve this question, but we suggest at this time that, as stated in our hypothesis (Section 2), the majority of efflux traces observed under salinity represent Na⁺ flow that is confined to the apoplastic matrix of the root. While this possibility is substantiated by the general lack of effect of metabolic inhibitors on the ostensibly energy-intensive efflux, it has also received some positive verification in experiments using the apoplastic fluorescent dye, trisodium, 3-hydroxy-5,8,10-pyrene trisulphonic acid (PTS). PTS is known not to cross plant cell membranes (Peterson et al., 1981; Yeo et al., 1987), and because of this property was used to establish the existence of an apoplastic bypass flow of Na⁺ from root to shoot in rice and other species (Peterson et al., 1981; Yeo

et al., 1987; Munns and Tester, 2008). Release of this dye from PTS-loaded roots of rice seedlings was also shown to have a kinetic half-time of 20–30 min (Yeo et al., 1987), which is very similar to the half-time of Na⁺-tracer release from labeled roots of rice and other species (e.g., Kronzucker et al., 2006; Malagoli et al., 2008), and much slower than release phases that are usually attributed to the apoplast (e.g., Kronzucker et al., 2005). In our own laboratory, we have also observed release of PTS from barley roots, with similar kinetics (unpublished work). However, it should be noted that it may not be realistic to compare the flux kinetics of a large (524 g/mol), polycyclic molecule such as PTS to those of a relatively small ion such as Na⁺. Nevertheless, these experiments indicate that slow phases of efflux from the apoplastic matrix can and do exist.

Clearly, more effort needs to be made to test the hypothesis presented here. One means could be to examine whether rapid sodium cycling is reduced in species that display a well-developed exodermis, the layer of cells that forms a barrier to apoplastic flow into the root cortex (Enstone et al., 2003; Schreiber and Franke, 2011). Indeed, it has been shown in rice that the development of apoplastic barriers occurs in response to salt stress, and reduces Na⁺ accumulation (Krishnamurthy et al., 2009). Another promising approach could be an integrative one, using not tracer analysis alone, but in conjunction with other technologies

such as multi-barrelled Na⁺-selective microelectrodes and Na⁺-specific fluorescent dyes (see Section 3). These latter methods could be used to provide independent measurements of apoplastic Na⁺ concentrations, which can then be compared with values derived from CATE, under the assumption that Na⁺-efflux traces are apoplastic in origin. Large disagreements between these values could invalidate the assumption, and thereby falsify our hypothesis.

Another question raised by the evidence presented in this review concerns the function of SOS1. What fraction of observed efflux traces is attributable to the transport of Na⁺ from the cytosol to the external medium by SOS1? The results of Ding and Zhu (1997) and Schulze (2013) suggest that it may be a fraction so small as to be hidden against a much larger flux background, which we hypothesize to originate from the apoplast, as in the case of K⁺ efflux at high K⁺ concentrations (Coskun et al., 2010 see Section 3). Because of this almost complete masking of the putative efflux from the symplast, it may be all but impossible to determine the true exchange kinetics of Na⁺ across the plasma membrane, and whether they differ from the dominant efflux trace, hypothetically from the apoplast.

This is not to say that SOS1 does not transport a significant amount of Na⁺ out of certain cell types, however, or that this transporter function is not related to the protective properties of SOS1 against salt stress; both of these ideas appear to be well substantiated (e.g., Qiu et al., 2002; Cuin et al., 2011). However, it is important to consider here the root-expression patterns of SOS1, in particular that it appears to be highly expressed only in two regions of the root: the xylem parenchyma, and the root tip (Shi et al., 2002). Xylem parenchyma cells appear to be involved in the translocation of Na⁺ to the shoot, or its retrieval (depending on the intensity of salt stress; Shi et al., 2002; Yadav et al., 2012; Mansour, 2014; Katschnig et al., 2015), but not in efflux to the cortical apoplast or external medium. On the other hand, the function of root-tip-localized SOS1 appears to be important for the protection of cells in the root apical meristem, which are neither vacuolated nor connected to vascular tissue and therefore cannot, like other root cells, engage in transport to the vacuole or to the stele for the maintenance of a low cytosolic [Na⁺] (Shi et al., 2002; Shi and Zhu, 2002; Chinnusamy et al., 2005; Maathuis et al., 2014). It should be noted, however, that in an extracellular microelectrode study, Shabala et al. (2005a) found that SOS mutations in *A. thaliana* affected the entire root, but unfortunately Na⁺ fluxes were not reported by these authors, due to poor selectivity of the liquid ion exchanger used in their electrodes.

