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REVIEW PAPER

Sodium in plants: perception, signalling, and regulation of sodium fluxes

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Abstract

Although not essential for most plants, sodium (Na^+) can be beneficial to plants in many conditions, particularly when potassium (K^+) is deficient. As such it can be regarded a 'non-essential' or 'functional' nutrient. By contrast, the many salinized areas around the globe force plants to deal with toxicity from high levels of Na^+ in the environment and within tissues. Progress has been made in identifying the relevant membrane transporters involved in the uptake and distribution of Na^+. The latter is important in the context of mitigating salinity stress but also for the optimization of Na^+ as an abundantly available functional nutrient. In both cases plants are likely to require mechanism(s) to monitor Na^+ concentration, possibly in multiple compartments, to regulate gene expression and transport activities. Extremely little is known about whether such mechanisms are present and if so, how they operate, either at the cellular or the tissue level. This paper gives an overview of the regulatory and potential sensing mechanisms that pertain to Na^+, in both the context of salt stress and Na^+ as a nutrient.

Key words: Calcium signal, cGMP, nutrient, regulation, salinity, sensing, sodium.

Sodium in plants; a blessing or a burden?

Sodium (Na^+) is an abundant element which makes up around 3% of the earth's crust. Furthermore it is found in almost all surface and subterranean water bodies and of course is plentiful in seas and oceans where it can reach over 5% (w/w). It is not surprising then that most plants will get in contact with Na^+ at some stage during their life cycle although the encountered levels may vary greatly. At the extreme end, terrestrial and aquatic plants can be exposed to levels of salinity (NaCl) that are two or three times higher than that of seawater (~540 mM) but very few species are capable of withstanding such an onslaught. Less extreme, and more widespread, are low and intermediate levels of salinization which occur all over the world. Salinization can arise through natural causes, such as the local geology or proximity to coastal areas, or be man-made, for example, through the use of irrigation water that contains high concentrations of salts. Although mostly Na^+ and Cl^-, saline soils often contain high concentrations of other inorganics such as calcium (Ca^{2+}), sulphate (SO_4^{2-}), and bicarbonate (HCO_3^-). On the other end of the spectrum, areas with high levels of precipitation may be almost devoid of Na^+ since it is relatively mobile in the soil. Consequently, plants living in these regions may only encounter trace levels of Na^+.

For almost all terrestrial plants, Na^+ is not essential for either growth and development or for reproduction. An exception is a subgroup of C_4 plants for which Na^+ has been shown to be essential. Examples are *Atriplex* spp, *Kochia childsii*, millet, and a number of other C_4 grasses. These C_4 species require Na^+ at trace levels to drive a particular transport process, the uptake of pyruvate into chloroplasts by a Na^+-pyruvate cotransporter (Furumoto *et al.*, 2011). In all other plants this function is mediated by a H^+ coupled pyruvate carrier. Although this small group of species is the only one for which Na^+ can be classified as an 'essential nutrient' (Maathuis, 2009), Na^+ can nevertheless be beneficial and 'nutritious' in other species. It has been observed many times that, during K^+ deficiency, many (glycophytic) plants respond positively to Na^+ fertilization (Subbarao *et al.*, 2003).

For salt-tolerant (halophytic) plants, even high concentrations of Na^+ promote growth. Good examples are species such as *Suaeda maritima* and *Salicornia* spp that show a drastic increase in growth when salt is present in the growth substrate. Even substrates with salinity levels that approach seawater are stimulatory showing that, in contrast to glycophytes, halophytes also benefit from Na^+ at high concentrations.

At low levels, Na^+ not only is harmless but can be very useful, particularly in low K^+ conditions. This is because, in hydrated form, Na^+ and K^+ are chemically and structurally very similar (Amtmann and Sanders, 1999). Thus, many of the roles that K^+ plays in plant cells, including some of the metabolic ones, can therefore be fulfilled by Na^+. However, this is unlikely to include the many enzymes that rely heavily on K^+ as cofactor; the different ionic sizes of K^+ and Na^+ mean binding energies and co-ordination within enzymes are quite different between the ions and this is precisely how large selectivity for one or the other is created. The latter is exemplified by starch synthetase (Lindhauer and De Fekete, 1990), an enzyme that tightly binds K^+ ions, which has a requirement of about 50 mM K^+ for normal activity (100%) with other, similarly sized cations such as Rb^+ and Cs^+ being about 80% as effective as K^+ and Na^+ only about 20%.

The similar efficacy of Na^+ and K^+ in a number of functions may be exploited in several ways: In soils where K^+ is scarce, increased Na^+ utilization could lower agriculture's dependence on expensive potash fertilizer. Uptake of Na^+ at the root:soil boundary could also help phytoremediate moderately saline soils. In addition, the presence of small amounts of Na^+ in the growth medium has been shown to improve the taste of many crops including asparagus, barley, broccoli, and beet. Increased Na^+ in plants can also be valuable to herbivores since it is essential to all animal life where it is a principal electrolyte for the ionic balance of tissues and fluids and for electrical signalling in nerve cells. This Na^+ requirement means that animals with plant-based diets often need supplements, for example, in the form of salt licks, which could be avoided with an increased Na^+ content in grasses and animal fodder.

