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Review article

Salt stress sensing and early signalling events in plant roots: Current knowledge and hypothesis

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ABSTRACT

Soil salinity is a major environmental constraint to crop production. While the molecular identity and functional expression of Na^+ transport systems mediating Na^+ exclusion from the cytosol has been studied in detail, far less is known about the mechanisms by which plants sense high Na^+ levels in the soil and the rapid signalling events that optimise plant performance under saline conditions. This review aims to fill this gap. We first discuss the nature of putative salt stress sensors, candidates which include Na^+ transport systems, mechanosensory proteins, proteins with regulatory Na^+ binding sites, sensing mediated by cyclic nucleotide-gated channels, purine receptors, annexin and voltage gating. We suggest that several transport proteins may be clustered together to form a microdomain in a lipid raft, allowing rapid changes in the activity of an individual protein to be translated into stress-induced Ca^{2+} and H_2O_2 signatures. The pathways of stress signalling to downstream targets are discussed, and the kinetics and specificity of salt stress signalling between glycophytes and halophytes is compared. We argue that these sensing mechanisms operate *in parallel*, providing plants with a robust system for decoding information about the specific nature and severity of the imposed salt stress.

Contents

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1. Salinity as an issue

Soil salinity is a major environmental stress, affecting crop production worldwide. Globally, about 950 million hectares of arable land are affected by salinity, 250 million hectares of which is irrigated land [1]. The result is a $27.3 billion annual loss in revenue [2]. Contrary to other "drastic" stresses such as drought or flooding, the impact of salinity is more concealed; it is far less apparent. Combined with an increased rate of urbanisation and the use of the primary land for non-food related production (e.g. fibres, biofuels), agricultural production is expected to be further forced into marginal areas unsuited for modern crops [3]. Consequently, to achieve the challenge of feeding 9.3 billion people by 2050 requires a qualitative breakthrough in attempts to develop salt-tolerant germplasm. A remarkable breakthrough in developing various cutting-edge molecular technologies have led to the sequencing or of the genome of many staple crops, with sequencing of those missing is imminent. The major hurdle however, is the lack of understanding of the fundamental mechanisms regarding plant salt stress sensing and early signalling in salinised plants. This paper updates recent progress in this field and summarises our current knowledge on the physiological and molecular mechanisms of salt stress sensing and early signalling events in plants. Given the complexity of the topic, we primarily focus on NaCl stress (central to most crop production systems and ecosystems), not covering soil sodicity or other types of salts present in the Earth's crust.

2. Salt sensing: basic concepts and definitions

The Oxford dictionary defines a sensor as "*a device which detects or measures a physical property and records, indicates, or otherwise responds to it*". This is a rather broad definition that does not specify the timescale of the sensing process. In biological systems, a cellular- or tissue-based sensor should define the protein or some other molecule that is able to respond to the environmental fluctuation (stress) and then encode (in a broad meaning of this term) this information into an orchestrated cascade of biophysical, biochemical and molecular events aimed to optimise an organism's performance under altered conditions. Applying this principle to NaCl salinity stress (the focus of this review), such sensing should infer the ability of plant cells and tissues to respond to and signal changes in either Na^+ and Cl^- concentrations in the soil solution, associate changes in the soil osmotic potential, or both. Strictly speaking, only the components (molecules/proteins) that will be *the first* to detect changes in the external environment satisfy the exact definition of the term "sensor". This limits the range of sensing events to the millisecond timescale, and implicates a biophysical nature for the sensor. However, it can be argued that given the lifespan of the plant and the fact that the detrimental effects of salinity on plant performance may often become evident only days or even weeks after exposure to NaCl, from an adaptive point of view it probably does not matter whether the altered environmental information is encoded using the millisecond (e.g. alterations in membrane tension) or second (e.g. changes in membrane voltage) timescale. A good example of this is the recent discovery of the root oxygen-sensing mechanism in plants, mediated by the N-end rule pathway. In 2011, two independent papers were published in the highly regarded journal Nature [4,5]. These studies showed that the stability of the VII ERFs (ethylene-responsive factors from Group VII) determines the switching on or off of downstream signalling networks involved in the acclimation response to oxygen deprivation, terming them "oxygen sensors". Although these process operate in a timescale of minutes (perhaps even hours), the idea of these ERFs operating as oxygen sensors was widely accepted by the research community and is now cited in nearly every paper in the field. From this point of view, we believe that our discussion of salt sensing should not be limited to just the molecular nature of a *true sensor per se*, but should also include very early signalling events associated with a plant's response to a hypersaline environment. It is also important to note that it is the coordinated response of the *entire plant* that enables it to adapt to a hypersaline environment. In this context, it is expected that different plant tissues will possess different sensing mechanisms, most likely operating at different timescales. Indeed, the specific Na^+ toxicity in the shoot becomes an issue only after weeks of exposure to salinity [6], while specific NaCl-induced program cell death of root cells is observed within several hours of stress exposure [reviewed in 7]. Such different timescales could imply a need for different sensing mechanisms.

Our current knowledge of how salt stress is sensed by plant tissues is severely limited. Plants experience multiple constraints under saline conditions. These include elevated Na^+ levels in the rhizosphere, reduced water availability due to the hyperosmotic stress imposed by salinity, a dramatic increase in reactive oxygen species (ROS) accumulation in plant tissues and a massive disturbance to the cytosolic Ca^{2+} and K^+ homeostasis [6,8,9]. Each of these constraints may theoretically be sensed by either membrane-bound or cytosolic sensors, and then translated into a broad array of physiological and genetic alterations that optimise plant performance under saline conditions. Moreover, it is highly probable that more than one of these sensory mechanisms may operate in the same cell at the same time, encoding specific information on stress severity, and sharing some common downstream signalling pathway(s). Understanding the molecular and physiological mechanisms of such signalling remains a great challenge. The forthcoming sections summarise our current understanding of the field and identify possible candidate genes as putative salt stress sensors.

3. Putative salt stress sensors and proteins involved in early signalling events

3.1. Na^+ transport systems and proteins with regulatory Na^+ binding sites

Although no Na^+-selective ion channels have so far been identified in plants [10], it was suggested by Maathuis [11] that "*plants may contain other proteins that have regulatory Na^+ binding sites such as those found in mammals*", e.g. Na^+-activated K^+ channels, which are widely expressed in various animal tissues [12]. Under such a scenario, changes in Na^+ levels would be translated into fluxes of other ions (such as Ca^{2+} or K^+), triggering a cascade of metabolic and genetic alterations.

