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REVIEW PAPER

Calcium signaling during salt stress and in the regulation of ion homeostasis

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Abstract

Soil composition largely defines the living conditions of plants and represents one of their most relevant, dynamic, and complex environmental cues. The effective concentrations of many either tolerated or essential ions and compounds in the soil usually differ from the optimum that would be most suitable for plants. In this regard, salinity—caused by excess NaCl—represents a widespread adverse growth condition, but shortage of ions such as K^+, NO_3^-, and Fe^{2+} also restrains plant growth. During the past years, many components and mechanisms that function in the sensing and establishment of ion homeostasis have been identified and characterized. Here, we reflect on recent insights that extended our understanding of components and mechanisms which govern and fine-tune plant salt stress tolerance and ion homeostasis. We put special emphasis on mechanisms that allow for interconnection of the salt overly sensitive pathway with plant development and discuss newly emerging functions of Ca^{2+} signaling in salinity tolerance. Moreover, we review and discuss accumulating evidence for a central and unifying role for Ca^{2+} signaling and Ca^{2+}-dependent protein phosphorylation in regulating sensing, uptake, transport, and storage processes of various ions. Finally, based on this cross-field inventory, we deduce emerging concepts and questions arising for future research.

Keywords: Calcium, CBL–CIPK, iron, metals, nitrate, nutrient sensing, potassium, salt stress, SOS.

Introduction

Because the sessile lifestyle of plants is inevitably connected with their rooting in the ground, the actual composition of the soil and especially fluctuations of its components such as minerals, metals, and ions are vitally important for plants. In nature, but also in artificial agricultural environments, an optimal soil composition is the exception rather than the rule. Instead, deviations of many soil components from the optimum either towards a shortage or alternatively towards excess supply represent the daily challenge for plant life. Suboptimal supply of essential nutrients and too high concentrations of normally tolerated ions in any case represent stress conditions to plants. The main soil stresses that affect plants and crops in the field include soil salinity, adverse pH, insufficient water availability, nutrient depletion, and anaerobic stresses connected with flooding (Suzuki *et al.*, 2014).

A steadily growing share of agriculturally used areas but also of natural ecosystems is facing salinity stress due to high salt in soils, currently affecting 20% of the total, and 33% of

irrigated agricultural lands worldwide (Munns and Tester, 2008; Cominelli *et al.*, 2013; Schroeder *et al.*, 2013). Due to the predominant role of NaCl, we refer within this article to the effects of high Na^+, when using the terms saline or salt. However, it should be considered that high concentrations of Cl^- anions are also toxic for plants (Tester and Davenport, 2003). The physiological and molecular implications of high Cl^- concentrations on plants as well as their Cl^- transport and evasion strategies have been most recently discussed in a comprehensive review article (Geilfus, 2018).

Salt accumulation in arable soils is mainly derived from irrigation water that contains trace amounts of NaCl, and from seawater (Munns and Tester, 2008; Deinlein *et al.*, 2014). Soils are considered saline when their electric conductivity reaches ≥4 dS m^{-1}, which is equivalent to 40 mM NaCl (Marschner, 1995). However, some crops, such as beans, are sensitive to much lower salt concentrations (Cabot *et al.*, 2014). In saline environments, the Na^+ concentration in soils has been determined to range from ~100 mM to 2380 mM Na^+ (Flowers 1985). Most crops will stall, die off, or at least not produce fruits or seeds under growth conditions exceeding 100 mM NaCl (Mass and Hoffman, 1977; Zhu, 2001; Flowers, 2004). High concentrations of Na^+ can alter the basic texture of the soil, resulting in decreased soil porosity and consequently reduced soil aeration and water conductance. High salt deposition in the soil generates a zone of low water potential, making it increasingly difficult for the roots to acquire both water and nutrients. One facet of salt stress for the plants is therefore the resulting water deficit mimicking a physiological drought in combination with nutrient deficiencies (Mahajan *et al.*, 2008). By causing osmotic stress, salt stress can inhibit the activity of many essential enzymes, cell division, and cell expansion, and can cause membrane disorganization and osmotic imbalance, which finally lead to growth inhibition (Marschner, 1995; Tuteja, 2007). Higher concentrations of Na^+ also provoke the stomatal limitation of photosynthesis and excess production of reactive oxygen species (ROS) (Chaves *et al.*, 2009; Flowers *et al.*, 2010). Irrespective of these aspects, the sole ionic component of salinity stress, namely the toxicity of Na^+, also has enormous consequences for the plant and exerts dramatic adverse effects on plant physiology including imbalances in the homeostasis of other ions such as K^+ and Ca^{2+} (Munns and Tester, 2008; Anschütz, *et al.*, 2014; Julkowska and Testerink, 2015).

The maintenance of intracellular, but also organismic scale, ionic homeostasis is therefore fundamental to plant physiology. The versatility and diversity of implications that salt stress has on plants is reflected by the complexity and variety of sensing and adaptation mechanisms that are triggered in challenged plants. These mechanisms involve, for example, the control of cellular water and ion homeostasis in different plant tissues as well as triggering scavenging mechanisms of toxic compounds (Hasegawa *et al.*, 2000). To mount an effective response to cope with salinity stress, land plants have developed the ability to sense and adapt to both osmotic stress and Na^+ (Deinlein *et al.*, 2014), which include Na^+/H^+ transporters at both the plasma membrane and tonoplast that enable efflux of Na^+ ions out of the cell into the apoplastic space or to sequester them in the vacuole, respectively. Also, transcriptional reprogramming and specific synthesis of osmolytes, as well as avoidance of salt stress by directing root growth away from high salt concentrations belong to the repertoire of plant response strategies. All these aspects of plant stress adaptation have been most informatively covered in a number of recent review articles (Julkowska and Testerink, 2015; Han *et al.*, 2017; Ismail and Horie, 2017; Köster *et al.*, 2018; Yang and Guo, 2018).

Similar to salt stress, lack of essential ions or nutrients (or in rarer cases their excess) triggers plant adaptation reactions to re-establish an optimal physiological homeostasis. These processes have been intensively studied for important nutrients and ions such as K^+, Mg^{2+}, Fe^{2+}, NO_3^-, and NH_4^+ (Nieves-Cordones *et al.*, 2016; Jeong *et al.*, 2017; Tang and Luan, 2017; Tegeder and Masclaux-Daubresse, 2018). Although our knowledge about the plant perception and adaptation of such environmental cues has been steadily increasing, for many of these substances it remains unclear how they are sensed, and details of their signal transduction and adaptation reactions are only beginning to unfold. Nevertheless, an emerging common theme of these fundamental plant response reactions appears to be the occurrence of Ca^{2+} signals somewhere during the chain of events resulting in plant physiological adjustment.