Although low levels of Na^+ can be beneficial in many conditions, moderate and high levels of salt are detrimental to the majority of plants which is classified as glycophytic. Indeed, soil salinity is one of the prime abiotic stresses limiting agricultural production in many areas of the world. There are several reasons why salinity decreases plant growth (Flowers and Colmer, 2008): First, high ambient concentrations of inorganics such as Na^+ and Cl^- lower the water potential and hence create osmotic stress. A logical response to counter this trauma is the uptake of these ions themselves, a strategy that is successfully employed by many salt-tolerant plant species. However, this response can lead to a second issue: a substantial increase in cellular ion contents, particularly of Na^+, that negatively affects cellular biochemistry. Thus, the potentially beneficial intake of inorganic osmotica needs careful monitoring and relies heavily on the efficient sequestration of potentially harmful ions in the vacuole. Salt-tolerant species are more capable of achieving this delicate balance than glycophytes, possibly by reducing their net uptake of Na^+, by limiting Na^+ translocation to the shoot, and by effective cellular partitioning. This is often translated into lower levels of Na^+ accumulation in tolerant species and ecotypes compared with their sensitive counterparts. The latter is exemplified by a comparison of tissue Na^+ in a salt-sensitive and a salt-tolerant *Plantago* species. At low salinity, the sensitive ecotype has a lower Na^+ tissue content (Fig. 1) but, at moderate to high levels of NaCl in the medium, the sensitive species 'loses control' and translocates large amounts of Na^+ to shoot tissues leading to plant death (Maathuis, 1991). However, there are many exceptions to this behaviour, particularly in so-called glycophytic 'includers' and in many halophytes, both categories that accumulate large amounts of Na^+ in their shoot tissue. A third aspect of salinity is the detrimental effect that Na^+ and Cl^- ions have on the acquisition and distribution of essential nutrients such as K^+, Ca^{2+}, and NO_3^-. These deleterious effects occur both at the cellular and whole tissue level and cause many terrestrial plants to struggle with even moderate salt levels. This is surprising because terrestrial plant life originated in marine environments from which it transferred to land around 450 million years ago. The early pioneer species obviously knew how to deal with high Na^+ concentrations, having been exposed to them permanently, but gradually appear to have lost their marine heritage.

Early perception of salt stress and Na^+ sensing

Mainly because of the damage caused by salinity on agriculture, vast resources have been spent to unravel the responses and mechanisms of tolerance that plants employ to counter this stress. The result is that we now have a reasonably comprehensive picture of how Na^+ enters plants and how it is

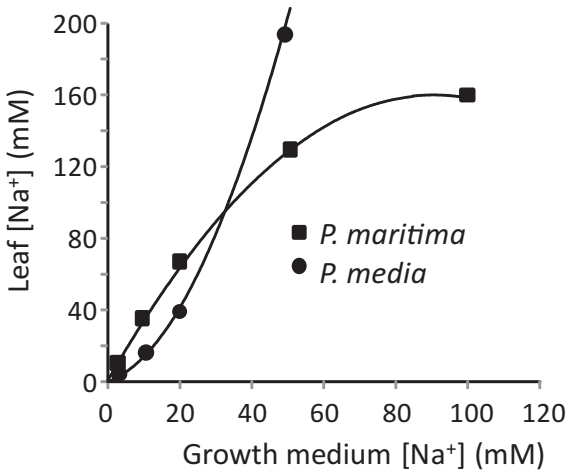

Fig. 1. Leaf [Na^+] as a function of external [Na^+] in two *Plantago* species. Shoot Na^+ levels at low and moderate external Na^+ concentrations remain lower in the salt-sensitive *P. media* compared with the salt-tolerant *P. maritima*. When external [Na^+] is increased further (>40 mM), leaf Na^+ in *P. media* rapidly rises and plants die when the external concentrations are higher than approximately 60 mM. In similar conditions, leaf Na^+ in *P. maritima* rises moderately and plateaus to around 150 mM.

distributed within cells and tissues (Fig. 2). Understanding how Na^+ enters plants, how it is moved between different tissues and within cell compartments is vital if we want to improve crop resistance to salt stress, but also to increase the value of Na^+ as a functional nutrient. In both cases we require insights into the identities and properties of membrane transporters that catalyse Na^+ movement. Some marine algae have unique transporters such as ATP-driven Na^+ efflux pumps (Popova *et al.*, 2006) but most studies show that all terrestrial plants, whether glycophytes, halophytes, C_3 or C_4 species, contain very similar complements of membrane transporters. Thus, it is tempting to conclude that transport regulation, rather than specific transport properties, are the key factors in explaining the ecophysiological differences between species. In the following sections, what is known about sensing, regulatory, and signalling mechanisms that impact on various aspects of Na^+ and how they potentially interact will be reviewed.

All plants tested so far have been shown to take up Na^+ in the low affinity range (Zhang *et al.*, 2010) and it appears that most plants also take up Na^+ in the high affinity range (Haro *et al.*, 2012). To start then, one can ask the question whether Na^+ levels are monitored for either or both of these processes and, if so, at what level, at what time scale, and whether this occurs in the apoplast, cytoplast or vacuole. At low levels, Na^+ is harmless and uptake in the high affinity range may be a purely 'passive' process. Different types of transporter may contribute to high affinity uptake (Haro *et al.*, 2010) but it is by now clear that some of the participants automatically switch on whenever K^+ becomes deficient (Garciadeblas *et al.*, 2003). A mechanistic basis for this can sometimes be found in the transport protein itself; members of the HKT subfamily 2 mediate Na^+ transport but some isoforms show a distinct inhibition whenever trace levels of K^+ are present. For example, K^+ inhibits barley HKT2;1 activity with a Kd of around 30 μM (Mian *et al.*, 2010) and hence Na^+ uptake only proceeds when K^+ is very low or absent. It is not clear whether other mechanisms, such as alteration in gene expression or post-translational regulation, play a role in modulating high affinity Na^+ uptake, nor whether such processes would require sensing of cellular Na^+ levels.