In the mammalian channels, the Na^+ sensor comprises the side chains of an aspartate and a histidine, which are located across from one another in a cytosolic loop [13] and form a DxR/KxxH motif [12]. This motif confers sensitivity to Na^+ in the previously insensitive mammalian Kir channels when engineered by mutagenesis [13]. Although plant equivalents of the mammalian Na^+-binding proteins are unknown, Maathuis has estimated that around 1200 candidate proteins containing this motif exist in *Arabidopsis* [11]. Among possible candidate genes, the cation-H^+ exchangers CHX5, CHX7, CHX16, CHX6, approximately 50 other transporters, ~27 transcription factors and ~120 kinases have emerged [11]. Further studies are needed to reveal their functional role(s) as possible Na^+ sensors by direct electrophysiological experiments on gain- or loss-of-function mutants. Importantly, the half-activation threshold for the Na^+-activated K^+ channels in mammalian systems were reported to be around 35 mM [12]; comparable with the cytosolic Na^+ levels typically found in plant cells.

Another potential candidate for Na^+ sensing in plants suggested in the literature is the SOS1 (salt overly sensitive (1) Na^+/H^+

antiporter [14]. It was argued by Zhu [15] that SOS1 protein has 10–12 transmembrane domains and a 700 amino acids-long tail that is predicted to reside in the cytoplasm and potentially sense Na^+. However, no direct experimental support for this hypothesis has been provided. Also, one can argue that SOS1 activity is regulated by a SOS3 (a myristoylated calcium binding protein) and SOS2 (a serine/threonine protein kinase) complex, which activates its C-terminus from the cytosolic site and relies on changes in cytosolic free Ca^{2+} [9; see also Section 4.1 for details], making it unlikely that SOS1 has a role as an initial sensor.

Another possible candidate for a Na^+ sensor may be the NCX Na^+/Ca^{2+} exchangers. NCX proteins have then been identified in the plasma membrane of many types of mammalian cells, and recent bioinformatics analysis [16] suggest the existence of a putative NCX gene in *Arabidopsis*. This gene was defined as NCX-like (AtNCL) and suggested to encode a protein with an NCX-like structure. NCX proteins have 9–11 transmembrane domains, with a large intracellular hydrophilic loop and are both structurally and functionally very different from the CAX Ca^{2+}/H^+ exchangers. By conducting sequence alignment analysis of NCXs and AtNCL, these authors have shown that some critical amino acids conserved in NCX (such as threonine, serine, or asparagine) and form the Na^+ site sensing domain on the intracellular side were also found in AtNCL [16]. The expression pattern of AtNCL examined by the GUS reporter gene fusion system revealed that AtNCL was expressed broadly in all tissues under normal growth conditions, with expression increased during abiotic stress such as salt or ABA treatment. Thus, the NCL exchanger may be ideally suited as a primary Na^+ sensor and be upstream of the cytosolic Ca^{2+} signalling, thus shaping salt stress-induced cytosolic free Ca^{2+} "signatures" and activating a broad range of metabolic pathways and transporters via modulating ROS levels (see below). It should be mentioned however that the *Atncl* mutant had higher survival rates under saline conditions compared with wild type, suggesting a possible redundancy in the NaCl sensing mechanisms.

3.2. NSCC/NADPH oxidases tandem

NADPH oxidase is a plasma-membrane-bound enzyme complex from the NOX family, which faces the extracellular space. When activated, NADPH oxidase produces the extracellular superoxide anion, $O_2^{\bullet -}$. The *Arabidopsis* genome contains 10 NOX genes, named RbohA-J [17]. NADPH oxidases are activated by salt stress, both at the transcriptional and functional level [18,19]. Two specific isoforms, RbohD and RbohF, seem to be critical to salt stress responses. Their expression is highly induced by salinity, and a double mutant in which the *AtrbohD* and *AtrbohF* genes are disrupted and generation of ROS was inhibited, was hypersensitive to salinity [19]. The functional role of NADPH oxidase and its activation by salt stress has been attributed to its involvement in the generation of the stress-induced Ca^{2+} "signatures" that mediate rapid systemic signalling [20], as well as to stabilisation of AtSOS1 transcripts [21]. Here we suggest that that the NADPH oxidase may also operate as a salt sensor in plants in tandem with Ca^{2+}-permeable channels (Fig. 1).

Plant NADPH oxidase Rbohs proteins have two N-terminal EF-hand motifs and are synergistically activated by Ca^{2+}-binding to the EF-hand motifs along with phosphorylation [17]. Ca^{2+} binding then triggers a conformational change that results in the activation of electron transfer originating from the interaction between the N-terminal Ca^{2+}-binding domain and the C-terminal superdomain [22]. The activity of the NADPH oxidase is half-saturated at about 1 μM of Ca^{2+}, which is consistent with reported values for the stress-induced cytosolic Ca^{2+} peak values [23,24]. Given the fact that such NaCl-induced cytosolic Ca^{2+} signals are detected within seconds [24], and that salt-induced ROS production is detected within minutes of an applied stress [25], involvement of the NADPH oxidase in salt sensing may be plausible.

A tentative model for such a sensing mechanism is depicted in Fig. 1. Plant plasma membranes harbor various non-selective cation channels (NSCC), which are permeable to Ca^{2+} and may be activated by both ROS and membrane depolarisation [26]. Importantly, these NSCCs cannot be blocked by either extracellular or intracellular Na^+ [27]. Salinity depolarises the plasma membrane almost instantaneously when Na^+ crosses the plasma membrane. This results in the instantaneous activation of NSCC, leading to a rapid elevation in the cytosolic Ca^{2+} (Fig. 1). Assuming NSCC are located in the immediate proximity of the NADPH oxidase (e.g. forms a microdomain in a lipid raft), this should also result in a very rapid activation of NADPH oxidase and a concurrent increase in ROS accumulation in the apoplastic space. These ROS will further activate NSCC and amplify stress-induced Ca^{2+} and ROS transients via self-amplification loops. The process can be further controlled or terminated by signals that could control NADPH oxidase activity. While the above model is only a hypothesis that requires experimental confirmation, published results on mammalian systems do make it plausible. Lipid rafts are cholesterol- and sphingolipid-enriched domains of the cell membrane [28]. It was shown that methyl β-cyclodextrin that depletes cholesterol from lipid rafts has abolished agonist-stimulated Ca^{2+} entry, while low concentrations of free cholesterol increased Ca^{2+} uptake into mammalian immune cells; changes that are tightly associated with the respiratory burst [29]. In plants, immune responses are intrinsically coupled with NOX-mediated oxidative burst [30]. In addition, the above cholesterol action in lipid rafts in mammalian cells was mediated by G proteins [29], proteins that also play a central role in controlling NOX activity in plants [30]. Measuring net Ca^{2+} fluxes and H_2O_2 production from plant tissues exposed to known cholesterol agonists will be crucial for validating this model.