Consequently, in this review, we first focus on recent findings in the field of salt stress-related Ca^{2+} signaling. In this context, we discuss emerging indications for the integration of the Ca^{2+}-triggered 'salt overly sensitive' (SOS) pathway with the co-ordination of developmental programs, nutritional status, and hormonal regulation to achieve plant developmental plasticity especially in stress conditions. From this, we expand our reflections towards the arising theme of Ca^{2+} signaling as a common and versatile mechanism shared by an increasingly appreciated number of plant nutrient and ion sensing and adaptation processes.

Calcium signaling in response to salt stress

Early studies on the possible involvement of Ca^{2+} signals in salt stress responses have already revealed strong indications that Ca^{2+} signaling indeed appears to be an early and prominent feature of the plant responses to this environmental cue (Lynch and Läuchli, 1988; Lynch *et al.*, 1989). It was found that plants exhibit a rapid increase in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) within seconds of being exposed to NaCl or mannitol (Knight *et al.*, 1997). The similarity of Ca^{2+} signatures induced by either NaCl or non-ionic osmotic triggers has up to now prevented conclusive discernment of whether Na^+ as an ion specifically triggers a Ca^{2+} response independently of its osmotic impact. Also, the still unknown identity of functional salt stress receptors up to now impedes the advancement of our understanding of these most important early steps in salt stress signaling (Köster *et al.*, 2018). Several studies observed that the salt-induced Ca^{2+} rise originated within the roots (Kiegle *et al.*, 2000; Tracy *et al.*, 2008). Moreover, more detailed studies on the location and dynamics of the initial Ca^{2+} signals already suggested cell specificity and tissue heterogenicity of this response. For example, Kiegle and colleagues observed a most pronounced increase in cytoplasmic Ca^{2+} concentration in endodermal cells throughout the root in response to acute salt stress (Kiegle *et al.*, 2000).

Independently, it was found that an initial Ca^{2+} signal after salt stress was formed close to the root apex and then appeared to disperse to basal parts of this organ (Moore *et al.*, 2002). More recently, the application of advanced Ca^{2+} reporter proteins has provided further important details about the Ca^{2+} signals that are triggered in response to NaCl application. Mazars and colleagues observed that exposure of roots to NaCl was sufficient to trigger systemic, wave-like Ca^{2+} elevations in plant leaves (Xiong *et al.*, 2014). Moreover, targeted local application of NaCl to distinct parts of Arabidopsis roots was found to trigger an initial locally restricted Ca^{2+} signal within a few seconds that subsequently expanded through the root and whole plant in a wave-like manner (Choi *et al.*, 2014). These important studies already suggest that Ca^{2+} signaling during salt stress represents a rather complex phenomenon and that its function and consequences are not restricted to the single-cell level. Moreover, these studies also provided initial evidence that Ca^{2+} signals fulfill a central role in orchestrating the whole-plant response to soil-borne signals that are formed by salt stress. Subsequently, it was reported that the tonoplast-localized cation channel TPC1 and the plasma membrane-localized NADPH oxidase RBOHD crucially contribute to the appropriate formation of salt-induced long-distance signals (Choi *et al.*, 2014; Evans *et al.*, 2016). These findings reinforce the emerging complexity of these processes, since they indicate the involvement of multiple cellular compartments and the contribution of several signaling systems to the full manifestation of Ca^{2+} signaling in response to salt stress. However, as important as the formation of Ca^{2+} signals for the implementation of appropriate physiological responses is their decoding and translation into downstream reactions.

Converting Ca^{2+} signals into salt tolerance: the SOS pathway

Our current knowledge on central processes and components of plant salt tolerance is to a large extent due to an elegant genetic screen for SOS mutants that identified (and allowed characterization of) central components of this pathway. Despite the non-biased character of this screen, the very first gene to be identified serendipitously turned out to be that encoding the Ca^{2+}-binding protein SOS3 that most probably functions to decode the above-described Ca^{2+} signals (Liu and Zhu, 1997). Subsequently, SOS3 was found to be the fourth member (designated as CBL4) of the family of calcineurin B-like proteins (CBLs) which function as Ca^{2+} sensor proteins in many fundamental processes in plants (Kudla *et al.*, 1999; Kolukisaoglu *et al.*, 2004). Subsequent research established that SOS3 interacts with and activates the CBL-interacting protein kinase (CIPK) SOS2/CIPK24 (Halfter *et al.*, 2000; Liu *et al.*, 2000). The resulting Ca^{2+} sensor–kinase complex then phosphorylates and activates the Na^+/H^+ antiporter SOS1, which functions in Na^+ extrusion and long-distance Na^+ transport in plants (Shi *et al.*, 2000, 2002).

The SOS pathway, consisting of a Ca^{2+} sensor, a protein kinase, and a substrate, represents the first completely identified CBL–CIPK pathway for maintaining ion homeostasis in plant cells (Qiu *et al.*, 2002) (Fig. 1). Although, the formation and activation of SOS3/CBL4–SOS2/CIPK24 complexes *in vitro* was not promoted by increased Ca^{2+} concentrations, it is generally accepted that the SOS pathway lies downstream of the salt-induced root $[Ca^{2+}]_{cyt}$ increases (Yang and Guo, 2018).

The SOS pathway appears to be conserved in every plant species that has been studied in this regard, and experimental evidence for its functional conservation has been obtained not only for higher plants, but also for mosses such as *Physcomitrella patens* (Benito and Rodríguez-Navarro, 2003; Fraile-Escanciano *et al.*, 2010). Strong evidence for such functional conservation has, for example, been obtained by heterologous complementation of Arabidopsis *sos* mutants with respective SOS cDNAs from different species, including, for example, the crops maize and rice (Martínez-Atienza *et al.*, 2007; Wang *et al.*, 2007; Olías *et al.*, 2009). Comparative evolutionary studies suggest that SOS3/CBL4 and SOS2/CIPK24 may even constitute the most basal representatives of their respective gene families with single homologs already present in green algae, suggesting that a precursor of this pathway and its regulation by Ca^{2+} may have been already functional very early in plant evolution (Edel and Kudla, 2015).