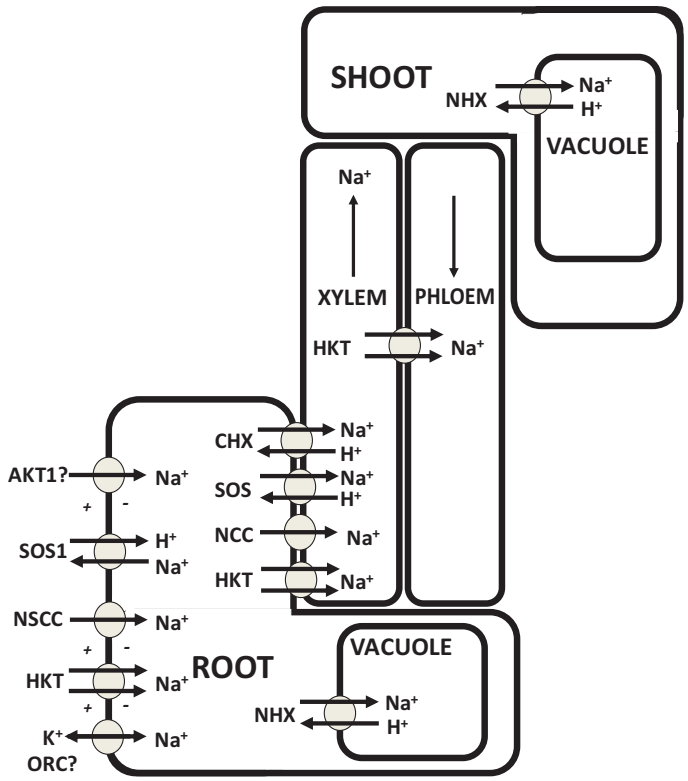

Fig. 2. Transport proteins that are involved in Na^+ uptake, efflux and distribution. Transport functions are shown for Na^+ uptake, efflux, long-distance transport, and cellular partitioning in roots and shoots. AKT1, inward rectifying K^+ channel; CHX, cation:H^+ exchanger; HKT, high affinity K^+ transporter; NHX, vacuolar Na^+:H^+ exchanger; NSCC, non-selective cation channel; ORC; outward rectifying K^+ channel; SOS1, plasma membrane Na^+:H^+ antiport.

At higher levels of ambient Na^+, large, low affinity fluxes occur that are mediated by HKT-type carriers (Laurie *et al.*, 2001; Garciadeblas *et al.*, 2003; James *et al.*, 2012), non-selective ion channels (for a review, see Amtmann and Sanders, 1999), and, possibly, K^+-selective ion channels such as AKT1 (Zhang *et al.*, 2010) and outward rectifying K^+ channels (Fig. 2; Amtmann and Sanders, 1999). Typically, this causes considerable amounts of Na^+ to accumulate in cells and tissues. There is overwhelming evidence that, in these conditions, plants do respond, for example, by modulating transmembrane Na^+ fluxes or by altering expression of salt-specific genes. In both cases, this would require some form of Na^+-sensing/monitoring. How Na^+ is monitored remains an enigma but some general mechanisms have been described to sense the onset of salinity stress; salinity lowers the external water potential, causing an almost immediate reduction of water delivery to plant tissues, and there are several ways plants can register and transduce this powerful stimulus. For example, membrane receptors can report the changed physical forces on membranes and cell walls that follow after the changes in turgor. One candidate is the *Arabidopsis* histidine kinase AtHK1 which has periplasmic domains that record changes in turgor by measuring the distance between the membrane and the cell wall. HK1 activation, in turn, leads to a MAPK signalling cascade and altered gene expression (Urao *et al.*, 1999; Chefdor *et al.*, 2006). Alternatively, distortion of cell wall-membrane geometry can be relayed by mechanosensitive ion channels which open in response to membrane stretching. The fairly non-selective properties of these transporters means that channel opening would cause large membrane depolarizations and, possibly, induce increased cytoplasmic Ca^{2+} levels, either of which is a potent signal to relay changes in environmental osmolarity further.

However, these relatively rapid mechanisms respond to *osmotic* perturbations and are therefore not specific to salt stress, let alone to Na^+. Also, ion toxicity due to the accumulation of Cl^- and Na^+ is unlikely to occur instantaneously so would probably require the perception of actual changes in ionic concentration at later stages. How plants register changes in ion levels is largely unknown except in the case of Ca^{2+} where specific binding domains and proteins

have been identified. In animals (where Na^+ plays many important physiological roles) mechanisms for sensing Na^+ appear to consist mostly of specific Na^+ selective ion channels and other Na^+ transporters. In the case of Na^+ channels, these can act as sensors to regulate body fluid Na^+ levels (Watanabe *et al.*, 2006) because their activity is controlled by a gating mechanism that depends on the Na^+ concentration itself. Similar mechanisms may sense excessive ambient Na^+ levels of nematodes; in the sensory cilia of these organisms, 'transmembrane channel like' (TMC) channels activate when [Na^+] is higher than around 140 mM causing an avoidance reaction (Chatzigeorgiou *et al.*, 2013). In taste buds, Na^+ (salt) is registered by Enac-type Na^+ channels that cause a depolarization proportionate to the amount of Na^+ that is present. The immediate effect of the depolarization is Ca^{2+} influx and subsequent neurotransmitter release. A further Ca^{2+}-dependent Na^+-sensing system occurs in kidney proximal tubule cells. Na^+ levels per se are not sensed but translated into a Ca^{2+} signal via a Na^+:Ca^{2+} antiport. The increased Ca^{2+} leads to the phosphorylation of SIK1 (salt-inducible kinase) via a calcium–calmodulin-dependent kinase. Subsequently, SIK1 interacts with and activates the plasma membrane Na-K-ATPase to increase Na^+ extrusion (Sjöström *et al.*, 2007).