3.3. Mechanosensory channels and transporters

Both animal and plant cells contain a large number of membrane receptors that sense and transduce changes in the physical force exerted on either cell walls or membranes [31]. These are likely candidates for sensing the salinity-induced changes in cell turgor observed immediately after the onset of stress [32], and some of them are shown to encode sodium-sensitive channels in mammalian systems [33]. These receptors are generally separated into two major groups: (1) mechanosensitive ion channels that open in response to membrane stretching [31,34], and (2) receptor-like (RLK) serine–threonine protein kinases imbedded into the plasma membrane that transduce mechanical forces to downstream targets [35] (Fig. 2).

Mechano-sensory channels (MSC) convert a mechanical force into electric trans-membrane variation within milliseconds. Thus, they are the most likely candidates for sensing changes in the external media imposed by a sudden change in the solution osmolality. They are ubiquitously expressed in various plant tissues including roots, leaves and guard cells. Mechano-sensory channels vary dramatically in their selectivity and may range from non-selective to Cl^-, Ca^{2+}, and K^+-selective channels and have a difference in their conductance as big as two orders of magnitude [31].

Three major types of MSC are known in plants [31,34,35]: (1) the MscS-like (MSL) protein family, (2) MCA protein family, and (3) the Piezo protein family. MscS mechano-sensory channels usually lack specificity and are permeable to various inorganic and organic substrates. Similar to all channels, they can rapidly transport massive amounts of ions (3 billion ions per second [35]), thus are ideally suited for acting both as a sensor and a safety valve. Ten MscS-like (MSL) homologues have been identified in *Arabidopsis*. At least two of them (MSL9 and MSL10) are expressed at the plasma membrane [31]. Both these channels have strong selectivity towards Cl^-, so can release anions and depolarise a membrane within millisec-

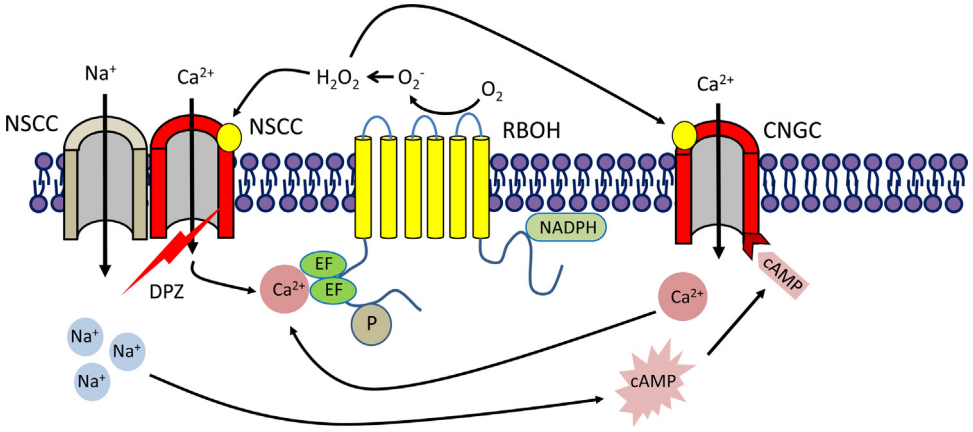

Fig. 1. Sensing salt stress by NADPH oxidases and cyclic nucleotide-gated channels. The Rboh protein is clustered together with either the Ca^{2+}-permeable depolarisation-activated non-selective cation channel (NSCC) or a cyclic nucleotide-gated channel (CNGC) to form a specific "microdomain" in a lipid raft. Na^+ entrance into the cell (via NSCC in the model) results in a rapid elevation of the cytosolic free Ca^{2+} [24] as a result of membrane depolarisation (DPZ). Ca^{2+} is then bound to one of the EF hands of the NADPH oxidase and (if phosphorylated) activates electron transfer, resulting in an increased apoplastic concentration of superoxide (O_2^-) that is rapidly converted into H_2O_2 by the cell wall superoxide dismutase (SOD). H_2O_2 then activates ROS-sensitive NSCC, leading to a further increase in the cytosolic Ca^{2+} concentration and amplifies stress-induced Ca^{2+} and ROS transients via a self-amplification loop. For CNGC, salt-stress induced increase in the cytosolic cNMP will be translated into a massive Ca^{2+} uptake into the cytosol, shaping Ca^{2+} "signatures" and activating gene network-mediated plant adaptive responses to salinity.

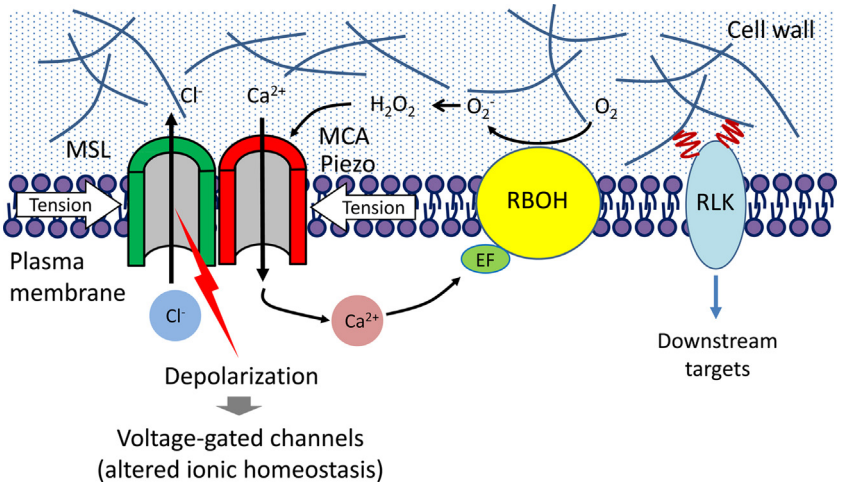

Fig. 2. Sensing salinity stress by mechanosensory channels and transporters. Changes in the mechanical force exerted on the plasma membrane due to the osmotic effect associated with salinity are translated into altered activities of either anion (chloride-permeable; MSL) or cation (Ca^{2+}-permeable; MCA or Piezo) channels. This activation is immediate (milliseconds range) and represents the fastest receptor system in plants. MSL activation and the associated Cl^- efflux results in a significant plasma membrane depolarisation, thus affects the uptake of all other ions and metabolites transported by voltage-gated systems. Activation of MCA leads to a rapid increase in the cytosolic free Ca^{2+} (resulting in NaCl-induced cytosolic Ca^{2+} spike [24]) and is then self-amplified by the PM-bound NADPH oxidase (Rboh in the model). In addition, stress-induced deformation of the cell wall is sensed by the receptor-like protein kinases (RLK) and then transmitted to the cell interior via kinase-dependent phosphorylation of target proteins.

onds. MSL2 and MSL3 are located in the plastid envelope [31], thus could potentially couple salt stress-induced changes in the xylem tension [36] with changes in the leaf photochemistry. MSL channels are also present in guard cells [37] and could allow the rapid coupling between the root-induced changes in the xylem pressure observed immediately after stress onset [36] and changes in the stomatal aperture required to adapt to new conditions.

