Regulation Of Root Nutrient Transporters By CIPK23: "One Kinase 1 To Rule Them All

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Running head : Regulation of root nutrient transporters by CIPK23

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Subject areas: Environmental and stress responses; Membrane and transport

Two color figures

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Abstract

Protein kinases constitute essential regulatory components in the majority of cellular processes in eukaryotic cells. The CBL-INTERACTING PROTEIN KINASE (CIPK) family of plant protein kinases function in calcium (Ca^{2+})-related signaling pathways, and are therefore involved in the response to a wide variety of signals in plants. By covalently linking phosphate groups to their target proteins, CIPKs regulate the activity of downstream targets, their localization, their stability and their ability to interact with other proteins. In Arabidopsis, the CIPK23 kinase has emerged as a major hub driving root responses to diverse environmental stresses including drought, salinity and nutrient imbalances such as potassium, nitrate and iron deficiencies as well as ammonium, magnesium and non-iron metals toxicities. This review will chiefly report on the prominent roles of CIPK23 in the regulation of plant nutrient transporters and on the underlying molecular mechanisms. We will also discuss the different scenarios explaining how a single promiscuous kinase such as CIPK23 may convey specific responses to a myriad of signals.

Keywords

Plant, Transporter, Kinase, Nutrition, CIPK23, Calcium.

Introduction

Higher plants are rooted to a specific location and must constantly monitor their natural environment to undertake adaptive changes in response to varying environmental inputs. Roots forage the soil in search for macronutrients and micronutrients that are essential to plant growth and development. However, roots frequently grow in soils with contrasting nutrient level/availability and have therefore evolved sophisticated responses to maintain cellular nutrient homeostasis. Calcium (Ca^{2+}) plays a crucial role in mediating such responses as well as in the regulation of many physiological and developmental processes in plants (Harper 2001; Knight and Knight 2000; Knight et al. 1996; Knight et al. 1991; Kudla et al. 2018). Generally, Ca^{2+}-dependent responses involve an increase in cytosolic Ca^{2+} and or oscillation in cytosolic Ca^{2+} concentrations called Ca^{2+} signatures. These Ca^{2+} signatures vary in amplitude, frequency, or duration and serve as a signal that is sensed, decoded and transmitted to downstream responses by Ca^{2+}-binding proteins acting as Ca^{2+} sensors (Dodd et al. 2010; Gong et al. 2002; Gong et al. 2004; McAinsh and Pittman 2009). In plants, one of the major calcium sensors are CALCINEURIN B-LIKE (CBL) proteins, which regulate the activity of their cognate CBL-INTERACTING PROTEIN KINASE (CIPK) proteins. Similar to most Ca^{2+} sensors, CBL proteins possess EF-hand structural motifs, consisting of two alpha helices linked by a short-loop region that usually binds Ca^{2+} (Batistic and Kudla 2004; Gifford et al. 2007; Gong et al. 2004; Kolukisaoglu et al. 2004). In Arabidopsis, 10 CBLs proteins specifically interact with 26 distinct CIPKs, forming a complex network involved in signaling pathways responding to salinity, osmotic stress, dehydration, cold, abscisic acid, and other abiotic and biotic stresses (Batistic and Kudla 2009; Halfter et al. 2000; Hashimoto et al. 2012; Jiang et al. 2019; Kim et al. 2007; Kudla et al. 2018; Kudla et al. 1999; Mao et al. 2016; Pandey et al. 2007; Steinhorst and Kudla 2013; Yang et al. 2019; Zhu et al. 1998; Zhu 2016). The CIPK protein family is a central regulator of several plant signaling pathways conveying endogenous information or in response to different biotic and abiotic stresses, generally by phosphorylating their target proteins such as transcription factors, phosphatases, or transporters/channels.

Among CIPKs proteins, CIPK23 stands out as a major signaling hub controlling the acquisition and homeostasis of different ions such as potassium (K^+), nitrate (NO_3^-), ammonium (NH_4^+), magnesium (Mg^{2+}), iron (Fe^{2+}), and non-iron metals including zinc (Zn^{2+}) or manganese (Mn^{2+}) (Dubeaux et al. 2018; Ho et al. 2009; Li et al. 2006; Maierhofer et al. 2014; Ragel et al. 2015; Straub et al. 2017; Tang et al. 2020; Tang et al. 2015; Tian et al. 2016; Xu et al. 2006). This review gathers the diverse physiological roles of CIPK23 in the response to external stress conditions and associated molecular mechanisms. Some prospects

about how a single kinase may convey specific outputs to tightly regulate plant iron homeostasis will also be presented.

CIPK23 as a central hub: from signals to regulatory mechanisms.

Over the past 15 years, several reports have brought to light the indispensable role of CIPK23 in nutrient acquisition by regulating transport proteins. CIPK23 was originally identified as a regulator of the inward rectifying K^+ channel ARABIDOPSIS K^+ TRANSPORTER 1 (AKT1) in Arabidopsis roots (Li et al. 2006; Xu et al. 2006) (Table 1, Figure 1). CIPK23 indeed interacts with CBL1 or CBL9 to phosphorylate AKT1 upon low K^+ conditions and activate AKT1-dependent K^+ uptake in root epidermal cells (Li et al. 2006; Xu et al. 2006). Activation of AKT1 has been shown to depend on Ca^{2+} signatures sensed by CBL1 upon K^+ deprivation (Behera et al. 2017). Interestingly, the role of the CIPK23-CBL1/9 regulatory module in controlling K^+ acquisition is not restricted to the sole activation of AKT1. CIPK23-CBL1 also phosphorylates the HIGH AFFINITY K+ TRANSPORTER 5 (HAK5) transport protein, increasing its K^+ uptake activity in roots (Ragel et al. 2015) (Table 1, Figure 1). K^+ UPTAKE TRANSPORTER 4 (KUP4), required for tip growth in *Arabidopsis* root hairs (Rigas et al. 2001), has also been identified as a substrate of CIPK23 in low-potassium-treated plants (Wang et al. 2020) (Table 1, Figure 1)., although the precise role of CIPK23 in KUP4 regulation has not been uncovered yet. CIPK23 kinase is not only involved in the regulation of K^+ acquisition but also in K^+ retrieving from the vacuole in response to K^+ deprivation (Tang et al. 2020). The Arabidopsis Two Pore K+ (TPK) channels are indeed activated when plants are facing low external concentrations of K^+, in a Ca^{2+}-dependent manner, by the four CIPK23/3/9/26 and the vacuolar-localized CBL2/3. TPK activation leads to K^+ efflux from the vacuole to the cytoplasm, thus contributing to K^+ homeostasis and responses to K^+ limitation (Tang et al. 2020) (Table 1, Figure 1).