No Na^+-selective ion channels have been identified in plants, making it very unlikely that plant Na^+ sensing works in a similar fashion. However, plants may contain other proteins that have regulatory Na^+ binding sites such as those found in mammals. For example, the activity of the mammalian protease thrombin is modulated allosterically by Na^+ with a Kd of around 20 mM (Huntington, 2008). In the protein, Na^+ is co-ordinated by carbonyl oxygens from lysine (K224) and arginine (R221) and four water molecules and the Na^+ binding loop is directly connected to the active site (Huntington, 2008). Na^+-activated K^+ channels are widely expressed in neurons, kidney, heart, and skeletal muscle cells and have Kd values for Na^+ binding between 50 and 70 mM. The Na^+ co-ordination domain in these channels has been located to the C-terminus (Zhang *et al.*, 2010).

As discussed above, tight binding to, predominantly, carbonyl oxygens is necessary to endow ion selectivity. For a proper Na^+ sensor, a Na^+-specific binding site would be expected in the sensing compound. As yet, no plant equivalents of the mammalian Na^+-binding proteins are known but, on the basis of mammalian examples, Na^+-binding sequences can be derived with which to probe plant sequences. For Na^+-activated K^+ channels the consensus motif is DxR/KxxH (Zhang *et al.*, 2010) and a search (http://www.genome.jp/tools/motif/MOTIF2.html) in *Arabidopsis* reveals around 1200 candidate proteins. Unfortunately, this is a rather unwieldy number but, nevertheless, some interesting candidates emerge from this search such as the cation H^+ exchangers CHX5, CHX7, CHX16, CHX6b, around 50 other transporters, ~27 transcription factors, and ~120 kinases. Clearly more research is needed to test whether any of these functions as a plant Na^+ sensor.

Regulation of Na^+ uptake

In plants that have not previously been exposed to salt, the initial net Na^+ influx can be very large. Typically, both net and unidirectional Na^+ influx are reduced when plants are exposed for a longer period. Indeed, a large reduction of the initial Na^+ uptake rate is evident within 10–20 min (Chen *et al.*, 2007). This is true when moderate or high external [NaCl] is used; for 'high affinity' Na^+ uptake, very few data are available. The down-regulation of Na^+ uptake may result from lower unidirectional influx, increased efflux or a mixture of both, and part of this process may be via coupling to the root membrane potential which usually becomes less negative when NaCl is present in the ambient medium (Amtmann and Sanders, 1999; Essah *et al.*, 2003; Mian *et al.*, 2010). A low membrane potential would reduce the driving force for Na^+ uptake.

More sophisticated regulatory mechanisms that rely on second messengers may also be important in modifying Na^+ uptake. Second messengers such as Ca^{2+}, cGMP, and reactive oxygen species (ROS) show rapid transient increases in cytoplasmic levels after elevation of the ambient salt concentration (Knight *et al.*, 1997; Kiegle *et al.*, 2000; Donaldson *et al.*, 2004). After the onset of salt and osmotic stress an increase in cellular cGMP can be measured within seconds (Donaldson *et al.*, 2004). Furthermore, studies on *Arabidopsis* seedlings (Maathuis and Sanders, 2001; Essah *et al.*, 2003) and on mature pepper plants (Rubio *et al.*, 2003) have shown that unidirectional Na^+ influx is affected by cyclic nucleotides, particularly cGMP (Fig. 3). In conjunction, a direct inhibitory effect of cGMP on non-selective ion channels was shown in *Arabidopsis* root protoplasts (Maathuis and Sanders,

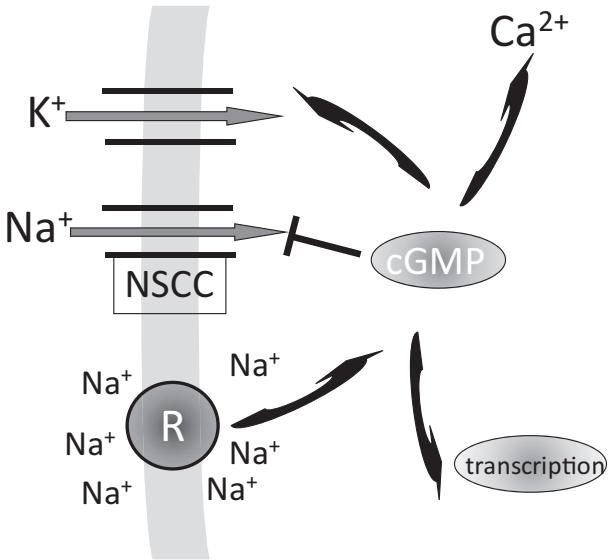

Fig. 3. Regulation of cation flux by cGMP. Transport functions are shown for Na^+ uptake, efflux, long-distance transport, and cellular partitioning in roots and shoots. AKT1, inward rectifying K^+ channel; CHX, cation:H^+ exchanger; HKT, high affinity K^+ transporter; NHX, vacuolar Na^+:H^+ exchanger; NSCC, non-selective cation channel; ORC; outward rectifying K^+ channel; SOS1, plasma membrane Na^+:H^+ antiport.