The MCA (Mid1-complenenting activity) protein family is present in all land plants [34]. MCA channels are predominantly Ca^{2+}-permeable and located at the plasma membrane. Overexpression of MCA1 and MCA2 correlates with increased Ca^{2+} influx in *Arabidopsis* in response to hypo-osmotic stress or mechanical stimulus, and roots of *mca1* null mutant were unable to grow into hard agar [31]. The MCA1 channel was also associated with an increased lignin production [38], and was suggested to signal cell-wall damage. Consequently, MCA channels may have a dual role in salt stress signalling and adaptation: sensing tension changes resulting from a rapid reduction of turgor upon cell-wall loosening and increasing lignin deposits, thus reducing cell wall permeability to elevated Na^+.

The Piezo family encodes cation non-selective mechano-sensitive channels. Piezo proteins form large homomeric complexes with 36 transmembrane helices per monomer [31]. It was predicted that at least one gene encodes a Piezo-like protein in *Arabidopsis*, but its characterisation has not yet been reported.

Specific details of activation of mechano-sensitive channels remain a topic for intensive discussion. At least two different models have been suggested [31]. The "intrinsic" model implies that a mechanical force imposed on the cell is directly transmitted to the channel through the lipid bilayer surrounding the channel (Fig. 2). The membrane is thinned as a consequence of this mechanical tension, so the pulling force exerted upon the channel by the bilayer lipids is increased and conformational changes occur, favouring the open state of the channel. The "trapdoor" model implies that the

mechanical force is transmitted to a domain of the channel via links to other cellular structures such as the cell wall or cytoskeleton. In this model deformation of the cell wall is transmitted to the cell interior via kinase-dependent phosphorylation of the target proteins by receptor-like protein kinases (RLK). Two major families of RLK are known in plants: wall-associated kinase (WAK) and RLK from the *Catharanthus roseus* subfamily [31]. Importantly, some of the RLK mutants showed reduced ROS production, suggesting a possible interaction with the NADPH oxidase-mediated sensing mechanism discussed above. RLK are also known to be specifically associated in various microdomains (lipid rafts) and mediate both ROS burst (via chitin-dependent binding) and also plasmodesmata closure [28], thus directly affecting Na^+ movement between cells.

3.4. Cyclic nucleotides receptors

Cyclic nucleotides are established signalling molecules in plant adaptive responses to environment [39,40]. Their cellular levels increase within seconds after onset of salt and osmotic stress [9]. In *Arabidopsis*, 20 non-selective cation channels belong to the cyclic nucleotide-gated channel (CNGC) family, which can be activated upon binding of cyclic nucleotides to the ligand-binding site [41,42]. These channels are permeable to Ca^{2+} [42], and in a tandem with NADPH oxidase, may comprise a self-amplifying functional unit (Fig. 1) in which salt stress-induced increases in the cytosolic cyclic nucleotides is translated into a massive Ca^{2+} uptake into cytosol, shaping Ca^{2+} "signatures" and activating gene network-mediated plant adaptive responses to salinity. Some CNGCs do not discriminate amongst monovalent cations [26], so may also constitute a pathway for initial Na^+ entry.

3.5. Purino-receptors

Extracellular purines such ATP and ADP function as signalling molecules, activating specific animal ionotropic (P2X) receptors [43] that may form functional ligand-gated non-selective (NSCC) ion channels. Application of extracellular ATP to plants causes plasma membrane depolarisation in *Arabidopsis* root hairs [44] as well as transient elevations in the cytosolic free Ca^{2+} [45]. These effects are blocked by antagonists of animal P2X receptors, suggesting that purinergic receptors also exist in plants [43], and that these receptors are involved in the signalling events during stress. The role of the extracellular ATP (eATP) in stress responses has been postulated on several occasions [46]. Acute salinity stress also induces a transient elevation in eATP in *Populus euphratica* roots [47]. Salt-induced eATP is thought to be sensed by the purinoceptors in the plasma membrane and then translated into downstream signals such as H_2O_2 and cytosolic Ca^{2+}, which are required for the upregulation of genes linked to K^+/Na^+ homeostasis and plasma membrane repair [47]. Evidence for eATP acting upstream of other signalling molecules such as cytosolic Ca^{2+}, reactive oxygen species and nitric oxide, have also been presented in other studies [45,48,49]. eATP signalling blockage by P2 receptor antagonists resulted in a loss of the acclimation ability to salinity in *P. euphratica* [47], and a causal link between eATP and salinity stress sensing may be postulated based on the fact that sustained eATP elevation causes programmed cell death in salt-tolerant poplar species [47].

Using a combination of electrophysiological and imaging techniques, we have determined that extracellular ATP is sensed at the plasma membrane rather than in the cell wall [43]. The perception of extracellular ATP causes the production of reactive oxygen species (ROS) through the activation of a specific plasma membrane NADPH oxidase. Thus, similar to the model described above for CNGC, it may be suggested that P2X receptors cluster in close proximity to NADPX oxidase and form a functional "tandem" that senses

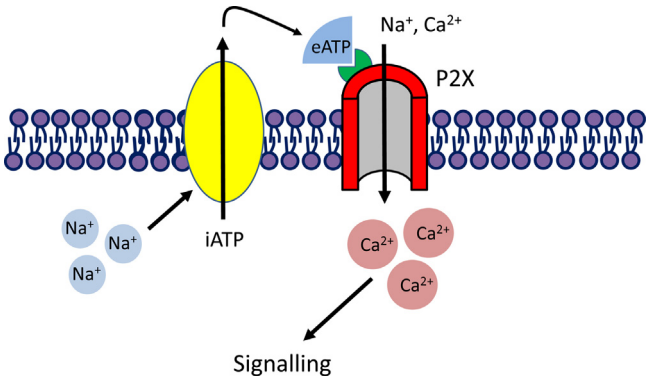

Fig. 3. Sensing via P2X purinoreceptors. Salinity stress activates some (currently unidentified) protein that transports ATP from the cytosol to the apoplast. The extracellular eATP is then bound to the P2X receptor that operates as a Ca^{2+} permeable channel and results in transient elevations in $[Ca^{2+}]_{cyt}$ [45].

salt and translates it into the elevation of cytosolic free Ca^{2+} (Fig. 3). The peak of eATP production in salt-stressed roots was observed within 5 min [47], making the system rather efficient in early stress recognition.