Besides the central role of CIPK23 in regulating cellular K^+ homeostasis, the same kinase has been coopted to drive responses to nitrate deficiency and especially though the NITRATE TRANSPORTER 1.1/CHLORATE RESISTANT 1 (NRT1.1/CHL1 also known as NPF6.3, NRT1/PTR FAMILY 6.3). NRT1.1/CHL1 has been reported to act as a 'transceptor' (Bouguyon et al. 2015; Gojon et al. 2011; Ho et al. 2009; Liu and Tsay 2003; O'Brien et al. 2016), directly sensing nitrate concentrations and switching from high-affinity to low-affinity nitrate uptake upon phosphorylation by CIPK23 (Leran et al. 2015; Liu and Tsay 2003). At low external nitrate concentrations, nitrate-binding to the high-affinity nitrate binding

site in NRT1.1/CHL1 triggers specific Ca^{2+} waves (Riveras et al. 2015) that are sensed by CBL9 and CBL1, promoting the activation of CIPK23 (Ho et al. 2009; Leran et al. 2015) (Table 1, Figure 1). Activated CIPK23 subsequently phosphorylates NRT1.1/CHL1 at threonine residue 101 (Thr101), leading to the dissociation of the dimer and stabilizing the NRT1.1/CHL1 monomer state. The latter shows a higher flexibility and nitrate affinity than the dimer (Sun et al. 2014). In addition to macronutrients, the CIPK23-CBL1/CBL9 regulatory module also drives responses to iron deficiency by regulating directly or indirectly the ferric chelate reductase activity in a calcium-dependent manner (Tian et al. 2016) (Table 1, Figure 1).

CIPK23 not only regulates responses to nutrient deprivation but also participates in transporter inactivation when plants experience potentially toxic ion levels. This is exemplified by the control of NH_4^+, Mg^{2+} and metal transporters in Arabidopsis. When plants face toxic external NH_4^+concentrations, CBL1 protein activates the CIPK23 kinase which in turn directly interacts with and phosphorylates the AMMONIUM TRANSPORTER 1;1 and 2;2 (AMT1;1 and AMT1;2), promoting their inactivation (Straub et al. 2017) (Table 1, Figure 1). The involvement of Ca^{2+} in AMT1;1 and AMT1;2 regulation upon high ammonium is however still unclear.

To protect plant cells from toxic levels of Mg^{2+}, CIPK23 kinase regulates Mg^{2+} transport to the vacuole for detoxification. The Ca^{2+} sensors CBL2/3, which are located to the tonoplast, interact with and recruit CIPK23/3/9/26 to regulate a yet to be characterized Mg^{2+} vacuolar transporter and alleviate the toxic effects of high external Mg^{2+} concentrations (Tang et al. 2015) (Table 1, Figure 1). Whether specific Ca^{2+} signatures decoded by CBL2/3 participate in the regulation of vacuolar Mg^{2+} transport will need further characterization. The MAGNESIUM PROTON (H^+) EXCHANGER MHX and the MAGNESIUM TRANSPORTERS MGT2/3 are unlikely to be the Mg^{2+} vacuolar transporters targeted by CIPK23/3/9/26, as the corresponding *mhx* and *mgt2mgt3* knock-out mutants show wild-type response to high Mg^{2+} conditions (Tang et al. 2015). Besides, under resting conditions, the plant vacuole exhibits a hyperpolarized membrane voltage due to two proton pumps, the Vacuolar H^+-ATPase (V-ATPase) and Vacuolar H^+-PPAse (V-PPase), that are responsible for generating a proton gradient and membrane potential to energize secondary transport processes across the vacuolar membrane (Krebs et al. 2010). Although CBL2 and CBL3 have been shown to modulate the tonoplast V-ATPase activity (Tang et al. 2012), and as such may impact the tonoplast membrane potential and consequently ion transport, the role of the CBL2/3-CIPK23/3/9/26 module on Mg^{2+} and K^+ vacuolar transport appears to be V-ATPase-independent (Tang et

al. 2020; Tang et al. 2015). The possible involvement of V-PPase in vacuolar Mg^{2+} detoxification remains to be investigated. The Slowly activated Vacuole channel (SV) TPC1, recognized initially as a nonselective Ca^{2+}-activated vacuolar channel (Hedrich et al. 1986; Hedrich and Schroeder 1989), is the major conductance system of the tonoplast and is permeable to Ca^{2+}, Mg^{2+}, K^+, sodium (Na^+) and NH_4^+ (Allen and Sanders 1996; Carpaneto et al. 2001; Coyaud et al. 1987; Pei et al. 1999; Pottosin et al. 1997; Ranf et al. 2008; Schulz-Lessdorf and Hedrich 1995). The activity of TPC1 is not only modulated by voltage and cytosolic Ca^{2+} levels, but also by elevated cytosolic Mg^{2+} concentrations through promoting voltage-activation gating in a synergistic manner with Ca^{2+} (Carpaneto et al. 2001; Hedrich and Marten 2011; Pei et al. 1999; Pottosin et al. 1997). Considering that plant tolerance to high external Mg^{2+} concentrations through CBL2/3 appears to be dependent on external Ca^{2+} concentrations (Tang et al. 2015), TPC1 may represent a target of CIPK23/3/9/26-CBL2/3 controlling Mg^{2+} sequestration into the vacuole. This is further substantiated by the fact that TPC1 acts in concert with TPK1/3 to confer electrical excitability to plant vacuole (Jaślan et al. 2019), TPK channels being themselves regulated by CIPK23/3/9/26-CBL2/3. Further examination will be required to identify the transport protein(s) that are regulated by Ca^{2+}, CBLs and CIPKs to drive the detoxification of Mg^{2+} ions.