2001), which could explain the inhibitory effect of externally applied cGMP on net Na^+ uptake. During salinity, cGMP promotes K^+ uptake and affects transcript levels of many genes, particularly those encoding membrane transporters (Maathuis 2006). The study by Donaldson *et al.* (2004) also implicated Ca^{2+} signalling as an intermediary in this process, downstream of the cGMP signal.

Salt stress promotes the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the superoxide anion (Miller *et al.*, 2010). Although ROS are toxic by nature and up-regulation of antioxidant enzymes (e.g. superoxide dismutases, catalases, peroxidases) often mitigates sensitivity, they can also serve as signalling molecules. Salt-induced ROS emerge within minutes of the applied stress (Hong *et al.*, 2009), mainly in the form of H_2O_2 and this may depend on the activity of NADPH oxidases. Although relatively little is known about downstream targets of ROS during salt stress, it is generally accepted that MAP kinase cascades are involved (Miller *et al.*, 2010). More recent studies show that specific ROS-sensitive transcription factors may be activated. One such transcription is ERF1 (ethylene response factor) in rice (Schmidt *et al.*, 2013) which, when activated, binds to multiple promoters including those of MAPKs. Increased ERF expression led to better salt tolerance (Schmidt *et al.*, 2013). ROS may also directly impact on ion fluxes as was shown *Arabidopsis* roots where outward rectifying K^+ channels were directly activated by ROS (Demidchik *et al.*, 2010) and hence could explain the often observed K^+ loss in plant roots during salt stress.

How ROS signalling is initiated remains unclear but one hypothesis suggests the participation of the SOS pathway (see below) since the SOS2 kinase may interact with nucleoside triphosphate kinase 2 (NDPK2) which is involved in ROS signalling. NDPK2 is induced in response to oxidative stress and was shown to be important for the H_2O_2-induced activation of MAPkinase (Verslues *et al.*, 2007).

These findings evoke several intriguing questions. Production of ROS occurs as a 'by-product' of almost any plant stress and, thus, it is not yet clear whether salt-induced ROS are specific and therefore capable of providing useful signals. Furthermore, Ca^{2+} signals occur in response to drought *and* salt stress, often with virtually identical signatures. Whole organ measurements may camouflage some signal variation between different cell types (Kiegle *et al.*, 2000) but convincing evidence for *NaCl-specific* rises in cytoplasmic Ca^{2+} is still lacking. Indeed, studies in yeast showed that rapid Ca^{2+} transients (approximately 0–120 s) are entirely due to osmotic effects and did not vary for different salts or between ionic and non-ionic osmotica (Denis and Cyert, 2002; Matsumoto *et al.*, 2002). Thus, the observed transients most probably report changes in osmotic conditions. Secondly, if the increased levels of ions per se generate cGMP or Ca^{2+} signals, are they specific for Na^+ and/or Cl^-? In other words, do they constitute a genuine salinity stress signal or merely a generic response to variations in ionic strength such as changes in surface charge or membrane depolarization.

Although Na^+ fluxes (and other ionic fluxes such as K^+ and Cl^-) can change almost instantaneously during salinity, it is improbable that flux magnitude itself is sensed. Rather, compartmental Na^+ concentrations would provide a much more physiologically important parameter. But in that case the physiological relevance of signals within seconds is unclear: It is very unlikely that cytoplasmic or vacuolar ion levels will change significantly during such a brief period, let alone cause significant stress. Donaldson *et al.* (2004) showed that the cGMP signal persists much longer (up to 15 min) and this would, therefore, be a more likely candidate for Na^+ flux regulation. But what is the identity of the cGMP downstream target(s)? Plants do not appear to have cGMP-dependent kinases (Martinez-Atienza *et al.*, 2007) and most plant proteins with a putative cGMP binding domain are membrane transporters (Maathuis, 2006), particularly non-selective ion channels from the CNGC family. However, nucleotide binding to CNGCs generally leads to channel activation rather than deactivation (Leng *et al.*, 1999; Balague *et al.*, 2003) making these unlikely candidates for cGMP-dependent inhibition of Na^+ uptake. The Na^+:H^+ antiporter, SOS1 (see next section) also contains a putative cyclic nucleotide binding domain (Maathuis, 2006) but ^{3}H-cGMP binding studies and yeast growth assays in the presence and absence of cyclic nucleotides did not alter SOS1 activity (F Maathuis *et al.*, unpublished data). Thus, the exact nature of cGMP targets remains to be identified.

There may be several other, less-well-characterized mechanisms that impact on Na^+ uptake and, therefore, participate in Na^+ sensing. These include amino acids and purines (Demidchik *et al.*, 2011) since both these compounds activate Na^+ permeable cation channels. Another parameter is the membrane potential itself. For example, the onset of salinity is typically followed by rapid depolarization of epidermal and cortical root cells (Maathuis *et al.*, 2006). By contrast, hyperosmotic shock generally causes a cell hyperpolarization. Thus, changes in membrane potential could act as signals to relay different stimuli at least where salinity and osmotic shock are concerned. However, cell depolarizations are obtained whenever cation concentrations in the root medium increase. Indeed, monovalent cations such as Na^+, K^+, Rb^+, and Cs^+, all give very similar depolarizations as a function of external concentration. This lack of specificity appears to make membrane potentials on their own unsuitable to report changes in salinity and/or Na^+.