3.6. Annexins

Annexins are soluble proteins that contain a four-fold repeat of an approximately 70 amino acid domain, a discrete calcium binding site, and with a molecular mass between 33 and 36 kD [50]. Plants contain several annexin isoforms possessing multiple roles including cation transport [50]. The most abundant *Arabidopsis* annexin, AtANN1, is a plasma membrane protein [51] capable of forming Ca^{2+}-permeable channels in planar lipid bilayers. It is responsible for root epidermal plasma membrane Ca^{2+}-permeable conductance *in planta* [52]. While AtANN1 does not contribute to root Na^+ uptake, it is a component of the cytosolic Ca^{2+} signal generated upon salt treatment and is suggested to be a key component in root cell adaptation to salinity [53]. The physiological role of AtANN1 is attributed to ROS-induced cytosolic Ca^{2+} elevation upon NaCl exposure and stabilisation of SOS1 Na^+/H^+ exchanger protein. AtANN1 is also a negative regulator of Na^+ influx that may influence activity of Na^+ uptake routes such as via AtCNGC3 and AtCNGC10 channels [54,55]. Annexin expression, abundance, and cellular position can respond to osmotic stress, salinity, drought and ABA (reviewed in [56]). Annexin 1 mRNA abundance increased by salinity stress in *Arabidopsis* [57], and both salinity and ABA-induced Annexin 12 expression in tobacco [58]. Importantly, stress-induced changes in annexin protein abundance may occur even though transcript levels are not affected (e.g. Annexin 1 protein changes upon osmotic stress treatment; [51]), making it a good candidate for very early signalling events in root responses to NaCl stress.

3.7. H^+-ATPase/GORK tandem

It has long been repeatedly proposed that a proton extrusion pump may function as a stress detector. According to this view, H^+ pump operation is directly affected by the hydrostatic pressure gradient across both sides of the plasma membrane. No supporting evidence has been provided however. More recently, it was shown that the plant plasma membrane H^+-ATPase is stimulated by potassium bound to the proton pump at a site involving Asp(617) in the cytoplasmic phosphorylation domain [59]. Binding of K^+ to this site induces dephosphorylation of the phosphorylated E(1)P reaction cycle intermediate by a mechanism involving Glu(184) in the conserved TGES motif of the pump actuator domain, suggesting a role for K^+ as an intrinsic uncoupler. A massive K^+ efflux is

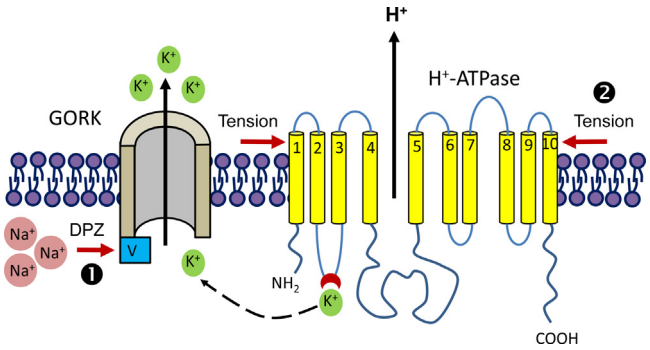

Fig. 4. Sensing via the H^+-ATPase/GORK tandem. Similar to previous models, H^+-ATPase and a depolarisation-activated GORK channel form a "microdomain" in a lipid raft. Membrane depolarisation caused by Na^+ crossing the plasma membrane results in an immediate depolarisation and opening of GORK channels, causing K^+ efflux [8,60]. As the GORK channels are located in close proximity to H^+-ATPase, the cytosolic K^+ concentration in the H^+-ATPase 'microdomain" will drop rapidly, modifying the activity of H^+-pump.

one of the earliest events measured in plant roots in response to salinity [7,8,60]. In *Arabidopsis*, this efflux is mediated by outward-rectifying depolarisation-activated (GORK) channels [60], resulting in rapid (within minutes) 2-fold decreases in cytosolic K^+ concentration [27]. Assuming GORK channels are located in close proximity of H^+-ATPase, a reduction in cytosolic K^+ concentration in the H^+-ATPase microdomain may be even more pronounced and rapid, prompting activation of the H^+-pump by release of inhibition at Glu(184) as described above (Fig. 4).

4. Salt stress transduction pathways

4.1. SOS signalling pathway

Maintenance of the optimal cytosolic K^+/Na^+ ratio is considered a key attribute of salinity stress tolerance in plants [7,8,60,61]. In animal cells, the cytosolic Na^+ content is regulated by a ubiquitous plasma membrane Na^+/K^+-ATPase. Such Na^+/K^+-ATPases are absent in higher plants that do not require Na^+ for growth [11] and where Na^+ exclusion is mediated by SOS1 (salt overly sensitive) Na^+/H^+ antiporters [14]. AtSOS1 belongs to the CPA1 (monovalent cation/proton antiporter) family and is located at the plasma membrane. The importance of SOS1-mediated Na^+ exclusion in crop salt tolerance is widely accepted (see [6,62] for reviews).

The activity of SOS1 is regulated by the CBL-CIPK complex. According to the proposed model, salt stress-induced Ca^{2+} signal is first detected by SOS3 (CBL4), a myristoylated calcium binding protein [63]. SOS3 is then activated by recruiting SOS2 (CIPK24), a serine/threonine protein kinase [63,64]. The resulting CBL-CIPK complex then increases the activity of SOS1 by phosphorylating its C-terminus [11,65]. Activated SOS2 proteins added to salt-treated isolated *Arabidopsis* membrane vesicles resulted in a two fold increase in Na^+/H^+ exchange activity [66]. Furthermore, SOS2 interacts with tonoplast Na^+/H^+ NHX exchangers [67], thus also controls Na^+ sequestration in vacuoles. There is also evidence that SOS2 regulates vacuolar V-ATPase activity [68], energising the tonoplast and enhancing salt tolerance. SOS3 may be involved in the regulation of other pathways and proteins that are related to salt stress, including AtHKT1- mediated Na^+ entry [69], ABA synthesis [70] and Ca^{2+}-dependent reorganisation of actin filaments during salt stress [71]. The putative calcium sensor CBL10 interacts with the SOS2 protein kinase and protects *Arabidopsis* shoots from salt stress [72]. CIPK-CBL complexes activate inward-rectifying K^+ channels in various plant systems [73]. The coordinated action of CBL-CIPK complexes produces transient phosphorylation, activating several signalling pathways in response to environmental stresses including salinity [74]. AtMyb73 is a negative regulator of SOS induction in response to salt stress in *Arabidopsis* [75]. Two other members of the SOS family have also been identified and characterised. AtSOS4, encoding a pyridoxal kinase, is involved in the pyridoxal phosphate (vitamin B6) synthesis and root hair development [76]. Another member of this family is AtSOS5, a cell-surface proteoglycan that regulates cell expansion and is essential for the root growth under saline conditions [77]. Therefore, a role of the SOS pathway in root architectural changes under salt stress has emerged in addition to its well-established role in maintaining ionic homeostasis [78].