The CIPK23 kinase is also involved in the regulation of the IRON-REGULATED TRANSPORTER 1 (IRT1) root transporter. IRT1 is a broad spectrum high affinity metal transporter uptaking not only Fe^{2+}, but also closely-related divalent metals such as Zn^{2+}, Mn^{2+}, Co^{2+} and Cd^{2+} (Barberon et al. 2011; Vert et al 2002). By boosting *IRT1* transcription, iron starvation promotes the overaccumulation of highly reactive Zn^{2+}, Mn^{2+}, cobalt (Co^{2+}) and cadmium (Cd^{2+}) ions that are harmful to plants and enter the food chain. IRT1 was recently shown to act as a transceptor, directly sensing non-iron metal flux through the plasma membrane using a histidine-rich motif present in its cytosolic loop (Dubeaux et al. 2018). Non-iron metal binding to such residues is sufficient to trigger the recruitment of CIPK23, which interacts with and phosphorylates IRT1 at serine and threonine residues located in close proximity to histidines. Phosphorylated IRT1 subsequently recruits the E3 ubiquitin ligase IDF1 that decorates IRT1 with K63-linked ubiquitin chains to tag it for degradation in the vacuole (Dubeaux et al. 2018) (Table 1, Figure 1). This regulation, initiated by IRT1 and culminating in IRT1 degradation, limits the accumulation of potentially noxious heavy metals in plant cells. Importantly, such negative regulation of IRT1 takes place at local level, only shooting IRT1 in non-iron metal rich soil patches, but maintaining IRT1-dependent Fe^{2+}

transport in the rest of the root system (Dubeaux et al. 2018). Whether Ca^{2+} and CBLs are required to activate CIPK23 and initiate IRT1 phosphorylation has not been established yet.

As presented above, CIPK23 regulates a wide variety of nutrient transport proteins. Besides nutrient responses, CIPK23 also participates in the osmotic stress response and in the regulation of ABA responsiveness of guard cells during their opening and closure, leading to reduced water loss under drought conditions (Cheong et al. 2007). CIPK23, in complex with CBL1 or CBL9, indeed phosphorylates and triggers the opening of the SLOW ANION CHANNEL 1 (SLAC1) and HOMOLOG 3 (SLAH3) guard cell anion channels (Maierhofer et al. 2014) (Table 1, Figure 1). Interestingly, CIPK23 also regulates K^+ uptake through AtHAK5 transporter under osmotic stress conditions; meanwhile CIPK9 kinase, that has recently been described as AtHAK5-mediated high affinity K^+ uptake regulator protein as well, is not involved in AtHAK5 regulation in the presence of osmotic stress (Lara et al. 2020).

CIPK23 in other plant species

Functionally, the role of CIPK23 is conserved in other plant species such as rice, grapevine, poplar and wheat. In the case of rice, the homologs OsCIPK23 and OsCBL1 have been demonstrated to interact with and to activate the AKT1 homolog OsAKT1 (Li et al. 2014). Moreover, OsCIPK23 is a positive regulator of drought responses (Hu et al. 2015; Yang et al. 2008), similar to its Arabidopsis counterpart (Maierhofer et al. 2014). Studies in grapevine (*Vitis Vinifera*) showed that AtCIPK23 homolog, VvCIPK04, also activates the AKT1 homologs VvKT1.1and VvKT1.2 during berry filling (Cuellar et al. 2010). The CIPK23 homolog from poplar (P*opulus euphratica*), PeCIPK24, is involved in K^+ nutrition by regulating the PeKC1 and PeKC2 K^+ channels (Zhang et al. 2010). The Venus flytrap *Dionaea muscipula* also harbors homologs of HAK5 and AKT1, with both channels being activated by a CIPK23 homolog (Scherzer et al. 2015). Other examples involving CIPK23 in nutrient responses or ion transport exist in the literature but in most cases, the CIPK23 targets are not known. For instance, TaCIPK23 interacts with TaCBL1 and positively regulates drought stress in wheat (Cui et al. 2018).

Overall, CIPK23 kinases control numerous nutrient transporters in various plant species and thus stands out as a major regulator of plant mineral nutrition. The fact that CIPK23 was also demonstrated to play a crucial role in crops highlights its potential in the development of varieties tolerant to multiple stresses to boost the quantity and quality of the crop production.

CIPK23 functional specificity

A wealth of information was gathered over the past two decades on the roles of CIPK23 is the regulation of plant transport proteins and plant responses to stress conditions. In most cases, a detailed picture of CIPK23 activation by CBLs and Ca^{2+} is available. However, the rationale for using a single kinase to regulate so diverse responses and how specificity is established remains elusive. This is absolutely essential to ensure that plants respond to a specific stimulus without firing the whole CIPK23-dependent regulatory network. This would indeed be rather detrimental and energy costly. Two levels of specificity are to be considered. The first relies in the exclusive use of CIPK23 and certain CBLs among families of 26 and 10 members, respectively, in Arabidopsis. The second level of specificity concerns the ability to restrict the action of the promiscuous CIPK23 kinase to a given target within a cell under a defined condition.

Specificity of the CIPK23 regulatory module

How do cells employ mostly CIPK23 and no other CIPK to control nutrient homeostasis in root epidermal cells? This is first achieved by the intrinsic ability of certain CIPKs to interact with a subset of CBLs, to be activated/deactivated, and to interact with their targets.

CBL-CIPK interaction. CIPK proteins are divided into two domains: a conserved N-terminal catalytic kinase domain and a C-terminal regulatory domain. The kinase domain contains the ATP binding site and a typical activation loop. The regulatory domain harbors the FISL or NAF domain involved in CBL binding (Albrecht et al. 2001; Guo et al. 2001), and a conserved protein phosphatase interaction (PPI) motif responsible for the interaction with PP2C-type phosphatases and presumably other proteins (Ohta et al. 2003). Several hydrophobic interactions are responsible for the recognition and the maintenance of the complex between CBLs and the helical FISL motif (Sanchez-Barrena et al. 2013). The sequence variations in the loop connecting the two helical segments of the FISL motif in CIPKs was proposed to confer specificity of CIPK-CBL interaction (Sanchez-Barrena et al. 2013). Several interaction studies notably showed that CIPK23 protein interacts strongly with CBLs 1,2,3,5, and 9 and weakly with CBL8 protein (Kolukisaoglu et al. 2004; Li et al. 2006; Xu et al. 2006).

The CBL-CIPK pairs impact on the biological process to be regulated. The CIPK23 kinase protein has been involved in the regulation of AKT1, HAK5 and AMT1, mostly through CBL1 (Li et al. 2006; Ragel et al.

