Trends in Plant Science

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Review

The CBL–CIPK Calcium Signaling Network: Unified Paradigm from 20 Years of Discoveries

Ren-Jie Tang,[1] Chao Wang,[1] Kunlun Li,[1] and Sheng Luan[1,*]

Calcium (Ca^{2+}) serves as an essential nutrient as well as a signaling agent in all eukaryotes. In plants, calcineurin B-like proteins (CBLs) are a unique group of Ca^{2+} sensors that decode Ca^{2+} signals by activating a family of plant-specific protein kinases known as CBL-interacting protein kinases (CIPKs). Interactions between CBLs and CIPKs constitute a signaling network that enables information integration and physiological coordination in response to a variety of extracellular cues such as nutrient deprivation and abiotic stresses. Studies in the past two decades have established a unified paradigm that illustrates the functions of CBL–CIPK complexes in controlling membrane transport through targeting transporters and channels in the plasma membrane and tonoplast.

Highlights

A novel type of Ca^{2+} sensors, termed as calcineurin B-like proteins (CBLs), were identified in plant cells 20 years ago. They specifically target a family of plant-specific CBL-interacting protein kinases (CIPKs).

To decode a Ca^{2+} signal, CBL binds Ca^{2+} and interacts with CIPK, leading to activation of the kinase. The CBL–CIPK complex phosphorylates downstream target proteins and changes their biological activities.

Most CBL proteins are localized to the cell membranes and, as a result, CBL–CIPK complexes are largely associated with membranes. This unique feature underlies the core function of the CBL–CIPK network in regulating various membrane transport processes in the plasma membrane and the tonoplast, thereby linking Ca^{2+} signaling to plant nutrient sensing and homeostasis.

Decoding Ca^{2+} Signals by Plant-Specific Sensor-Kinase Modules

Cellular calcium (Ca^{2+}) signals regulate nearly every aspect of eukaryotic physiology. In all cases, **Ca^{2+} signaling** (see Glossary) features a complex toolkit that includes an array of receptors sensing extracellular cues and Ca^{2+}-permeable channels that deliver Ca^{2+} into the cell across the plasma membrane or release Ca^{2+} from intracellular stores, formulating a specific Ca^{2+} signature [1]. Downstream of cellular Ca^{2+} signatures are Ca^{2+}-binding proteins that interact with effector proteins to trigger specific biochemical reactions leading to cellular responses [2,3]. In plants, Ca^{2+} signaling appears particularly important because it provides an indispensable mechanism for the sessile organisms to rapidly respond and adapt to the ever-changing environments through modifying their flexible developmental programs [2,4]. A transient and defined pattern of cytosolic Ca^{2+} elevation in plant cells is believed to serve as a '**second messenger**' that is required and sufficient for downstream responses [5–8]. Numerous environmental cues such as biotic and abiotic stress conditions often elicit Ca^{2+} second messengers with specific temporal and spatial characteristics. These various Ca^{2+} signals can be coded in the form of spikes, waves, and oscillations that are interpreted by **Ca^{2+} sensors** and effectors leading to specific responses. Several families of Ca^{2+} sensors have been identified in higher plants, including calmodulin (CaM) and CaM-like proteins (CMLs) [6,9,10], Ca^{2+}-dependent protein kinases (CDPKs) [11–13], and the more enigmatic **calcineurin B-like proteins (CBLs)** [6,14,15]. To define the functional identity of CBL family Ca^{2+} sensors, a novel family of plant-specific **CBL-interacting protein kinases (CIPKs)** serve as major downstream signaling components [16]. Further genetic work has established a central role of the CBL–CIPK signaling system in fine-tuning plant adaptive responses to adverse environmental conditions. Since the discovery of CBL–CIPK network in 1999, studies in the past 20 years have defined molecular mechanisms governing the actions of the CBL–CIPK modules in Ca^{2+} signal transduction and uncovered various physiological processes in which this Ca^{2+} signaling network facilitates plant response and adaption to changing environments, particularly in the context of **membrane transport** in plant cells. We review some unified paradigms from the recent studies and present questions that require further investigations.

[1]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA

*Correspondence: sluan@berkeley.edu (S. Luan).