Recent research has further corroborated the central role of the kinase SOS2 in salt tolerance, but has also extended the complexity of the Ca^{2+} sensing components that contribute to the SOS pathway. The Ca^{2+} sensor CBL10/SCaBP8 has also been found to form complexes with SOS2. In contrast to the root-expressed CBL4, CBL10 is predominantly expressed in the aerial tissues of Arabidopsis. Here, CBL10–SOS2 complexes can again target SOS1 and in this way contribute to salt extrusion over the plasma membrane (Quan *et al.*, 2007). Moreover, CBL10–SOS2 complexes are also localized at the tonoplast membrane and appear to activate an as yet unknown transport protein for Na^+ sequestration in the vacuole (Fig. 1). In line with this latter function, *cbl10* mutants accumulate significantly less Na^+ during salt stress despite their strong salt sensitivity (Kim *et al.*, 2007). Most recently, analyses of a tomato T-DNA insertion mutant in combination with complementation of an Arabidopsis *cbl10* mutant with a tomato CBL10 cDNA provided strong evidence for a conserved function of tomato CBL10 in salt tolerance by regulating Na^+ fluxes in the vacuole (Egea *et al.*, 2018). Also, in poplar (*Populus trichocarpa*), which expresses two CBL10 homologs, both isoforms have been localized at the tonoplast and have been shown to function in shoot salt tolerance via interaction with *P. trichocarpa* SOS2 (Tang *et al.*, 2014). While CBL4 function has so far only been specifically associated with salt tolerance, CBL10 appears to be a multifunctional Ca^{2+} sensor. In tomato, CBL10 together with CIPK6 were found to interact with RBOHB at the plasma membrane and to contribute to the regulation of ROS generation during effector-triggered immunity (de la Torre *et al.*, 2013). Moreover, in rice, variations in the OsCBL10 promoter have been reported crucially to determine the flooding tolerance of the respective rice cultivars by affecting OsCIPK15 protein accumulation (Ye *et al.*, 2018). These cases already clearly illustrate the integrative capacity of Ca^{2+} signaling to interconnect salt stress responses with additional physiological processes at the level of Ca^{2+} sensor function. Finally, in Arabidopsis, it was found that CBL10 can also interact with the K^+ channel Arabidopsis K^+ Transporter 1 (AKT1) and, in this case, counteract effective accumulation