Regulation of Na^+ efflux

The use of forward genetics identified several salt oversensitive (SOS) loci (Shi *et al.*, 2000). One of these was SOS1 which encodes a plasma membrane-located Na^+:H^+ antiporter that is involved in the extrusion of Na^+ from the cytoplasm, an important aspect of salinity tolerance. SOS1 is expressed in many tissues, but particularly in the root epidermis and around the vascular tissue, and transcript levels are elevated after several hours or days of salt stress. SOS1 activity is directly responsive to phosphorylation by the kinase CIPK24 (SOS2) (Chinusamy *et al.*, 2004; Fig. 4), a member of the CIPK (calcium-induced protein kinase) family. CIPK24 is activated

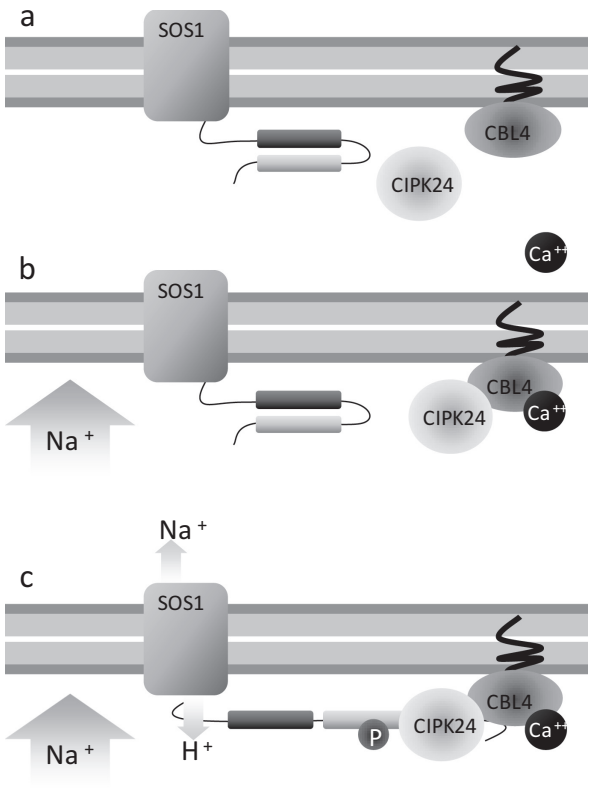

Fig. 4. The SOS (salt over sensitive) regulatory pathway. (a) In the absence of a salt stimulus, SOS1 is inactive. (b) In the presence of Na^+, CBL4 (SOS3) binds Ca^{2+} which, in turn, causes recruitment of the kinase CIPK24 (SOS2). (c) The CBL-CIPK complex phosphorylates the SOS1 C-terminus. This leads to a conformational change which relieves SOS1 auto-inhibition and instigates antiport activity.

when it associates with the calcineurin-B like (CBL) calcium sensor CBL4 (SOS3). After binding of Ca^{2+}, CBL4 dimerizes allowing its interaction with a NAF (asparagine, alanine, phenylalanine) domain on CIPK24. CBL binding releases the C-terminal autoinhibition domain of CIPK24, thereby activating this kinase. CIPK-CBL-mediated phosphorylation at the SOS1 C-terminus, in turn, removes the SOS1 autoinhibitory domain (Quintero *et al.*, 2011) activating the antiporter. Thus a linear pathway can be envisaged (Fig. 3) that starts with a salt-induced Ca^{2+} transient and terminates in the increased activity of an antiporter that limits cytoplasmic Na^+ accumulation. In addition, CIPK24 may also affect the activity of other transporters such as HKT1 which is involved in Na^+ uptake (Laurie *et al.*, 2002; Rus *et al.*, 2001) and, via interaction with CBL10, NHX1 which is responsible for the extrusion of Na^+ into the vacuole (Apse *et al.*, 1999; Weinl and Kudla, 2009).

Loss of function in any of the SOS genes leads to increased salt sensitivity but also to changes in homeostasis of other cations, particularly K^+. Interpretation of CBL4 (SOS3) and CIPK24 (SOS2) knockouts is further complicated because both are capable of interacting with many other proteins. For example, CBL4 also impacts on K^+ transport by initiating ER to plasma membrane trafficking of the K^+ channel AKT2 (Held *et al.*, 2010).

In this case too, many questions remain about the physiological relevance of a Ca^{2+}-initiated regulatory cascade to activate SOS1: As argued above, most studies suggest that the Ca^{2+} signal reports osmotic rather than ionic perturbation and the role of SOS1 in osmotic stress is unclear. Secondly, salt/osmotic-induced Ca^{2+} transients are short-lived and cytoplasmic Ca^{2+} is restored to resting levels within 1 or 2 min. Binding of Ca^{2+} to CBL4 can be expected to be instantaneous followed by dimerization and then binding to CIPK24. Although the dynamics of trafficking of the CBL-CIPK complex to SOS1 are unknown, the whole cascade is likely to be completed within minutes, when cytoplasmic and vacuolar Na^+ concentration may have risen but probably not to harmful levels.