As well as its essential role as a Na^+ efflux system, SOS1 may also mediate Na^+ loading into the xylem [3,79]. SOS1 has a role in protecting K^+ uptake by maintaining plasma membrane K^+ permeability in the presence of Na^+ [80] and also interacts with ROS; SOS1 mRNA stability is elevated by H_2O_2 treatment [21]. SOS2 also interacts with the nucleoside diphosphate kinase 2 (NDPK2), a H_2O_2 signalling protein, and catalases [81]. Taken together, these results suggest possible crosstalk between the SOS pathway and ROS signalling during salt stress. More explicit proofs for this model may come from experiments quantifying tissue-specific ROS production in NaCl- challenged *Atsos* roots.

4.2. MAPK

Mitogen-activated protein kinases (MAPKs) are a specific class of serine/threonine protein kinases that have key roles in the transduction of external stress signals to a range of cellular responses in eukaryotes. A MAPK cascade minimally consists of a MAPKKK-MAPKK-MAPK module that is linked in various ways to upstream receptors and downstream targets [82]. Generally, under environmental stresses, the stimulus-triggered activation of a MAPKKK phosphorylates MAPKKs, which in turn phosphorylate MAPKs. Plants have two large subfamilies of MAPKKKs (also known as MAP3Ks and MEKKs), and four groups (A–D) of both MAPKKs (also known as MAP2Ks, MEKS, and MKKs) and MAPKs (also known as MPKs) [83]. All plants MAPKs have a TEY or TDY phosphorylation motif at the active site, while in contrast to TEY MAPKs, all TDY MAPKs have long C-terminal extensions [82]. The phosphorylation and activation of MAPKs can lead to the phosphorylation of transcriptional effectors, thereby reprogramming gene expression [84].

High salinity (250 mM NaCl) rapidly induces activation of AtMPK4 and AtMPK6 in *Arabidopsis* [85]. Also, the transgenic overexpression of rice *OsMAPK5* increases salt tolerance, in a contrast to the significantly reduced salt tolerance of dsRNAi lines [86]. *Arabidopsis* MKK2 is specifically activated by cold and salt stresses and by the stress-induced MEKK1. Plants overexpressing MKK2 have constitutively high MPK4 and MPK6 activity and increased salt tolerance [87]. Salt-induced activation of SIMK (stress induced MAPK) is thought to be mediated by the dual-specificity protein kinase SIMKK (stress induced MAPK Kinases) [88]. SIMKK and SIMK are co-localised in the cytoplasm and in the nucleus in an inactive state. A substantial part of the nuclear pool of both is relocated to the cytoplasmic compartments upon salt stress [89]. Overexpressing SIMKK enhanced the activity of both MPK3 and MPK6 in *Arabidopsis,* but plants had increased sensitivity to salt stress at the seedling stage [90]. This apparent controversy for the role of MPK6 in the overall salt tolerance suggests that not only MAPKs but also MAPKKs play an important role in response to salt stress.

In addition to its involvement in salt stress response, MPK6 also phosphorylates ACS (1-aminocyclopropane-1-carboxylic acid synthase), inducing ethylene biosynthesis in *Arabidopsis* [91]. Interestingly, NaCl activated MPK6 also phosphorylates the C-terminal of SOS1 in *Arabidopsis* [92]. This suggests crosstalk between MAPK and SOS salt stress signalling pathway [83].

4.3. Other signalling cascades

Elevated NaCl levels in the soil solution impose a hyperosmotic stress on plants. Thus, the pathway for the osmotic stress signalling might be partially invoked during salt stress signalling transduction. SnRK2 (sucrose nonfermenting 1-related protein kinase 2) has emerged as a component of the osmotic signalling mechanism [9]. Indeed, *snrk2.1/2/3/4/5/6/7/8/9/10* mutants (carrying mutations in all 10 members of the SnRK2 family in *Arabidopsis*) grew poorly under hyperosmotic stress conditions but were similar to the wild type in a culture media without osmotic stress, demonstrating critical functions of the SnRK2s in mediating osmotic stress signalling and tolerance [93]. SnRK2.4 and SnRK2.10 are involved in the maintenance of the *Arabidopsis* root system architecture during salt stress [94], suggesting a possible role of the SnRK2 family in the signalling response to salt stress. Another kinase from this family, SnRK2.6, modulates activation of the respiratory burst oxidase homologue RbohF [9], thus controls salt stress-induce ROS production, with implications for long-distance ROS signalling between the root and shoot [95]. Other members of SnRK2 family are also activated under saline stress. Some (SnRK2.4 and snRK2.10) bind to signalling phospholipids [9], which have the capacity to assemble complexes with both RbohF and potassium channel subunits. This may integrate various signalling pathways and also enable a cross-talk between various sensing mechanisms. A salt stress signalling pathway that resembles an endoplasmic reticulum stress response was also identified and characterised in *Arabidopsis*. It includes AtS1P (subtilisin-like serine protease) and AtbZIP17 (membrane-localised b-ZIP transcription factor) components [96]. According to suggested model [96], up-regulation of salt-inducible genes involves AtbZIP17 processing and its translocation to the nucleus.

5. Specificity of salt stress signalling in halophytes

Halophytes naturally inhabit saline environments and benefit from having substantial amounts of salt in the growth media [97,98]. Optimal halophyte growth is achieved between 50 mM and 150 mM NaCl [98]. Some halophyte species do not have a significant yield reduction even when irrigated with seawater (e.g. *Suaeda maritima*). Despite this, the general consensus is that there is nothing really unique to halophytes, neither in their anatomical features, nor in their physiological mechanisms. The major difference to glycophytes is how efficiently these mechanisms are controlled in these two plant groups [3,98]. The key to improving salinity stress tolerance in crops may lie in understanding how salt stress-responsive mechanisms are *regulated* in halophytes. For example, is there anything special about Ca^{2+} and ROS "signatures" in halophyte species?

Similar to glycophytes, halophytes utilise Ca^{2+} sensors (CaM, CMLs, CDPKs, and CBL/CIPKs), which mediate adaptive responses to salinity, but are modulated in a different manner. For example, CIPK1 was upregulated to a greater extent in halophytic *P. euphratica* than in salt-sensitive *Populus tomentose* [99]. A CIPK from the halophyte *Hordeum brevisubulatum* (HbCIPK2) rescued salt hypersensitivity in the *Arabidopsis sos2-1* mutant and further enhanced salt tolerance in wild type *Arabidopsis* [100]. This indicates a similarity between the SOS pathway in glycophytes and halophytes. CBL10 and CIPK3 were upregulated not only in roots but also in individual epidermal bladder cells in halophytic *Mesembryanthemum crystallinum* [101]. The presence of salt bladders is one of the anatomical hallmarks of halophytes that have a major role in protecting mesophyll cells from the excessive Na^+ [102]. Understanding the role of Ca^{2+} signalling pathways and Ca^{2+} sensing molecules in these unique salt storage organs may thus be pivotal for trafficking Na^+ load away from metabolically-active photosynthetic tissues in conventional crops.