214 2015; Straub et al. 2017; Xu et al. 2006). On the other hand, the regulation of NRT1.1/CHL1 by nitrate
215 availability involves mainly CBL9 (Ho et al. 2009). Whether this is driven by differences in Ca^{2+} signatures
216 will be discussed below. While some CBLs are required to activate CIPK23 and to specify its target, other
217 CBLs seem to antagonize CIPK23 action. CBL10 competes with CIPK23 for direct binding to AKT1,
218 therefore negating the CBL1/9-CIPK23 activation of AKT1 (Ren et al. 2013). Future work will be needed
219 to elucidate the intricate mechanisms allowing of some CBLs to antagonize CBL-CIPK modules.

220 *Reciprocal regulation of CIPK23, cognate CBLs and targets*. The different CIPK23-CBL pairs mentioned
221 above, together with the mechanisms involved in CIPK23 kinase activation and inhibition, will ultimately
222 influence target modification. Structural and biochemical data indicate that CIPK23 is intrinsically inactive
223 and requires an external stimulation comprising CBL binding and phosphorylation by upstream kinases for
224 full activation (Chaves-Sanjuan et al. 2014). Whereas AKT1 activation is possible in the presence of the
225 CIPK23 kinase domain alone (Lee et al. 2007), HAK5 needs full length CIPK23 and the presence of a CBL
226 (Ragel et al. 2015). The activation loop phosphorylation by an unknown kinase might be an alternate
227 mechanism of CIPK activation and possibly independent of CBL interaction (Gong et al. 2002; Guo et al.
228 2001). Recently, MAPK6 kinase was identified as interacting with CIPK23 under K^+ deprivation (Wang et
229 al. 2020), and may be involved in the specific activation of CIPK23. In addition to activation by upstream
230 kinases, PP2C phosphatases seem to play important roles in modulating the CBL–CIPK module and several
231 inactivation mechanisms have been proposed. A first mechanism suggests that CIPK inactivation by PP2C
232 phosphatases that bind to the CIPK PPI domain negatively regulates substrate phosphorylation. This is
233 exemplified by CIPK24 inhibition through ABI2 phosphatase in the salt stress signaling pathway (Ohta et
234 al. 2003). On the other hand, a PP2C phosphatase known as AIP1, dephosphorylates and reduces AKT1
235 inward-rectifying activity in electrophysiological assays in *Xenopus* oocytes (Lee et al. 2007). Since AIP1
236 also interacts with CIPK23, it has been suggested that AIP1 could use CIPK23 as a scaffolding protein and
237 directly interact with AKT1, preventing channel activation by the CBL–CIPK23 module (Lee et al. 2007).
238 A third mechanism involves a PP2C phosphatase acting through CIPK kinase domain. PP2CA inhibits
239 AKT1 activation induced by CIPK6 by directly interacting with its kinase domain, independently of its
240 phosphatase activity (Lan et al. 2011). Furthermore, CBL1 and CBL2 proteins also contribute to the CIPK6-
241 AKT1-PP2CA regulatory network by directly interacting with and acting as negative regulators of PP2CA
242 (Lan et al. 2011). Finally, AP2C1 phosphatase was shown to regulate CIPK9 by dephosphorylating the
243 autophosphorylated form of CIPK9 (Singh et al. 2018). Surprisingly, CIPKs kinases not only phosphorylate

their target but also CBL proteins, giving further complexity to this regulatory module, as described for CBL1 phosphorylation by CIPK23 during AKT1 activation (Hashimoto et al. 2012). Phosphorylation of CBLs by CIPKs occurs at a serine residue within a conserved C-terminal motif called PFPF (Du et al. 2011), and affects the stability of the CBL-CIPK complex (Du et al. 2011; Hashimoto et al. 2012).

CIPK23 target specificity. Parallel to the formation of CBL-CIPK pairs, the response specificity also relies in the ability of CIPKs to interact with dedicated targets. This is first highlighted by the phenotypes of *cipk* mutants. For example, the *cipk9cipk23* double mutant showed a much stronger phenotype than the *cipk3cipk26* double mutant under low K^+ conditions during seed germination, while the same *cipk9cipk23* mutant shows only a mild phenotype under Mg^{2+} excess (Tang et al. 2015). This suggests that these CIPKs target different proteins and thus do not contribute to the two nutrient stresses in the same manner, although they partner with the same CBLs (Tang et al. 2020).

At the molecular level, CIPK23 interacts with and phosphorylates several transporters at the cell surface. The mechanistic basis of such promiscuous behavior for CIPK23 is unclear, but CIPK23 interaction with its target appears not to be mediated by a specific interaction domain. The recently solved X-ray structure of the ankyrin domain of the AKT1 K^+ channel together with biochemical and structural analyses have shown that the physical interaction between CIPK23 and AKT1 ankyrin domain regulates kinase docking and channel activation. The properties of the CIPK23 binding site to the ankyrin domain thus provide specificity for this kinase versus other CIPKs (Sánchez-Barrena et al. 2020). On the other hand, HAK5 K^+ transporter activation by CIPK23 is carried out by phosphorylation of its N-terminal domain that is devoid of ankyrin domain (Ragel et al. 2015). Furthermore, in the case of IRT1, direct non-iron metal binding to a histidine-rich stretch in an unstructured cytosolic loop of the transporter is sufficient to promote the association with CIPK23 (Dubeaux et al. 2018). Whether this involves the folding of the loop upon non-iron metal binding or rather the direct interaction of CIPK23 with these metals when coordinated by histidines remains elusive and will need further attention. Besides the recruitment of CIPK23 *per se*, the use of particular phosphorylation sites by CIPKs can also discriminate between targets. Specific phosphorylation sites have been identified, such as Thr101 residue of NRT1.1/CHL1, Thr460 residue of AMT1;1 and Thr472 residue of AMT1;2 (Ho et al. 2009; Menz et al. 2016; Straub et al. 2017). Surprisingly, sequence conservation of the phosphorylation target site does not guarantee activation by CIPK23, as observed for the AMT1;3 ammonium transporter (Straub et al. 2017), indicating that other factors may also provide the specificity to CIPK23 to discriminate among targets.

The second major determinant for the specific requirement of CIPK23 in the regulation of nutrient transporters relies on its tissue/organ/cell-type expression profile and subcellular localization.