Long-distance Na^+ transport

Most glycophytes can be classified as salt excluders, i.e. species that prevent a large accumulation of salt in photosynthesizing tissues (Marschner, 1995). Such species show a relatively high K^+:Na^+ selectivity where salt translocation is concerned, possibly via the reabsorption of salts in the basal parts of the root vasculature (Lessani and Marschner, 1978). In addition, retranslocation of Na^+ from the shoot to the root has also been described and such mechanisms would also contribute to low shoot salt loads (Marschner, 1995).

Long-distance transport to the shoot relies on loading into the xylem. The exact transport mechanisms for xylem loading of Na^+ have yet to be determined but may comprise both passive loading (mediated by Na^+-permeable ion channels located at the xylem–parenchyma interface: Wegner and De Boer, 1997), and active loading mediated by Na^+/H^+ exchangers. SOS1 (see previous section) is not only expressed in the root epidermis but also in xylem parenchyma and, in *Arabidopsis*, impacts on Na^+ loading into the xylem sap during moderate salt stress (Shi *et al.*, 2000). However, its exact function may depend on the severity of the salinity stress and include the removal of Na^+ from the xylem stream when salt stress is excessive. The cation antiporter CHX21 has also been implicated as a mechanism to move Na^+ into the xylem (Hall *et al.*, 2006). AtCHX21 is mainly expressed in the root endodermis and loss of function in this gene reduced levels of Na^+ in the xylem sap without affecting phloem Na^+ concentrations, however, CHX21 and its homologue CHX23 are also involved in K^+ homeostasis (Evans *et al.*, 2012).

Very convincing data are pointing to an important role of HKT-type transporters in controlling net Na^+ translocation to the shoot, via Na^+ retrieval from the xylem. Na^+ retrieval from the xylem had been postulated as a mechanism back in the 1970s (Lessani and Marschner, 1978) but now has a firmly established molecular basis. In *Arabidopsis*, loss-of-function mutations in the HKT1 gene led to the over-accumulation of Na^+ in shoots and rendered the plant hypersensitive to Na^+ (Berthomieu *et al.*, 2003; Moller *et al.*, 2009). In other species too, HKT isoforms have been implicated in long-distance

Na^+ movement, especially in cereals. In rice, OsHKT1:5 is a plasma membrane Na^+ transporter expressed in xylem parenchyma cells that retrieves Na^+ from the xylem sap (Ren *et al.*, 2005). The activity of OsHKT1;5 results in less Na^+ load in shoot tissue and, therefore, a considerably higher K^+:Na^+ ratio in leaf tissue. In wheat, the HKTs NAX1 and NAX2 fulfil similar roles (Lindsay *et al.*, 2004).

So, it appears that a relatively small number of proteins controls translocation of Na^+ from the root to the shoot. SOS1 may be regulated as explained above, with Ca^{2+} signals presumably leading to increased Na^+ loading into the xylem. Indeed, salinity provoked Ca^{2+} transients can be recorded in the endodermis and pericycle (Kiegle *et al.*, 2000). Many CHXs and HKTs are transcriptionally regulated in response to salt stress (e.g. https://www.genevestigator.ethz.ch/) suggesting that some sort of feedback mechanism operates between an altered Na^+ status and transport activity but nothing is known regarding the post-transcriptional regulation of HKTs and CHXs. However, as explained above, the activity of several HKTs directly responds to alterations in Na:K ratios, an important determinant of salt stress (see the second section on the early perception of salt stress and Na^+ sensing). Such kinetic mechanisms may be involved in regulating the quantity of Na^+ that is delivered to the shoot and/or the modulation of Na:K ratios in the transpiration stream. How and where control mechanisms operate can only be speculated on but the xylem parenchyma, or stelar tissue in general, would provide an excellent location for a Na^+-sensing moiety to control the important process of root to shoot flux.

Cellular partitioning of Na^+

In contrast to K^+, Na^+ can easily accumulate to toxic levels within the cell cytosol and various mechanisms are present to prevent this. Na^+ compartmentation in the vacuole probably occurs in all tissues and is a main strategy to detoxify Na^+ while still retaining its contribution as a 'cheap' osmolyte to lower the water potential. Antiport mechanisms, especially from the NHX family, were identified early on as H^+-driven exchangers at the tonoplast that load Na^+ into the vacuole (Fig. 1). AtNHX1 over-expression significantly improved salinity tolerance in *Arabidopsis* (Apse *et al.*, 1999) and subsequent manipulation of the expression of NHX1 orthologues in other species such as wheat (Xue *et al.*, 2004), rice (Fukuda *et al.*, 2004), and tomato (Zhang and Blumwald, 2001) showed the fundamental role this protein plays in salt tolerance and explains why it is a major focus for genetic engineering. Under normal, low Na^+, growth conditions, NHX exchangers mainly mediate K^+:H^+ exchange rather than Na^+:H^+ exchange (Zhang and Blumwald, 2001; Barragan *et al.*, 2012). This dual selectivity means that the exact HNX function during salinity is sometimes difficult to discern because it directly impacts on cytoplasmic Na^+ and K^+, stomatal function (Barragan *et al.*, 2012) and may indirectly modify Na^+ and K^+ translocation. For example, increased NHX1 expression led to improved wheat salt tolerance (Xue *et al.*, 2004). This was suggested to be the result of higher levels of shoot K^+ which were caused, indirectly, by increased Na^+ sequestration in root vacuoles.