The kinetics of Ca^{2+} signalling is another important difference between halophytes and glycophytes. NaCl-induced cytoplasmic Ca^{2+} elevation in the halophytic quince (*Cydonia oblonga* Mill) species were more rapid, higher in the amplitude, and returned to a lower cytoplasmic concentration than the basal Ca^{2+} level [103] than in glycophytic rice [104]. Interestingly, Na^+ entry into the cytoplasm was essential for the cytoplasmic Ca^{2+} elevation in rice [104] but not in quince [103], suggesting that Na^+ is sensed in a halophyte species at the *apoplastic* side and is subsequently translated into elevation of the cytoplasmic Ca^{2+}.

The kinetics of H_2O_2 signalling appears to be much faster in halophytes than in glycophytes [105]. For example, the salt-induced H_2O_2 accumulation peaked at 4 h upon salt stress onset in the leaves of a halophyte *Cakile maritime* and declined rapidly afterwards. In the glycophyte *Arabidopsis thaliana*, however, H_2O_2 continued to accumulate even after 72 h after salt application [106]. Similarly, salt stress-induced H_2O_2 production was higher in the leaves of a halophyte (*P. euphratica*) in comparison with the leaves of a glycophyte (*P. popularis*) after 24 h of salinity treatment [107], suggesting that elevated H_2O_2 levels upon stress exposure are *essential* to confer salt stress signalling and adaptation to stress in halophytes. Enhanced H_2O_2 production in the chloroplasts of *Thellungiella salsuginea* is pivotal for the "salt stress preparedness" mechanism in this halophyte species [108], consistent with this notion.

While (transiently) elevated H_2O_2 appears to be essential for salt-stress signalling in halophytes, interaction between H_2O_2 and transition metals (Fe^{2+} or Cu^{2+}) present in cell walls may result in the generation of highly reactive hydroxyl radicals via the Fenton reaction. Hydroxyl radicals damage cellular structures, decrease cytosolic K^+/Na^+ ratio by directly activating a range of Na^+, K^+ and Ca^{2+}-permeable cation channels [109,110] and eventually cause cell death [109]. The abundance of the metal binding proteins (e.g. ferritin) in specific tissues or cellular compartments controls the concentration of the transition metal ions available for the Fenton reaction, hence regulates the H_2O_2-mediated signal propagation in plants. Salt stress increases ferritin deposits in the leaves of the halophytic *M. crystallinum* [111]. Similarly, a green micro-alga (*Dunaliella salina*) that grows only under highly saline conditions accumulates large quantities of a triplicated transferrin-like protein [112]. Furthermore, there is a transient increase in the transcripts of ferritin gene (*Fer1*) in the leaves of a mangrove *Avicennia marina* 12 h after salt stress [113]. These observations suggest that halophytes can indeed regulate the propagation of H_2O_2-mediated signalling by increasing the abundance of metal binding proteins in all the tissue/cellular organelles where H_2O_2 accumulates, preventing it from being converted into hydroxyl radicals.

Another physiological hallmark of halophytes is a highly efficient membrane potential maintenance and voltage gating. Plasma membrane depolarisation is amongst the earliest (few seconds) responses observed upon salinity treatment, e.g. [114]. The extent of the plasma membrane depolarisation is clearly salt dose dependent [105], suggesting that the plasma membrane potential itself can be a salt sensing mechanism (e.g. Fig. 5). The downstream targets for the plasma membrane depolarisation could be depolarisation-activated potassium channels (e.g. GORK) and Ca^{2+} -permeable cation channels because: (i) regulation of GORK through the membrane potential is important for K^+ homeostasis during salt stress [7,8], and (ii) Ca^{2+} entry into the cytoplasm through depolarisation activated cation channels can act as a signal that activates SOS1 (discussed in Section 4.1). A comparison between halophytes (*Atriplex lentiformis*, *Chenopodium quinoa* and *Thellungiella halophile*) and glycophytes (e.g. *Arabidopsis*, barley, lucerne) revealed that roots of halophytes maintained membrane potential at more negative values than glycophytes, at a given

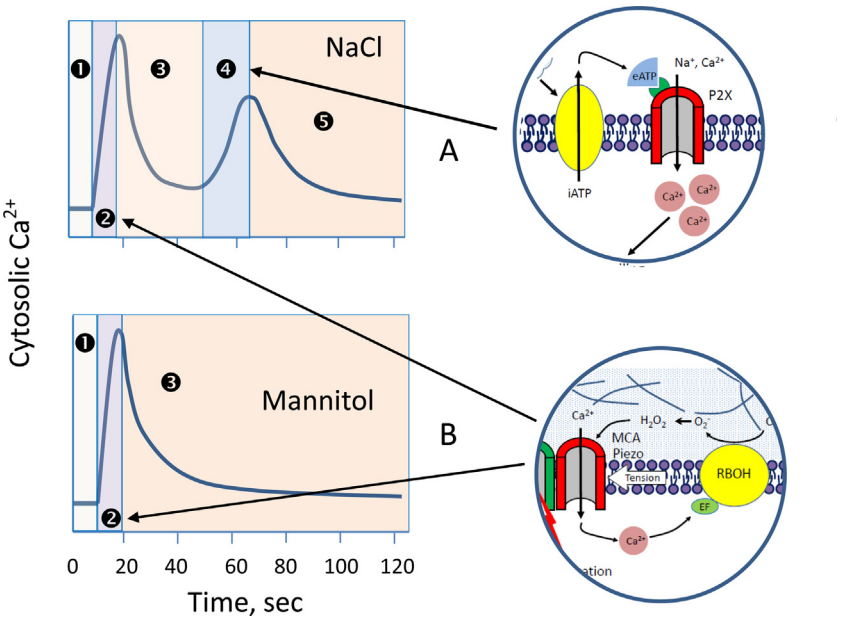

Fig. 5. Integration of salt-sensing mechanisms to distinguishing between NaCl-specific and non-ionic (mannitol) osmotic stresses. Panels A and B depict the qualitative course of transient changes in the cytosolic free Ca^{2+} concentrations in response to each stimulus, as reported in [118]. ❶ - prior to a stimulus, the cytosolic Ca^{2+} concentration is set around the 100 nM level. ❷ - stress onset (at 10 s) activates MCA mechano-sensing Ca^{2+} channels and results in a rapid transient elevation in the cytosolic free Ca^{2+}. This elevation is identical for both NaCl- and mannitol-induced stress. ❸ - Ca^{2+} efflux systems are then activated to return cytosolic free Ca^{2+} to its basal level [119,122]. ❹ - by this time, sufficient amounts of eATP is accumulated in the apoplast as a result of Na^+ accumulation in the cytosol and activation of some ATP transport protein. P2X receptors are activated, resulting in a second peak in $[Ca^{2+}]_{cyt}$. This peak is absent in a case of the mannitol treatment. ❺ - additional Ca^{2+} efflux systems are then activated to return cytosolic free Ca^{2+} to its basal level.

external salt concentration [[114] and references within]. Consequently, the observed K^+ loss through GORK was several fold smaller in halophytes than in glycophytes [114]. Taken together, it is tempting to suggest that the difference between halophytes and glycophytes in salt stress sensing comes from the operation of the H^+-ATPase/GORK tandem sensing mechanism.