Subcellular distribution of CIPK23 and cognate CBLs. At the cellular level, CBL proteins show diverse localization spanning the cytosol, the nucleus or membranes, allowing to recruit and activate CIPKs at different locations (Batistic et al. 2010; Cheong et al. 2007; Waadt et al. 2008; Xu et al. 2006). Their association to membranes is mediated by the presence of a N-terminal conserved MGCXXS/T motif, responsible for lipid modification by myristoylation and S-acylation. CBL1 and CBL9 are targeted to plasma membrane, and participate to the CIPK23-mediated responses of cell surface transporters such as AKT1, HAK5, NRT1.1/CHL1 among others (Cheong et al. 2007; Ho et al. 2009; Ragel et al. 2015; Xu et al. 2006). CBL2 and CBL3 are specifically localized to the tonoplast allowing CIPK23-dependent Mg^{2+} detoxification from the cytoplasm to the vacuole (Tang et al. 2015), and also in K^+ movement to cytoplasm through TPK vacuolar channel under K^+ deprivation conditions (Tang et al. 2020).

Expression profile of CIPK23. The spatial and temporal expression of *CIPK23* in plants also participates to the specific use of CIPK23 in the regulation of nutrient transporters. This also holds true for the CIPK23-interacting CBLs for which the levels and cell type specificity will also contribute to mounting the proper response. The *CIPK23* promoter is active the root epidermis and the cortex where nutrient uptake is highly active and needs to be finely tuned (Xu et al. 2006). The expression of *CIPK23*, *CBL1* and *CBL9* has been reported to be overlapping in roots, in stomatal guard cells and in vascular tissues of leaves (Cheong et al. 2003; Cheong et al. 2007; Pandey et al. 2004) (Figure 2A). CBL2 and CBL3, also described as components of a regulatory complex with CIPK23, are expressed in roots, leaves and flowers (Kudla et al. 1999; Tang et al. 2012). Transcriptional regulation of *CIPK23* by nutritional cues may also increase the specificity towards the use of CIPK23. For example, an increase in *CIPK23* expression is observed 30 minutes after NH_4^+ shock in nitrogen-starved plants, and transcripts levels are still increasing after 2 hours of supply (Straub et al. 2017). *CIPK23* expression is also up-regulated under K^+ deprivation (Cheong et al. 2007; Lara et al. 2020) to fuel CIPK23-dependent low K^+ responses. Moreover, under high NH_4^+/K^+ ratios, *CIPK23* is strongly upregulated in leaves and roots, which efficiently reduces the leaf chlorosis by regulating the contents of NH_4^+ and K^+ in plant shoots (Shi et al. 2020). However, this clearly points to the need for additional mechanisms to avoid crosstalk between responses to K^+ and NH_4^+, and more globally

between all CIPK23-mediated responses (see section below). Part of the answer may also come for different mode of transcriptional regulation for *CIPK23*. As mentioned above, nutrient stress responses in the root epidermis are often dually regulated by both local and long-distance signals (Remans et al. 2006; Ruffel et al. 2011; Vert et al. 2003). Depending on the nutritional cue, CIPK23 may be under systemic or local nutrient control, providing further spatial specificity to the response. For instance, we already depicted, that CIPK23-dependent regulation of *IRT1* is a local response restricted to soil patches showing high non-iron metal concentrations (Dubeaux et al. 2018). Whether *CIPK23* is transcriptionally induced only in these same patches is not known but that would clearly allow for a targeted response to non-iron metals without interfering with CIPK23-dependent responses in other parts of the root system.

The third mechanism to confer specificity target to the CIPK-CBL regulatory module is through the temporal signature of the signal-decoder couple. Unfavorable environmental conditions, such as drought, salt, extreme temperatures, nutrient deficiencies and pathogen infections are indeed known to modulate cytosolic Ca^{2+} concentrations in plant cells.

Ca^{2+} signatures. Several evidence support the existence of a highly specialized spatio-temporal pattern of the Ca^{2+} rise that confers the specificity to the corresponding response (Dodd et al. 2010; McAinsh and Pittman 2009). For example, it has been established that salt stress triggers a transient increase in cytosolic Ca^{2+} concentration with a duration of approximately 2 minutes (Knight et al. 1997; Tracy et al. 2008), while a distinct Ca^{2+} signature profile has been described in response to glutamate application (Behera et al. 2015). In the context of CIPK23-dependent responses, two successive and distinct Ca^{2+} signatures are generated in response to K^+ deprivation (Behera et al. 2017). The CBL1-dependent activation of CIPK23 and the subsequent increase in AKT1 K^+ channel activity upon K^+ deprivation have been shown to result from the generation of a primary Ca^{2+} signal. On the other hand, a secondary signal occurs after 18 hours of K^+ shortage and under the form of a sustained Ca^{2+} elevation that lasts for several hours (Behera et al. 2017). Considering that *HAK5* expression is induced after 24 hours of K^+ deprivation, HAK5 activity may be under the influence of the secondary Ca^{2+} signal. Therefore, the existence of two distinct Ca^{2+} signals in roots separated in time could be in part responsible for CIPK23 specificity towards AKT1 or HAK5 in response to the same stimulus. Altogether, these observations clearly point to the importance of a spatial and/or temporal separation of Ca^{2+} signatures to maintain specificity in CIPK23-dependent responses.

Requirement for CBL, CBL Ca^{2+} affinity, and more. As mentioned above, CBL sensor proteins are responsible for decoding the Ca^{2+} signatures generated during stress conditions, and for transducing the signal by interacting and activating the right CIPKs. CBLs have four canonical or non-canonical EF-hand motifs showing helix-loop-helix structure that are responsible for Ca^{2+} binding. In Arabidopsis, CBL2, CBL3, CBL4, CBL5, and CBL8 possess no canonical EF-hands, whereas CBL6, CBL7, and CBL10 possess one; and CBL1 and CBL9 contain two canonical EF-hands (Batistic and Kudla 2004; Kolukisaoglu et al. 2004). Variation exists in Ca^{2+} coordination by CBLs, and this is also modulated by interaction with CIPKs. Despite of no having canonical EF-hands, CBL4 was shown to bind Ca^{2+} ions in all four EF-hands, while when in complex with CIPK24, only two Ca^{2+} ions are bound (Sanchez-Barrena et al. 2007; Sánchez-Barrena et al. 2005). On the contrary, CBL2 alone binds two Ca^{2+} ions with EF1 and EF4 while the CBL2-CIPK14 complex binds four Ca^{2+} ions (Akaboshi et al. 2008; Nagae et al. 2003). Furthermore, CBL proteins can undergo dimerization in response to Ca^{2+} binding (Sánchez-Barrena et al. 2005), influencing further its ability to coordinate Ca^{2+}. Such differences in calcium coordination and complex composition within the CBL-CIPK module likely translates into different affinities for Ca^{2+} or kinase activation, yielding different signaling properties.