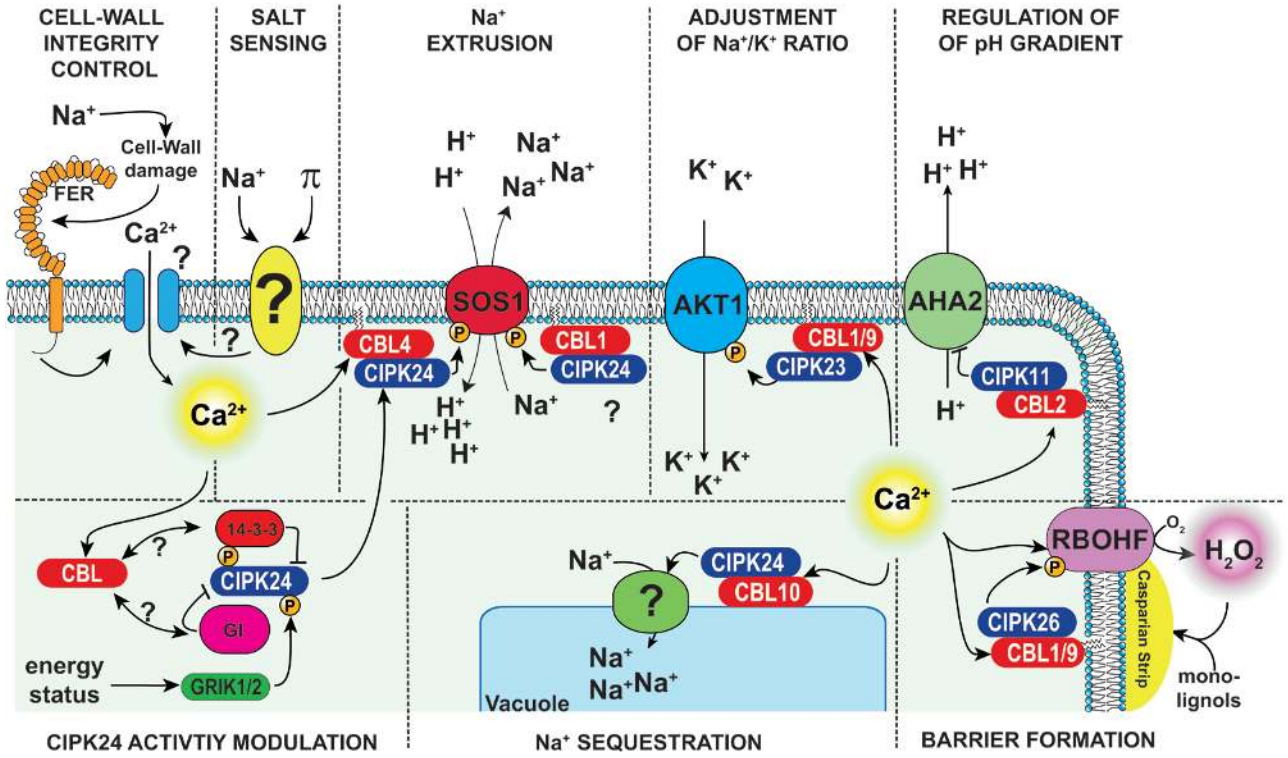

Fig. 1. Ca^{2+} signaling crucially functions in various facets of plant salt stress responses. The receptor-like kinase FERONIA (FER) is required for single cell-specific Ca^{2+} signaling that mediates cell wall integrity control during late salt stress responses, which are linked to re-establishment of growth. The recognition of Na^+-induced cell wall softening probably involves physical interaction between the extracellular domain of FER, and pectin- and FER-dependent signaling elicits cell-specific Ca^{2+} signals within this process. The molecular nature of the receptor(s) for both the ionic (Na^+) and the osmotic (π) component of salt stress, as well as the identity of the downstream Ca^{2+} channel(s) responsible for the well-documented cellular Ca^{2+} influx upon salt stress remain to be uncovered. Na^+ extrusion from the cytosol is achieved by the activity of the Na^+/H^+ antiporter SOS1. Upon salt stress, the transporter is phosphorylated and thereby activated by the kinase SOS2/CIPK24, which is in turn regulated by the interacting Ca^{2+} sensor protein SOS3/CBL4. Besides CBL4, other members of the CBL protein family can potentially form functional complexes with SOS2/CIPK24, such as CBL10 and CBL1. Along with activation by CBL Ca^{2+} sensors, additional CIPK24 activity modulators have been recently identified. The geminivirus Rep-interacting kinases (GRIKs) 1 and 2, which both play critical roles in sugar/energy signaling by phosphorylating SnRK1-type kinases, were identified also to phosphorylate CIPK24 within its activation loop at Thr168. This phosphorylation is required for full SOS2 activation and accurate responses during salt stress. In the absence of salt stress, CIPK24 is kept in an inactive state by both the flowering time regulator GIGANTEA and the 14-3-3 proteins λ/κ. Whether these inhibitory interactions interfere with CBL binding to CIPK24, or if they represent CBL-independent mechanisms, remains to be addressed. Within the aerial parts of the plant, upon salt stress Na^+ sequestration into the vacuole is a strategy to retain cytoplasmic Na^+ concentrations in a tolerable range. Here, CBL10/CIPK24 complexes probably activate a still unknown mechanism of Na^+ transport into the vacuole. One of the toxic effects of Na^+ within the cells is the perturbance of the Na^+/K^+ ratio. To counteract this effect triggered by Na^+ influx into cells during salt stress, the import of K^+ ions can be enhanced to achieve adjustment of the Na^+/K^+ ratio by Ca^{2+} elevations through activation of the plasma membrane-localized K^+ transporter AKT1 by CBL1/9—CIPK23 complexes. The regulation of the pH gradient across the plasma membrane is crucial for salt tolerance responses, as this gradient represents the driving force for the SOS1-mediated Na^+ extrusion from the cytosol. In the absence of salt stress, the AHA2 proton pump is negatively regulated by CBL2 and/or CIPK11 to prevent excess ATP consumption. During the onset of stress, this negative regulation is released. During endodermal differentiation, a ring-like, lignified tertiary cell wall deposition called the Casparian strip is formed to block the apoplastic and the coupled trans-cellular pathway into the stele to prevent excess uptake of toxic elements such as Na^+ into, as well as leakage of valuable metabolites from, the vasculature. For this barrier formation, precisely localized activity of the NADPH oxidase RBOHF is required to produce H_2O_2 needed for lignin cross-linking by peroxidases. RBOHF is known to be directly regulated by Ca^{2+} binding and phosphorylation by CBL/CIPK26 complexes. *rbohF* mutants with disturbed Casparian strip formation display a specific transpiration-dependent salt stress phenotype.

of AKT1 at the plasma membrane to regulate K^+ uptake negatively (potentially in the absence of salt stress) (Ren *et al.*, 2013; Yang and Guo, 2018). Such a function would be in line with the importance of maintaining an optimal K^+/Na^+ ratio in plants for sustaining salt tolerance and may also explain the salt-sensitive phenotype of *cbl1* mutants that has been reported previously (Albrecht *et al.*, 2003; Cheong *et al.*, 2003; D'Angelo *et al.*, 2006). The Ca^{2+} sensor CBL1 constitutes a major activator of the K^+ uptake channel AKT1, and the impairment of AKT1 activation in *cbl1* mutants may underlie their salt-sensitive phenotype (Cheong *et al.*, 2003; Xu *et al.*, 2006) (Fig. 1). In the future, it will be most interesting to address whether other CBL proteins or alternative Ca^{2+} sensors contribute to the fine-tuning or functional flexibility of the core SOS pathway.

Integrating the SOS pathway with a plant's daily routine

Salt stress can hit plants by chance at any given time and under any given physiological condition. Consequently, the ability for a prompt and appropriate activation of the SOS pathway (in particular) and its co-ordination with either

ongoing developmental processes, physiological programs, or other stress responses is crucial for a plant's fitness. Recent research has provided first important insights into such interconnections.

One emerging principle of SOS2 regulation and co-ordination appears to be phosphorylation by other kinases (and of course the corresponding dephosphorylation). In this regard, it was found that SOS2 directly interacts with the protein phosphatase ABI2, which prominently functions in ABA signaling (Ohta *et al.*, 2003). Unfortunately, the physiological and mechanistic consequences of this interaction still await further elucidation. However, endodermis-specific expression of the abscisic acid (ABA)-insensitive 1-1 mutant protein (ABI1-1), a dominant-negative version of the ABA-regulated phosphatase ABI1, which is most closely related to ABI2, negatively affected plant salt responses, underscoring the important role of ABA signaling and its likely interconnection with Ca^{2+} signaling in plant salt tolerance (Duan *et al.*, 2013; Dinneny 2015).

More recently, Yan Guo and colleagues identified and investigated the consequences of phosphorylation at amino acid Ser294, which is located in the junction domain of SOS2 (Zhou *et al.*, 2014). In non-stressed conditions, this residue is phosphorylated and allows for interaction with the 14-3-3 proteins λ and κ. This binding of 14-3-3 proteins to SOS2 renders this kinase inactive and thereby maintains the SOS pathway in a dormant status (Fig. 1). Salt stress probably triggers dephosphorylation of S294 (by an as yet unknown phosphatase), resulting in 14-3-3 dissociation from SOS2 and thereby allowing for activation of the SOS pathway. Moreover, salt stress has been found to accelerate 26S–proteasome-mediated degradation of both 14-3-3 proteins (Tan *et al.*, 2016). These conclusions were further corroborated by genetic analyses. A non-phosphorylatable S294A version of SOS2 failed to complement the salt-sensitive phenotype of *sos2* mutants. Also, *14-3-3 λ/κ* double mutants displayed an enhanced salt tolerance when compared with the wild type, a phenotype in line with their negative regulatory role in this pathway. This mechanism potentially ensures rapid SOS pathway activation immediately after the onset of the stress, as well as pathway stabilization. The dephosphorylation of SOS2 instantly turns on the pathway, while simultaneous degradation of 14-3-3s facilitates its continuous function.

Another trans-phosphorylation site, Thr168, was found to interconnect the SOS pathway with the energy status of the plant (Barajas-Lopez *et al.*, 2018). This residue was determined to be phosphorylated by the kinases GRIK1 and 2 which previously have been reported to activate SnRK1s that function centrally in metabolic regulation in eukaryotes. GRIK phosphorylation appeared to enhance SOS2 activity and, accordingly, mutation of Thr168 prevented complementation of salt sensitivity of a *sos2* mutant. Moreover, *grik1/2* double mutants were sensitive to Na^+ stress. Reconstitution analysis of the SOS pathway in an appropriate yeast mutant background also indicated that GRIK co-expression increased the capacity of SOS proteins to confer salt tolerance. Considering the dual role of GRIKs, these data identify a mechanism that may allow for a co-ordinated modulation of the plant sugar-sensing/energy status via SnRK1 phosphorylation and salt stress tolerance via SOS2 phosphorylation.