Some insights about NHX1 regulation are available: Na^+:H^+ activity is regulated by binding of a calmodulin-like protein (CaM15) to the C terminus. NHX1-calmodulin interaction inhibits Na^+:H^+ exchange by lowering the enzyme V_{max} but, remarkably, does not affect K^+:H^+ exchange (Yamaguchi *et al.*, 2005). This provides a regulatory framework not only to modulate vacuolar Na^+ deposition but is also an elegant way to alter the cytoplasmic K^+:Na^+ ratio. Calmodulin itself requires Ca^{2+} binding for activation and to allow binding to other proteins. Intriguingly, this regulatory mechanism takes place in the lumen of the vacuole, ruling out a direct link to salinity-associated Ca^{2+} signals. By contrast, CIPK24 (SOS2) has also been put forward as a positive regulator of NHX1; its interaction with CBL10 is proposed to lead to phosphorylation of the NHX1 C-terminus although direct evidence for this has yet to be reported. In all, (different) Ca^{2+} signals may be involved in both up- and down-regulation of NHX1.

To prevent reflux of Na^+ from the vacuole to the cytoplasm, tonoplast Na^+ permeability appears to be lowered in saline conditions. For example, the activity of both SV (slow vacuolar) and FV (fast vacuolar) channels, both of which are Na^+ permeable is down-regulated in salt-grown plants (Maathuis and Prins, 1990; Bonales Alatorre *et al.*, 2013).

Cytosolic Na^+ is further controlled by H^+-driven antiporters that are expressed at the plasma membrane. For example, SOS1 expression is prominent in root tip cells. Since these cells are predominantly evacuolate and therefore lack the option of vacuolar Na^+ compartmentation, SOS1 is believed to be essential in this tissue for the extrusion of cytoplasmic Na^+ into the apoplast. Na^+ extrusion into the apoplast is assumed to take place in most plant tissues, particularly at the root–soil boundary. However, the participating proteins have not been identified and, whether such a mechanism can substantially contribute to salt tolerance, particularly in field conditions, has yet to be established.

Conclusion

Plants need to respond to the osmotic and ionic components of salt stress. This is important to maintain the correct functioning of the roots and shoots in the presence of water deficits and high concentrations of potentially toxic ions such as Na^+ and Cl^-. There are now plausible scenarios to explain how osmotic stress is sensed, for example, via specific kinases or mechanosensitive channels. There are also many other elements known to have roles in the response to salt stress, including Ca^{2+}, cyclic nucleotides, reactive oxygen species, and membrane lipids, but many of these appear to be either responders to the osmotic components of salinity or play roles in multiple abiotic stresses. Thus, how plants sense changes in Na^+ concentrations remains totally obscure. Regulation of Na^+ flux could rely on an entirely 'passive' mechanism via the activation of transporters such as SOS1 and NHX1. The K_m values for Na^+ of both SOS1 and NHX1 are in the tens of millimolar (Qiu *et al.*, 2004; Núñez-Ramírez *et al.*, 2012)

which means their activity would only become significant when cytoplasmic Na^+ levels exceed around 10 mM. But, the observation that expression of many genes is responsive to salinity strongly suggests more sophisticated signalling pathways are in place and Ca^{2+}, cGMP, and phosphorylation may be early components of these pathways although their upstream effectors and downstream targets are mostly unknown. If Na^+ specific, such pathways necessarily include an initial sensing moiety to report Na^+ concentration, either externally or in one or more intracellular compartments. Precedence from animal studies suggests that membrane transporters are the most likely candidates for this phenomenon. For K^+ (an ion very similar to Na^+) several K^+ channels have been proposed to function as sensor in plants (Geiger *et al.*, 2002; Johannson *et al.*, 2006; Liu *et al.*, 2006). Indeed, electrophysiological experimentation shows that the activity of several plant K^+ channels responds to either external $[K^+]$ or E_{K+} (representing the trans-membrane K^+ gradient; for a review see Dreyer and Blatt, 2009) and as such could report on the K^+ status of inside and/or external compartments. Unfortunately, as yet, there is no clarity whether this channel property only serves to regulate local K^+ channel activity or whether it plays a role at a more integrated level and is part of 'nutritional' K^+ sensing. Other obvious candidates to monitor ions are transcription factors, many of which have been shown directly to sense metal(oid)s such as Cu (Qi *et al.*, 2012) Zn (Li *et al.*, 2008), and As (Ali *et al.*, 2009). To date, many transcription factors that respond to salinity have been reported, such as members of the NAC family in wheat (Takasaki *et al.*, 2010), but it appears none of these responds specifically to Na^+.

So how do we get our hands on plant Na^+ sensors? With the increasing number of Na^+-co-ordinating and Na^+-sensing proteins identified in animals, it is tempting to use such domains for a homology search in plants. Na^+-co-ordinating motif searches could identify promising candidates although this is likely to be non-trivial because the binding domains are often ill-defined and/or rely on complicated tertiary structure. A bioinformatics approach could also be helpful. There are now many plant transcriptomics data available, making it feasible to identify co-regulated gene clusters that specifically respond to Na^+. These may be under the control of a relatively small number of Na^+-specific transcription factors that can be tested for DNA-binding efficacy as a function of Na^+. Alternatively, promoters of genes that respond selectively to Na^+, within an appropriate time-frame, can be fused to reporters such as luciferase to carry out forward genetics. Such an analysis may identify upstream signalling and/or sensing components.

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