CIPK kinases have also been reported to work independently of Ca^{2+} and CBLs, at least *in vitro* (Hashimoto et al. 2012). The dispensable use of the signal/decoder pair can also in theory contribute to specificity for some of CIPK23-mediated responses. There is currently no example of how CIPK23, or CIPKs in general, may be acting in different pathway depending on whether they interact with CBLs or not. That said, plants facing low iron conditions show an increase in cytosolic Ca^{2+} levels and a requirement for CBL1 to drive CIPK23-dependent regulation of the ferric chelate reductase (Tian et al. 2016) (Figure 2B). Surprisingly, under similar conditions of iron shortage, combined with an excess of non-iron metals such as Zn^{2+} or Mn^{2+}, no role for Ca^{2+} or CBLs was reported in the regulation of IRT1 by CIPK23 (Dubeaux et al. 2018) (Figure 2C). Provided that Ca^{2+} and CBLs are proven to be non-essential for the negative regulation IRT1 upon low iron and non-iron metal excess conditions, this would greatly support the idea that the requirement for Ca^{2+} and CBLs contributes to the specification of CIPK-dependent responses.

Keeping CIPK23 action local

Despite all the above-mentioned mechanisms contributing to the recruitment/activation of CIPK23 in nutritional responses, CIPK23 activity must be confined to a specific target within the cell. This requires to only activate CIPK23 locally, to avoid spurious activation of CIPK23 targets, together with the spatial separation of such targets.

Local activation of the CBL-CIPK23 module. The spatial control and the possibility of a local activation of CIPK23 and its cognate CBLs are still elusive. However, there are several puzzling observations that suggest a possible spatially-restricted activation of the CBL-CIPK module, at least in response to certain stimuli. First, unlike other kinases, CIPKs use Mn^{2+} over Mg^{2+} as cofactor to coordinate the phosphate groups of the nucleotide triphosphate substrate (Hashimoto et al. 2012). It is conceivable that, in the context of its role in non-iron metal response, CIPK23 is locally activated in the vicinity of IRT1 using the IRT1-bound Mn^{2+} ions to reach full kinase activity, yielding a specific phosphorylation of IRT1 (Figure 2C). While this scenario is tempting, there is currently no evidence for CIPK23 to use Zn^{2+} as a cofactor, considering that Zn^{2+} ions are also coordinated on IRT1 histidines and also trigger the CIPK23-dependent degradation of IRT1 (Dubeaux et al. 2018). Second, structural and thermodynamic studies have revealed that the EF-hand motifs can coordinate several other ions that Ca^{2+}, such as Mg^{2+}, strontium (Sr^{2+}), lanthanum (La^{3+}), barium (Ba^{2+}), lead (Pb^{2+}), Mn^{2+} and Zn^{2+} (Kumar et al. 2012; Lepsík and Field 2007; Senguen and Grabarek 2012). Considering that the CBLs are involved in the response to changes in the availability of some of these ions, we can speculate that binding of Mg^{2+}, Mn^{2+} or Zn^{2+} to CBLs may also participate to these responses (Figure 2C). For example, Mn^{2+} or Zn^{2+} binding by CBLs at the exit of the permeation domain of IRT1 could also contribute to the initiation of a local response targeting IRT1.

Spatial restriction of CIPK23 targets. The plasma membrane is a mosaic of domains with different lipid or protein composition. Recent reports highlighted the spatial separation of plant plasma membrane proteins sharing similar partners. For example, the BRI1 and FLS2 cell surface receptors for the brassinoteroid hormones and flagellin, respectively, localize to different nanodomains marked by specific remorins (Bücherl et al. 2017). Since most CIPK23-targets are localized at the plasma membrane of root epidermal cells (AKT1, HAK5, NRT1.1/CHL1, AMTs, IRT1, etc), localization to different nanodomains would clearly constrain locally-activated CIPK23 to a given target (Figure 2D). Single-particle tracking analysis revealed that plasma membrane domains contribute to the partitioning of the phosphorylated form of NRT1.1/CHL1 transporter, affecting NRT1.1/CHL1 spatiotemporal dynamics (Zhang et al. 2019). NRT1.1/CHL1 motility and co-localization with remorins is indeed influenced by nitrate provision or its

phosphorylation state. AMT1;3 organization at the cell surface is also control by substrate availability, with AMT1;3 clustering upon NH_4^+ excess (Wang et al. 2013). These clusters have been associated with internalization of AMT1;3 through clathrin-mediated endocytosis, and possibly microdomain-associated endocytosis through flotillin1 (Wang et al. 2013). Whether CBLs and activated-CIPK23 also show restricted localization at the cell surface is unknown.

Conclusion

CIPK23 kinase protein arises as a master regulator of nutrient acquisition and homeostasis, performing multifunctional roles in response to different stress conditions. How CIPK23 is capable of distinguishing the specific stimuli and regulate the appropriate target protein seems to be dependent on several aspects inherent to the signal and to the target. As such CIPK23 offers the unique opportunity to tackle how specificity in signaling is achieved. Only with such knowledge in hands will we be able to manipulate CIPK23 at will to generate modified crops with improved performance to stress. Much work is still needed, taking advantage of biochemical, imaging and structural approaches to grasp the functional specificities that allow a single protein to regulate such diverse transport systems.

Funding

This work was supported by grants from the Agence Nationale de la Recherche [ANR-13-JSV2-0004-01 to G.V.]; the French Laboratory of Excellence project "TULIP" [ANR-10-LABX-41 and ANR-11-IDEX-0002-02 to G.V.]; and a postdoctoral fellowship from the Alfonso Martín Escudero Foundation to R.R.C.

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Table 1. Mechanisms of nutrient transporter regulation by CIPK23 in *Arabidopsis thaliana* plants.