Intriguing insights into how a plant's salt tolerance can be directly interconnected with the regulation of fundamental developmental programs such as plant flowering were enabled by the finding that the well-known circadian clock, flowering time, and light signaling regulator GIGANTEA (GI) directly interacts with SOS2 (Kim *et al.*, 2013). Interaction of SOS2 with GI appears to interfere with SOS2 function in the SOS pathway and cages this kinase in the nucleus and cytoplasm (Fig. 1). Salt stress triggers GI degradation and thereby releases SOS2, which most probably allows for SOS3 interaction and, in this way, enhances its function in the salt stress pathway. This process of SOS2 caging and release is reflected in the corresponding phenotypes. GI-overexpressing plants are more salt sensitive than wild-type plants, whereas GI mutants are markedly salt tolerant. Together these findings identify GI as a transitory modulator of the salt stress response that enables integration of stress responses with flowering time regulation and provides a mechanistic explanation for the long-observed impact of salt stress on flowering time regulation in plants.

Multifaceted roles of Ca^{2+} in salt stress responses

Despite the obvious central role of Ca^{2+} signaling in activating the core SOS pathway, recent findings suggest that the function of this second messenger in salt stress responses may be even more versatile than so far appreciated. In this regard, accumulating evidence points to the involvement of a diverse array of Ca^{2+} sensor proteins in the various aspects of salinity tolerance. For example, CML9 [a member of the calmodulin-like (CML) gene family] was found to be up-regulated during salt stress, and *cml9* loss-of-function mutants displayed hypersensitivity in germination assays on medium containing either NaCl or ABA, whereas adult *cml9* plants show enhanced tolerance towards irrigation with salt water (Magnan *et al.*, 2008). While transcriptomic analysis revealed altered expression of several stress marker genes in *cml9* mutants, the target proteins of CML9 and the exact nature of the regulated process(es) remain to be uncovered. Moreover, the Ca^{2+}-dependent protein kinase 3 (CDPK3, designated as CPK3 in Arabidopsis) was found to interact with the vacuolar two-pore K^+ channel 1 (TPK1) *in planta* and to phosphorylate it in a Ca^{2+}-dependent manner *in vitro* (Latz *et al.*, 2013). As this phosphorylation is specifically induced *in planta* upon salt stress and as both *tpk1* and *cpk3* loss-of-function mutants show salt-sensitive phenotypes, this study points to a function of CPKs in the regulation of the K^+/Na^+ ratio during saline conditions (Latz *et al.*, 2013).

Another example in this regard is the function of the NADPH oxidase RBOHF in transpiration-dependent salt stress signaling (Jiang *et al.*, 2012; Köster *et al.*, 2018). The genomes of higher plants encode NADPH oxidases as gene families of considerable size with, for example, 10 members in Arabidopsis. In plants, these proteins are usually referred to as 'respiratory burst oxidase homolog' (RBOH) proteins, with alphabetic nomenclature. These proteins are localized at the plasma membrane and have been reported to regulate various biological processes including pathogen responses, plant development, and abiotic stress tolerance by their ability for

a fine-tuned production of ROS as second messengers. (Sagi and Fluhr, 2006; Miller *et al.*, 2009; Steinhorst and Kudla, 2013)

A common feature specific to plant RBOH proteins, that directly links Ca^{2+} signaling to ROS signaling, is the occurrence of two Ca^{2+}-binding EF-hands in their N-terminal, cytoplasmic domain. These elements of Ca^{2+} regulation are also present in RBOHF, which has been shown to be strongly expressed in the endodermal cell layer and to play a crucial role in the formation of the Casparian strip, which functions as a diffusion barrier, preventing both uncontrolled access of solutes into the vasculature and unwanted leakage out of the stele (Jiang *et al.*, 2012; Lee *et al.*, 2013). This function in plant development coincides with a second striking phenotype in that *rbohF* mutants are hypersensitive to salt stress when they are grown on soil, but indistinguishable from the wild type in their salt tolerance when grown *in vitro* on agar plates. The reason for this phenotypic difference lies in the transpiration dependence of enhanced Na^+ accumulation in the stele, the xylem sap, and shoot tissues, that is causing this mutant phenotype (Jiang *et al.*, 2012). Although the available data suggest that the transport and accumulation of multiple ions appear to be affected in mutants defective in endodermal barrier formation, the supposed agronomical relevance of this transpiration-dependent salt tolerance pathway argues for a consideration of this process in the context of plant salt tolerance (Pfister *et al.*, 2014; Doblas *et al.*, 2017; Köster *et al.*, 2018). Currently, it cannot be fully distinguished whether the morphological alterations in *rbohF* mutants or the modulation of ROS generation (or even both aspects together) are causative for the resulting salt sensitivity. Independent studies identified RBOHF as a target of Ca^{2+}-activated CBL1–CIPK26 complexes (Drerup *et al.*, 2013). Unfortunately, a potential role for CBLs or CIPKs in general, or more specifically for CBL1 and CIPK26, in transpiration-dependent salt tolerance has not been addressed. Nevertheless, the dual indication for a regulatory role for Ca^{2+} in ROS production (via Ca^{2+} binding to the EF-hands and via Ca^{2+}-dependent phosphorylation) by RBOHF leads to a tempting hypothesis that this second messenger plays a considerable role in endodermal differentiation and transpiration-dependent salt tolerance. This facet of Ca^{2+} signaling clearly deserves more attention in the future.

Establishment of salt tolerance not only requires proper adjustment to the acute stress, but also 'damage surveillance' and implementation of growth recovery. One of the primary targets of toxic Na^+ ions is the cell wall. Novel findings have linked Ca^{2+} signaling to these aspects of salt tolerance. Feng and colleagues investigated the contribution of the FERONIA (FER) receptor kinase to salt stress tolerance in Arabidopsis (Feng *et al.*, 2018). Quite intriguingly, they identified a novel class of Ca^{2+} signals that occurred as late-stage, stress-induced, local transients mostly in the early elongation zone for up to 15 h. These transients were localized to individual cells, persisted for <1 min, and were spatially and temporally correlated with growth recovery of the root. Importantly, these transients were strongly reduced in frequency in *fer* mutants, which correlated with their inability to recover growth and the frequent occurrence of salt stress-induced cell bursts in this mutant. Altogether, this study identified FER-dependent Ca^{2+} signaling as a consequence of salinity-induced softening of the cell wall and provided evidence for a FER-dependent process of cell wall integrity restoration and root growth. In this context, the study also uncovered a novel extracellular toxicity of salinity and again highlighted that salt tolerance is manifested not only at the cellular level, but also on the level of tissue and organ integrity.