In a detailed study by Oh et al. (2009), a loss of SOS1 function in the *Arabidopsis* relative *T. salsuginea* transformed this halophyte into a highly Na⁺-sensitive plant. It was found that cellular damage due to abnormally high Na⁺ intracellular accumulation in the *sos1* mutant was initiated in the root tip before spreading to the root hair zone, possibly via increased and uncontrolled apoplastic Na⁺ flow brought about by membrane destruction (also see Munns, 2002). The central importance of the root tip in both sensing ion stresses and counteracting them is well established for toxicant ions such as NH₄⁺, aluminum and iron (Jones and Kochian, 1995; Li et al., 2010, 2015), and has also been demonstrated, with a focus on lateral root formation, in the case of Na⁺ (Duan et al., 2013). In these studies, when specifically tested, exposure of the root tip was shown to both be essential and sufficient for the development of the ion stresses not only within the root system, but at the level of the whole plant, highlighting the extraordinary importance of this section of the root system to plant performance under edaphic stress. It is not unreasonable, therefore, to postulate a main (and perhaps the only) role of SOS1 in this zone of the root. Clearly, much more study on Na⁺ efflux as a function of root zonation is required to satisfactorily resolve this issue.

8. Conclusion

We have discussed several key aspects of Na⁺ efflux from plant roots under saline conditions here, in the context of the current RTSC model, and an alternative, apoplastic hypothesis. We find that the evidence supporting the RTSC model is in general very weak. This is largely due to the fact that, in the great majority of tracer studies demonstrating the rapid cycling of Na⁺, the malleability of the flux was not tested (Table 1). In the few studies where such tests were carried out, the results are ambiguous; changing the ionic composition of the root medium resulted in changes in efflux in some studies but not others, while, all but universally, efflux traces showed no response to powerful inhibitors of metabolism and transport, in sharp contradiction to the model. In only three studies were the effects of mutations in the SOS pathway considered; these do not form a consensus. Moreover, the high rates of Na⁺ movement inherent in the RTSC model are tied to inexplicably high energy demands and cytosolic Na⁺ pools. These problems suggest alternative hypotheses, one of which we have provided here (Fig. 3): that the bulk of the Na⁺ tracer released from labeled roots does not cross the plasma membrane, but cycles through the apoplast before returning to the external medium; a small amount cycles across the plasma membranes of root-tip cells, via SOS1. While only a small amount of positive evidence is available in support of this alternative hypothesis, however, it is not without precedent, and moreover, it can help explain many of the contradictory results presented in this review, as well as broader issues such as excessively high flux energetics and cytosolic Na⁺ concentrations. Interestingly, a redefinition of the compartmental origin of Na⁺ tracer release is not without precedent; Ritchie and Larkum (1984) showed that more than 99% of the ²²Na⁺ released from labelled cultures of the green alga *Enteromorpha intestinalis* (now known as *Ulva intestinalis*) was wrongly attributed to efflux from the cytoplasm.

Lastly, we briefly consider the impact of a new model for Na⁺ efflux on the large body of experimental work measuring Na⁺ influx under salinity conditions. If the RTSC model is rejected, new doubt will be cast on influx measurements in the literature, particularly those which present very high flux estimates and rapid-cycling scenarios (see Britto and Kronzucker, 2009). These include measurements made in our own laboratory, where we found that the discrepancy between pronounced Na⁺ influx into rice roots could not be reconciled with the energetic requirements inherent in the model of rapid transmembrane cycling (Malagoli et al., 2008). Given the arguments here, the possibility that influx values are over-estimated due to tracer accumulation in the apoplast cannot be ignored, and new caution should be applied to the common practice of using very short tracer-loading and -desorption times in Na⁺ transport studies (e.g., Essah et al., 2003; Wang et al., 2009). Indeed, the apoplastic artefact discussed here may be at least in part responsible for the lack of progress in identifying membrane-transport systems for Na⁺ in plant roots, and recognition of this problem may lead to new insights and means of enhancing plant Na⁺ tolerance.

We conclude that much fundamental physiological work remains to be done to test both the current model and alternative possibilities regarding the problem of Na⁺ cycling, including more rigorous testing of the malleability of efflux, particularly in the context of the limited expression pattern of SOS1, and examinations of apoplastic routes of Na⁺ movement through the root, which have as yet been poorly studied.

Acknowledgment

We wish to thank the Canada Research Chairs program for funding this work.

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