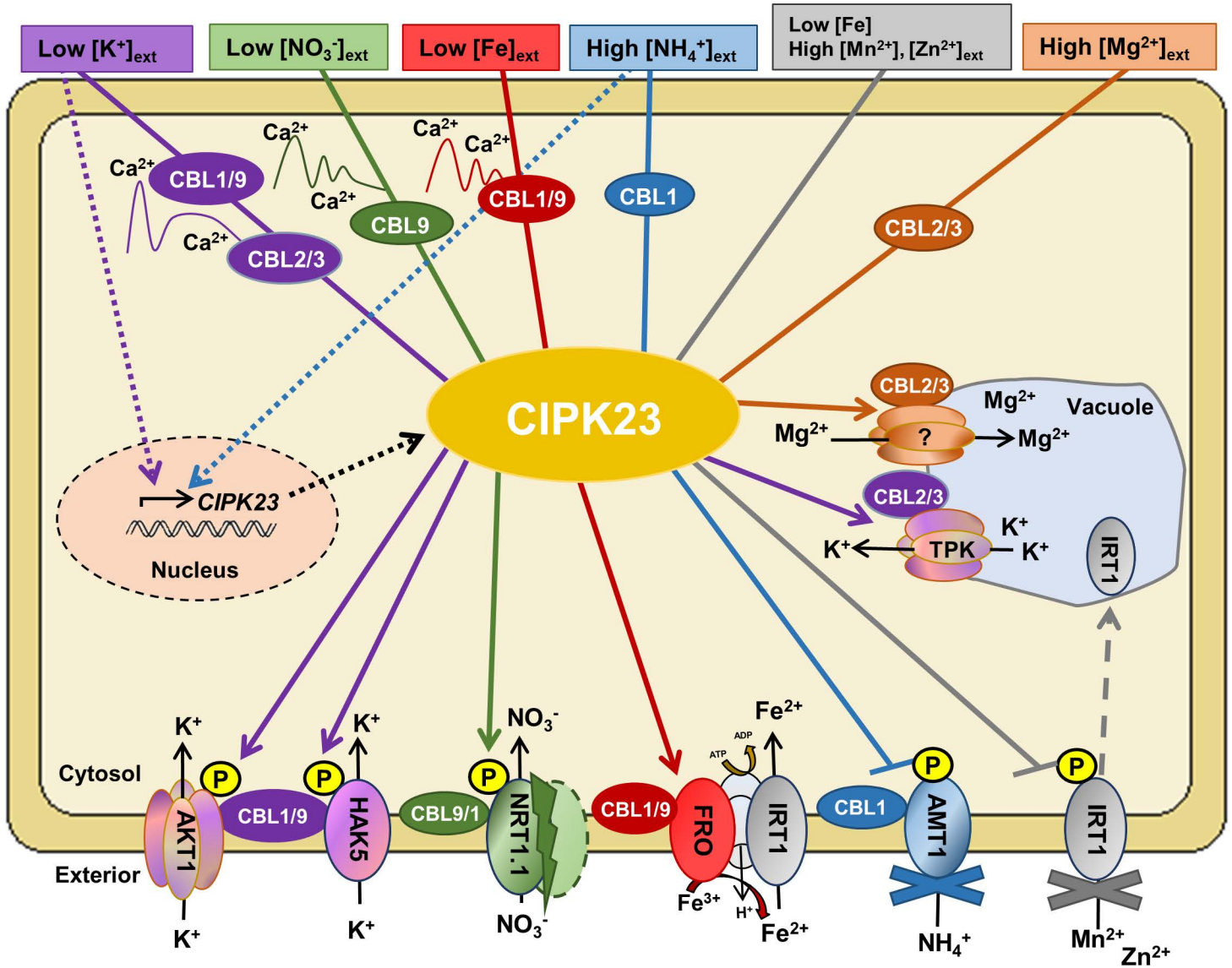
Stress	CIPK23 target	Mechanism of CIPK23 action	Localization	CBLs involved	Calcium signatures	References
Low K^+	AKT1	AKT1 activation by phosphorylation	PM / Root epidermis	CBL1/9	Yes	(Li et al. 2006) (Xu et al. 2006) (Behera et al. 2017)
Low K^+	AtHAK5	AtHAK5 activation by phosphorylation	PM / Root	CBL1/9	ND	(Ragel et al. 2015)
Low K^+	AtKUP4	ND	PM / Root	ND	ND	(Wang et al. 2020)
Low K^+	TPKs	TPKs activation	T/ Root and shoot	CBL2/3	Yes	(Tang et al. 2020)
Low NO_3^-	NRT1.1	NRT1.1 activation by phosphorylation	PM / Root	CBL9/1	Yes	(Liu and Tsay 2003) (Ho et al. 2009) (Leran et al. 2015) (Riveras et al. 2015)
Low Fe	FRO2	Enhances FRO2 activity	PM / Root epidermis	CBL1/9	Yes	(Tian et al. 2016)
High NH_4^+	AMT1.1 AMT1.2	AMT1.1/2 inactivation by phosphorylation	PM / Root	CBL1	ND	(Straub et al. 2017)
High Mg^{2+}	Unknown	Not described	T / Root and shoot	CBL2/3	ND	(Tang et al. 2015)
High non-iron metals	IRT1	Promotes IRT1 degradation by phosphorylation	PM / Root epidermis	ND	ND	(Dubeaux et al. 2018)
Drought	SLAC1 SLAH3	SLAC1 and SLAH3 activation by phosphorylation	PM / Guard cells	CBL1/9	ND	(Maierhofer et al. 2014)
Low K^+ and hyperosmotic	AtHAK5	ND	PM / Root	ND	ND	(Lara et al. 2020)

PM= Plasma Membrane; T=Tonoplast; ND= Not Described

659 **Figures legend**

660 **Figure 1. Schematic illustration of CIPK23 role in response to different stresses by regulating several**
661 **transporters/channels.** Low external potassium concentrations (in purple), low external nitrate (green),
662 low external iron (red), high external ammonium (blue), low iron and high non-iron metals (manganese and
663 zinc) external concentrations (grey) and high external magnesium (orange). Arrows in solid lines denote
664 positive regulation of targets by CIPK23, and blunt-headed arrows indicate negative regulation of targets.
665 Arrows in broken lines denote CIPK23 upregulation described in response to potassium deficiency and in
666 response to high ammonium external concentrations. The involvement of cytoplasmic calcium signals and
667 CBL proteins are indicated when known.
668