A co-ordinating role for Ca^{2+} signaling in plant nutrition

Any snapshot depicting the distribution of nutrients and essential ions within a plant would reveal spatially defined concentration differences that record the physiological situation of the plant, and in any case the accumulation of nutrients and ions would highly exceed their concentration in the soil. This situation defines nutrient and ion transport processes as central for plant biology. Consequently, the identification and characterization of such transporters has been a central subject of plant research for many years. However, during the past decade, the regulation of such transporter and channel proteins has taken center stage of research. One emerging outcome of these investigations is that Ca^{2+}-regulated phosphorylation (often in combination with phosphorylation/dephosphorylation processes that are triggered by hormones or initiated by receptors such as kinases) appears to be a common theme of transport regulation (Osakabe *et al.*, 2014; Kudla *et al.*, 2018). Ca^{2+}-dependent phosphorylation can be brought about by Ca^{2+}-dependent kinases (CDPKs designated as CPKs in Arabidopsis) or by a Ca^{2+} decoding network that is formed by CBLs (with 10 members in Arabidopsis), which interact with CIPKs (with 26 members in Arabidopsis) (Kolukisaoglu *et al.*, 2004; Weinl and Kudla, 2009; Wilkins *et al.*, 2016). As described above CBL–CIPK complexes were first implemented in salt tolerance. However, more and more research reveals that they appear to be involved in the regulation of nutrient uptake and ion transport of any molecule that has been studied intensively enough. Quite remarkably, out of the 26 CIPKs, one particular kinase, namely CIPK23, appears to function as a kind of central nutrient regulator, since it functions in a remarkably large number of nutrient-related processes (Fig. 2). This situation will be discussed in more detail below.

Plant potassium homeostasis

K^+ represents the most abundant inorganic ion in plants which accumulates at cytoplasmic concentrations of ~100 mM, while the abundance of K^+ in the soil is usually within the micromolar range (Wang and Wu, 2013). When challenged with salt stress, plants attempt to maintain a high K^+ to Na^+ ratio in the cytosol. They do this by regulating the expression and activity of K^+ and Na^+ transporters and H^+ pumps that generate the driving force for transport. Plants must maintain cytosolic K^+ at ~80 mM for optimal growth even under adverse conditions (Shabala and Pottosin, 2014). It has been long known that plasma membrane hyperpolarization represents a fast response to K^+ deprivation (Demidchik *et al.*, 2002). However, despite the reported Ca^{2+}-dependent regulation of K^+ channels >10 years ago, only recently has the occurrence of specific Ca^{2+} signals in defined regions of the roots in response to K^+ depletion been unambiguously detected (Xu *et al.*, 2006; Behera *et al.*, 2017).

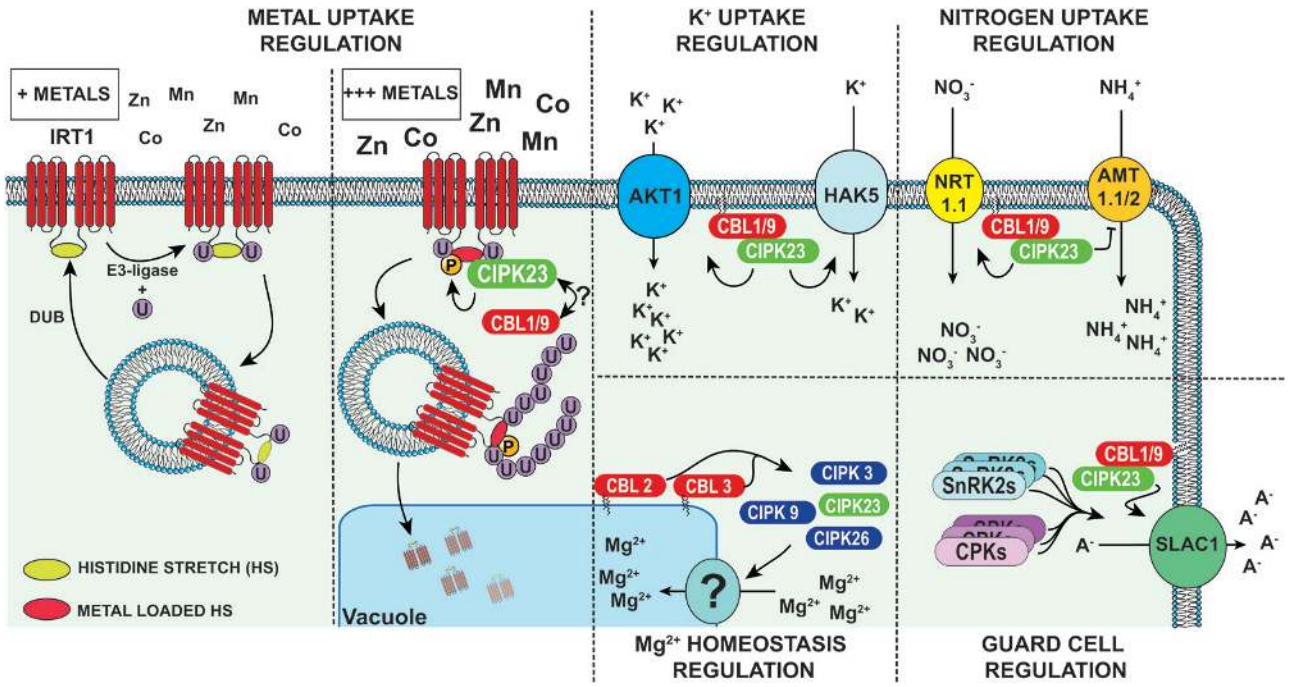

Fig. 2. Arabidopsis CIPK23 represents a general regulator of ion homeostasis and nutrient uptake. The transport protein IRT1 is the major uptake facilitator for iron in Arabidopsis. Under low iron conditions, the transporter's expression is up-regulated, and it accumulates at the plasma membrane. In addition to iron, other metals Zn, Mn, and Co can also enter the root through IRT1. Since overaccumulation of the latter ions is highly toxic, the accumulation and stability of IRT1 in the plasma membrane is tightly regulated on the post-transcriptional level. In the presence of low concentrations of non-iron metals (+ METALS), the cytoplasmic loop of IRT1 is monoubiquitinated (U) at two lysine residues. This modification initiates endocytosis of IRT1 to reduce the pool of active metal transporters at the plasma membrane. De-ubiquitination (DUB) of IRT1 allows recycling of those endosomes back to the membrane. Upon higher (even more toxic) non-iron metal concentrations (+++ METALS), these metals, especially Mn, directly bind to a histidine-rich stretch within the cytoplasmic loop. Subsequent recruitment of the kinase CIPK23 and CIPK23-mediated phosphorylation within the cytoplasmic loop of IRT1 initiates polyubiquitination. Dual post-transcriptional modification by phosphorylation and polyubiquitination triggers subsequent sorting of IRT1 towards late endosomes and degradation within the vacuole. Whether CBL1 and/or CBL9 have any influence on this CIPK23-regulated process remains to be explored. Both the Arabidopsis high affinity K^+ uptake transporter HAK5 and the low affinity channel AKT1 are activated by complexes consisting of the kinase CIPK23 and interacting CBL1/9 Ca^{2+} sensors, which are probably activated by Ca^{2+} signals triggered during K^+-deficient conditions. Besides the macronutrient K^+, the uptake of nitrogen is also regulated by CBL1/9–CIPK23 complexes. The nitrate transporter NRT1.1/NPF6.3/CHL1 is switched from low affinity mode to high affinity mode by phosphorylation mediated by the CBL1/9–CIPK23 complexes, enabling nitrate uptake at low external concentrations of this nutrient. The ammonium transporters AMT1.1 and AMT1.2 were shown to be negatively regulated by the same sensor–kinase complexes. Regulation of Mg^{2+} homeostasis under excess concentrations of these ions is impaired in mutants lacking the tonoplast-localized Ca^{2+} sensors CBL2 and CBL3, as well as in *cipk3/9/23/26* quadruple mutants. Note that the targets of the CBL2/3–CIPK complexes still await identification. Besides SnRK2 kinases and CPKs, CBL1/9–CIPK23 complexes represent regulatory elements that can activate the anion channel SLAC1, which represents an essential component of stomatal closure. Together, these findings strongly support the conclusion that CIPK23 represents a central integration and co-ordination hub of plant ion homeostasis.