A salt stress-induced increase in H^+-ATPase activity was observed in the roots of *C. quinoa* and in calluses from *Spartina patens* [114,115] even though H^+-ATPase transcript levels remained unchanged. It was suggested that Na^+ directly inactivates the autoinhibitory domain of the C-terminal portion of H^+-ATPase, thereby stimulating H^+-ATPase activity [115]. However, in vitro experiments revealed that NaCl inhibits the H^+-ATPase activity in two different halophytes, *S. patens* and *Salicornia bigelovii* [116]. H^+-ATPase activity is also dependent on cytosolic K^+ levels ([59]; Fig. 4). The binding of the regulatory 14-3-3 proteins to the autoinhibitory C-terminal domain of the pump is the key mechanism that controls H^+-ATPases activity [117]. In this context, it is absolutely essential to compare the kinetics of 14-3-3 protein production in halophytes and glycophytes under saline conditions and to link it with the observed alterations in cytosolic K^+ and Na^+ content.

Finally, as discussed in Section 3.2, a putative *Arabidopsis* Na^+/Ca^{2+} exchanger (AtNCL) was suggested to operate as a Na^+ sensor [16], acting upstream of cytosolic Ca^{2+} signalling in plants. The veracity of this suggestion is further supported by the observation that the NCL gene is much more strongly upregulated in a halophyte *P. euphratica* than in salt-sensitive *P. tomentose* [99]. A broader range comparison between halophytes and glycophytes will shed light on the essentiality of NCL protein activity in salt sensing and signalling.

6. Different salt sensing mechanisms are integrated and shape stress-specific Ca^{2+} and H_2O_2 "signatures"

As shown above, plant membranes harbour a large number of transport proteins, each of which may potentially operate as a salt sensor. Importantly, all of them operate over different time scales. The fastest are mechano-sensing systems that are activated almost instantaneously (millisecond range) upon changes in the solute osmotic potential. This is then followed by sensing via tentative proteins with regulatory Na^+ binding sites (such as SOS1) and voltage-gated H^+-ATPase/GORK tandem system (seconds range), and then by slower sensing systems that rely on changes in either cytosolic or apoplastic concentrations of second messengers (e.g. cyclic nucleotides or eATP). All these sensing mechanisms may operate *in parallel* and are integrated via common components, namely changes in cytosolic free Ca^{2+} and H_2O_2. Taken together, such a sophisticated sensing mechanism may provide plants with a robust system of decoding information about the specific nature and severity of the salt stress, converting it into stress-specific Ca^{2+} and H_2O_2 "signatures". This model also explains how plants can distinguish between NaCl-specific and non-ionic osmotic stresses (and adjust accordingly). For example, 200 mM NaCl treatment results in a two-peak transient cytosolic Ca^{2+} elevation in *Arabidopsis* [118], as revealed by an aequorin-based bioluminescence assay. The first peak appeared almost instantaneously (within 10 sec of treatment), while the second peak came 50–80 s later. This second peak was absent when isotonic mannitol treatment was used [118]. This difference may be explained by assuming that two different systems operate concurrently in NaCl sensing by *Arabidopsis* roots. One relies on mechano-sensing proteins and results in a rapid (within few seconds) transient elevation in the cytosolic free Ca^{2+} (the

first reported peak; Fig. 5). The second mechanism may include, for example, sensing via P2X receptors that are activated some time later (e.g.1–2 min; Fig. 5; hence, a second peak). This second mechanism will not operate when mannitol treatment is used, thus explaining the difference in transient kinetics between isotonic NaCl and mannitol treatments, as reported in [118]. The concurrent use of several sensing systems may also explain the presence of the widely reported stress-induced oscillations in cytosolic Ca^{2+} [119].

Why do plants need such a sophisticated sensing network and do not rely on only one system? The most likely explanation is that by relying on systems operating on very different principles, the sensing process is more robust and makes it fail-safe. This suggestion is well in line with the operating principles of general signalling networks in plants. The closure of the Venus flytrap lobes occurs only when trigger hairs are activated by two consecutive mechanical stimulations within a short time frame [120]. Propagating Ca^{2+} and ROS waves in systemic acquired acclimation signalling in plants are believed to "prime" tissues to the occurrence of a localised abiotic stress stimulus [121]. Communication between roots and shoots relies on a broad array of chemical (assimilates, hormones, nutrients, peptides), physical (electric and hydraulic signals, propagating Ca^{2+} and ROS waves), and molecular (RNA and proteins) signals [95]. Each of these signalling systems operates with a different time scale (e.g. hydraulic—almost instantaneous; electric—rapid; hormonal—slow), but carries various layers of information about the specific nature of the stimulus. Here we suggest that a similar principle is also applicable to root sensing of salt stress.

Understanding the tissue-specific mechanisms of salt sensing and signalling is essential for exploiting the potential of molecular and genetic tools to create salt-tolerant germplasm. For example, numerous attempts have been undertaken to improve salinity stress tolerance by increasing the activity of antioxidant enzymes. However, as commented by Bose et al. [105], "*a simple search on Web of Science for three keywords "antioxidant", "breeding or improvement", and "plant" reveals 791 entry. Unfortunately, while half of these reports claim a positive association between antioxidant production in plant tissues and plant salinity tolerance, other showed no, or even negative, correlation between these two traits*". The likely explanation for this controversy may be the fact that if these antioxidant genes are expressed in a wrong tissue they may interfere with ROS signalling and handicap plant sensing (and adapting to) salinity stress. Another example may be the post-translational regulation of the SOS1 Na^+/H^+ exchanger. While higher SOS1 activity is essential for Na^+ removal from the root cell cytosol back into apoplast, SOS1 transporters are also expressed in the xylem parenchyma and are likely involved in xylem Na^+ loading (see [3] for arguments and thermodynamical calculations). Thus, increasing SOS1 activity to pump Na^+ back into the soil media has the caveat of increasing xylem Na^+ loading and its delivery to the shoot, unless activation of SOS1 transporters in root epidermis and xylem parenchyma is sensed and controlled by different mechanisms.

In conclusion, as stated in the recent review by Maathuis [11], the molecular identity and mechanisms of plant Na^+ sensing remain obscure. Moreover, it is highly likely that plant cells do not possess one specialised Na^+ sensor in the strict sense of this term, but rather sense and respond to salinity stress by activating multiple sensing networks operating over different timescales. Further experimental studies combining electrophysiological and molecular approaches are needed to establish a solid experimental basis for this hypothesis and arm breeders with clear instructions of the genes and tissues that have to be targeted in order to improve salinity stress tolerance in crops.

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