669 **Figure 2. Examples of mechanism conferring specificity to CIPK23-dependent responses.** A)
670 Expression pattern specificity. Expression of CIPK23 (in yellow) and CBL1/9 (in orange) in guard cells
671 allows the regulation of SLAC1 and SLH3 anion channels and stomatal opening. B) Requirement for
672 calcium and CBLs. CIPK23 regulates the root ferric reductase activity in response to low iron (red circles),
673 in a CBL1/9 and calcium-dependent manner. C) Alternative mode of CIPK23 activation. IRT1 transporter
674 is degraded after phosphorylation by CIPK23 (phosphate group in yellow) when plants experience both
675 low iron and non-iron metal excess (grey circles). Activation of CIPK23 (phosphate group in red) may
676 occur independently of CBLs with Mn^{2+}-bound IRT1 serving as Mn^{2+} donor to locally activate CIPK23
677 kinase activity. CBLs may also bind non-iron metals transported by IRT1 and in turn activate CIPK23 in
678 the vicinity of IRT1. CIPK23 may also be activated by an unknown protein (in brown). D) Spatial and
679 temporal regulation of CIPK23 activation. The kinetic of the calcium increase likely controls the early and
680 late responses to potassium (purple circles) deprivation with the activation of AKT1 (early), as well as
681 HAK5 and TPK, respectively. CBLs subcellular localization confers specificity by recruiting CIPK23 to
682 different compartments, as exemplified by the regulation of plasma membrane and vacuolar transport
683 proteins. The distribution of transporters in different micro/nanodomains (in yellow at plasma membrane)
684 also allows for a local activation of CIPK23 and specificity in response.


Low [K+]ext
Low [NO3-]ext
Low [Fe]ext
High [NH4+]ext
Low [Fe]
High [Mn2+], [Zn2+]ext
High [Mg2+]ext
Ca2+
CBL1/9
CBL9
CBL1/9
CBL1
CBL2/3
CBL2/3
CIPK23
CIPK23
Nucleus
CBL2/3
Mg2+
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Mg2+
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Mg2+
CBL2/3
K+
K+
TPK
K+
IRT1
Cytosol
Exterior
K+
P
AKT1
CBL1/9
K+
P
HAK5
K+
NO3-
P
CBL9/1
NRT1.1
NO3-
CBL1/9
FRO
Fe3+
ADP
ATP
H+
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IRT1
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CBL1
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AMT1
NH4+
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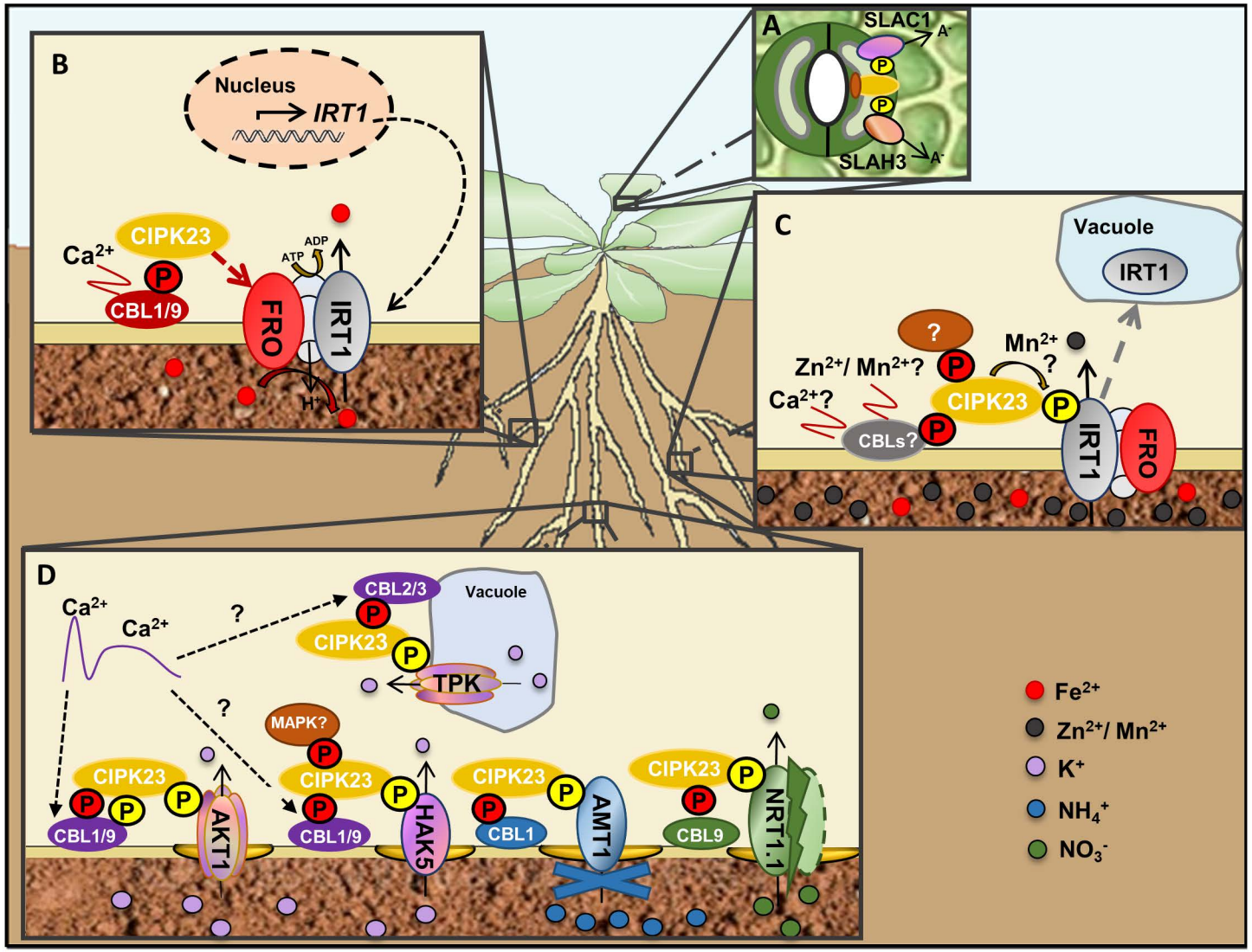
IRT1
Mn2+
Zn2+


A
SLAC1
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IRT1
CIPK23
Ca2+
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ADP
ATP
FRO
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NRT1.1
Fe2+
Zn2+/ Mn2+
K+
NH4+
NO3-