Central for plant supply with K^+, especially under limiting conditions, are the K^+ channel AKT1 and the *Arabidopsis thaliana* High Affinity K^+ transporter 5 (HAK5). A breakthrough study in 2006 identified AKT1 as subject to regulation by CBL1/9–CIPK23 complexes (Xu *et al.*, 2006). Phosphorylation of AKT1 by this Ca^{2+} sensor–kinase complex was found to be absolutely essential for channel activity, and later studies identified the protein phosphatase 2C (PP2C)-type phosphatase AIP1 as counteracting this activation (Xu *et al.*, 2006; Cheong *et al.*, 2007; Lan *et al.*, 2011). This regulation of K^+ uptake is recapitulated in rice roots, where OsCBL1–CIPK23 complexes activate OsAKT1 (Li *et al.*, 2014). Low K^+ conditions also induce the expression of HAK5. *HAK5* encodes a high-affinity K^+ transporter, which facilitates K^+ uptake at low external concentrations that are thermodynamically unfavorable for channel-mediated influx (Nieves-Cordones *et al.*, 2016). Quite remarkably, it was recently found that HAK5 activity in Arabidopsis roots is also positively regulated by CIPK23-mediated phosphorylation in conjunction with CBL1 and CBL9 (Ragel *et al.*, 2015). This simultaneous regulation of both K^+ uptake components by the very same kinase obviously provides the opportunity for co-ordinating these processes. However, details of the underlying mechanisms are still awaiting further elucidation. Moreover, it will be most interesting to clarify how alternative activation of either AKT1 or HAK5, which may be favorable under specific conditions, could be brought about by one and the same kinase.

Uptake and homeostasis of nitrogen-containing molecules

Like K^+, nitrogen also represents an essential component of plant nutrition. Most plants can obtain this nutrient mainly in the form of inorganic compounds such as NO_3^- and NH_4^+. The inter-relationships of NO_3^- and NH_4^+ in plant nutrition are manifold and complex (Xuan *et al.*, 2017). Simply formulated,

whenever possible, a plant would prefer to obtain NO_3^-, instead of NH_4^+. In contrast to K^+, where concentration decreases have been found to induce Ca^{2+} signals in roots, for NO_3^- it has been observed that an increase in concentration triggers Ca^{2+} signals (Riveras *et al.*, 2015; Liu *et al.*, 2017). NO_3^- uptake and distribution within plants are facilitated by a complex family of NO_3^- transporters. One important transporter that mediates NO_3^- uptake in plants is NRT1.1/NPF6.3. Remarkably, this protein appears not only to function in NO_3^- transport, but simultaneously exerts a function as a receptor (transceptor) for this ion (Ho *et al.*, 2009; Noguero and Lacombe, 2016). Ca^{2+}-dependent phosphorylation has been reported to regulate the NO_3^- uptake activity negatively and to affect the sensing function of NPF6.3. Subsequent structure–function analysis of the CIPK23 target residue Thr101 revealed that NPF6.3 activates several distinct signaling responses and that the phosphorylated and non-phosphorylated forms at Thr101 have distinct signaling functions (Léran *et al.*, 2015). Moreover, the Ca^{2+} sensor CBL1 and the PP2C ABI2 were identified as additional components regulating NPF6.3, which is inhibited by the stress response hormone ABA. ABI2-mediated dephosphorylation appeared to enhance NPF6.3-dependent NO_3^- transport sensing and signaling (Léran *et al.*, 2015). Consequently, these results not only highlight the general function of CIPK23 in nutrient homeostasis, but they also suggest that co-regulation of ion transport by Ca^{2+} and ABA may functionally link stress-regulated control of growth with energy-expensive nitrate utilization.

Intriguingly, CIPK23 was found to bring about negative regulation of NH_4^+ uptake (again in combination with CBL1 and CBL9) by negatively regulating the ammonium transporters AMT1.1 and AMT1.2 (Straub *et al.*, 2017). In addition to providing the obvious opportunity to regulate NO_3^- and NH_4^+ influx antagonistically, this finding, however, raises the difficult question of how one pair of Ca^{2+} sensors (CBL1 and CBL9) can bring about the specificity in decoding Ca^{2+} signals that allows the execution of such divergent response reactions.

Quite remarkably, the other major family of Ca^{2+} signal decoding kinases—the CDPKs—has also been found to function crucially in regulating NO_3^- signaling and primary NO_3^- responses. Extensive mutant studies involving *cpk* triple mutants together with the elegant engineering of chemical switchable *cpk* mutant versions identified CPK10, CPK30, and CPK32 as central components of nitrate signaling (Liu *et al.*, 2017). These kinases phosphorylated conserved NIN-like protein (NLP) transcription factors to adjust specifically the expression of downstream genes for NO_3^- assimilation and metabolism. Together with the work on Ca^{2+}-regulated NO_3^- transport, these findings underscore the conclusion that Ca^{2+} signaling networks integrate many processes of plant physiology to adjust plant growth and development appropriately to nutrient availability, thereby ensuring the developmental plasticity of plants.

Magnesium ion homeostasis

Mg^{2+} is also an essential ion for plants; it accumulates in relatively high cytoplasmic concentrations and regulates a multitude of cellular processes. However, excess Mg^{2+}, under specific conditions, such as, for example, in serpentine soils, can also be toxic to plants, underscoring the importance of a well-balanced Mg^{2+} homeostasis for appropriate plant development. Consequently, as for other ions, an elaborate network of Mg^{2+} transporters mediates uptake, distribution, and subcellular sequestration of Mg^{2+} in plants (Schmitz *et al.*, 2013; Tang and Luan, 2017). In contrast to the situation with other ions, our knowledge about a potential role for Ca^{2+} signaling or more specifically Ca^{2+}-dependent phosphorylation in regulating Mg^{2+} uptake is still very limited. However, recently, important insights into the role of Ca^{2+} in regulating Mg^{2+} sequestration have been gained. Here again, a crucial function of the CBL–CIPK signaling network in vacuole-mediated detoxification of high external Mg^{2+} was uncovered (Tang *et al.*, 2015). Analysis of *cbl2/3* double mutants revealed that these Ca^{2+} sensors are regulating vacuole-mediated Mg^{2+} ion homeostasis in cells (Tang *et al.*, 2015). The *cbl2/3* double mutant was hypersensitive to high concentrations of external Mg^{2+}, and ionic profile analyses also showed a reduced amount of Mg^{2+} accumulation in *cbl2/3* double mutant plants. Tang and colleagues found that the kinases CIPK3/9/23/26 physically interacted with CBL2 and CBL3 at the tonoplast, that *cipk3/9/23/26* quadruple mutants displayed severe hypersensitivity towards excess Mg^{2+}, and that these mutants exhibited a similar ionic profile to *cbl2/3* mutants (Tang *et al.*, 2015). These results strongly suggest that CIPK3/9/23/26 work together with CBL2/3 at the tonoplast to alleviate the toxic effects of external high Mg^{2+} concentrations via vacuolar sequestration. An independent study by K. Shinozaki and co-workers not only simultaneously identified the very same CIPKs as crucial components of Mg^{2+} homeostasis but also provided important hints that interconnect the regulation of Mg^{2+} distribution with the implementation of stress tolerance in plants (Mogami *et al.*, 2015). These authors did not only find that ABA synthesis-deficient mutants or ABA signaling mutants of SnRK2s displayed sensitivity to high external Mg^{2+} concentrations. Most importantly, this study also provided biochemical and genetic evidence for a direct interaction between SnRK2-type and CIPK-type kinases. Consequently, these insights again uncover molecular and genetic interconnections between abiotic stress tolerance and ion accumulation, and in this way (considering the results from research in the nitrate field) point to the fundamental importance of this signaling integration for plant biology.

Ca^{2+} signaling in metal uptake regulation

Many less abundant ions such as, for example, metals fulfill essential functions in plants including as cofactors. Among these, iron is of outstanding importance to plants due to its ability to change redox states and its central role in photosynthesis and respiration (Brumbarova *et al.*, 2015). Although abundant in nature, iron is often poorly available for plants, and diverse mechanisms for optimizing plant supply of iron have evolved. Also in this field, increasing evidence indicates that not only transcriptional regulation but also post-transcriptional regulation of iron transport processes is of crucial importance. In this regard, most recent findings have shed new light on the regulation of IRT1. IRT1 is a broad-spectrum transporter driving the

uptake of iron into plants, but also of non-iron essential heavy metals such as manganese, zinc, and cobalt (Vert *et al.*, 2002). A recent study by Dubeaux and colleagues has uncovered that IRT1 not only acts as an iron transporter, but also functions as a receptor (transceptor) in a rather sophisticated manner. For its latter function, IRT1 appears to sense directly the excess of non-iron metals in the cytoplasm, and this sensing appears to regulate its own degradation. Direct metal binding to a histidine-rich region in IRT1 triggers interaction with and phosphorylation by the Ca^{2+}-activated kinase CIPK23 (Dubeaux *et al.*, 2018) (Fig. 2). Although this work did not address the potential role of CBL proteins in this process, it is tempting to conclude that this mechanism indicates Ca^{2+} control of metal homeostasis in plants. Phosphorylation of IRT1 facilitates the recruitment of an E3 ligase, and phosphorylation and lysine polyubiquitination jointly drive endocytosis and vacuolar degradation of IRT1. In this way, IRT1 directly senses metal concentration and integrates this information for optimized iron uptake. This study not only provides new insights into the potential complexity of how Ca^{2+} can impact on iron homeostasis regulation, but it also raises the intriguing question of whether such an intracellular sensing mechanism of plants will be specific for IRT1 or instead may be considered as an aspect which may also be relevant for other plant transceptor proteins.

Last but not least: Ca^{2+}-controlled anion fluxes for ensuring plant nutrition

Although gaseous, the nutrient function of CO_2 for plants is as important as the above discussed ions. Opposite to the previous cases, CO_2 uptake via the roots can be neglected and here specific pores in leaves that are designated as stomata, which are formed and regulated by the aperture of guard cells, represent the dominant uptake pathway (Hetherington, 2001; Jezek and Blatt, 2017). Nevertheless, as in roots, Ca^{2+} signaling (and its interactions with ABA signaling) also represents a reoccurring theme in this case. Centrally functioning in regulating guard cell aperture is the anion-conducting ion channel SLAC1 (Negi *et al.*, 2008; Vahisalu *et al.*, 2008; Geiger *et al.*, 2009). This channel has become a model case for studying and illustrating the complexity of processes that converge on the regulation of ion conductance (Jeworutzki *et al.*, 2010; Kim *et al.*, 2010; Krol *et al.*, 2010; Hedrich and Geiger, 2017). In general, multiple kinases from at least three distinct families and PP2C phosphatases convey the fine-tuning of this channel (Fig. 2). Again, CDPKs, CBL–CIPKs, and SnRK2s appear to form an alliance counteracting the function of ABA-inhibited phosphatases. Details of this regulation have been worked out in many very informative original publications (Geiger *et al.*, 2009, 2010; Lee *et al.*, 2009; Maierhofer *et al.*, 2014; Yu *et al.*, 2012; Brandt *et al.*, 2012, 2015), and have been recently reviewed (Munemasa *et al.*, 2015; Edel and Kudla, 2015). Therefore, in the context of this article, we are not elaborating on these details again. Instead we draw the reader's attention to the most complex regulation of SLAC1 with the intention to underscore the broad biological significance of Ca^{2+} signaling and Ca^{2+}-dependent phosphorylation for plant biology. Obviously, this regulatory principle has not remained restricted to the regulation of ion transport and homeostasis, but also governs other distinct biological processes. By elucidating commonalities which are shared on the level of the biological challenges to the plant and simultaneously deciphering similarities and differences in their mechanistic solution, we may advance in our quest for a comprehensive appreciation of the grace and sophistication of plants.

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