The Historical and Evolutionary Aspects of CBL–CIPK Network

Among numerous Ca^{2+} sensor-effector combinations that constitute Ca^{2+}-dependent molecular switches in eukaryotes, calcineurin functions as a Ca^{2+}- and CaM-dependent serine/threonine protein phosphatase [17]. Calcineurin consists of a catalytic subunit (calcineurin A) and a Ca^{2+}-binding regulatory subunit (calcineurin B) and serves as a critical regulator in a number of Ca^{2+}-dependent signaling processes [18]. Although the sequence of both subunits and the heterodimeric quaternary structure remain highly conserved from yeast to mammals [19–21], an authentic homolog has never been isolated from plants. Upon identification of calcineurin as the target for immunosuppression by cyclosporin A and FK506 [22], pharmacological approaches have been used to provide initial evidence of similar activity existing in plants for regulation of ion channel activities in the plasma membrane or the tonoplast of guard cells [23,24]. Along the way to exploring the molecular nature of plant calcineurin-like activity, genes encoding CBLs are identified from arabidopsis (*Arabidopsis thaliana*) [14]. Later studies on their crystal structures clearly indicate that plant CBLs display a more similar folding pattern to that of calcineurin B than any other class of Ca^{2+} sensors, such as CaMs (Figure 1) [25,26], supporting the nomenclature of this family of plant-derived Ca^{2+} sensors. However, genes for calcineurin A-type phosphatases have never been found in plants. More surprisingly, subsequent studies indicated that, unlike the dogma in animals and fungi where calcineurin B proteins associate with the phosphatase calcineurin A, plant CBL proteins specifically interact with and regulate a family of protein kinases termed 'CIPKs' [16]. Meanwhile, genetic characterization of the salt overly sensitive (SOS) pathway in arabidopsis identified SOS3–SOS2 as a typical CBL–CIPK module [27,28], also known as CBL4–CIPK24 for nomenclature integration. Because both CBLs and CIPKs are regarded to be plant-specific, the discovery on the CBL–CIPK system not only identified a new mechanism for calcium signaling in plants but also revealed a 'paradigm shift' in calcium signaling transduction from fungi and animals to plant species (reviewed in [2,6]; Figure 1).

Soon after the discovery of some CBL–CIPK modules in arabidopsis, whole genome sequence became available for this model plant [29]. Using genomic tools, researchers have identified a total of 10 members of CBLs and 26 members of CIPKs encoded in the arabidopsis genome. Following sequencing of other plant genomes, the genomic composition of CBL–CIPK network has been established in various plant species throughout the plant kingdom [6,30–32]. A single CBL–CIPK pair is typically present in green algae, suggesting that the prototype of this signaling module may date back to single-cell plant ancestors. Remarkable expansion of CBL and CIPK genes in several early land plant lineages is probably driven by gene duplication and whole genome duplication events, supporting the hypothesis that the CBL–CIPK network plays a prominent role in plant adaptation to land environment [32]. Further expansion and increasing complexity of the CBL–CIPK system appear to synchronize with the evolution of land plants in morphology, development, life cycle, and adaptation to diverse and challenging habitats [31,32]. For example, multiple CBL–CIPK modules function in pollen tube growth [33,34], a specialized developmental process unique to flowering plants. Several modules are involved in coping with nutrient deficiency in the soil [35,36], a very dynamic growing environment for land plants. The large membership of the CBL and CIPK gene families in flowering plants thus constitute a highly convoluted and sophisticated signaling network. Among the ten CBLs and 26 CIPKs in arabidopsis, each CBL interacts with a subset of CIPKs and each CIPK interacts with one or more CBLs [37]. As a result, some CBLs have common CIPK partners and some CIPKs share common CBL regulators. Such specificity and overlap in protein–protein interactions may confer both signaling specificity and functional synergism of CBL–CIPK complexes *in vivo*. The functionality of the CBL–CIPK network may be regulated at multiple levels, including gene expression pattern, Ca^{2+}-binding affinity, and protein stability, which demands further genetic and biochemical analyses to dissect the complexity of the CBL–CIPK network in plant cell signaling.

Glossary

Ca^{2+} sensor: a type of protein that participates in Ca^{2+} signaling pathways by perceiving dynamic changes in intracellular Ca^{2+} concentrations. Capable of binding Ca^{2+} in different affinities, it undergoes Ca^{2+}-induced conformational changes to regulate downstream proteins and switches on a specific cellular response.

Ca^{2+} signaling: a process in the cell where calcium levels fluctuate to exert allosteric regulatory effects on proteins and enzymes so as to govern basic activities of the cell and coordinate proper cellular actions.

Calcineurin B-Like protein (CBL): a family of Ca^{2+} sensor proteins that share closest similarity with the regulatory subunit (CNB) of yeast and animal calcineurin. However, CBLs are generally found in plants but not in animals or fungi. CBL proteins perceive Ca^{2+} signals in response to stress signals and enable plant adaptation to the environmental changes.

CBL-interacting protein kinases (CIPK): a family of plant-specific protein kinases that physically interact with CBL-type Ca^{2+} sensors. Their activities and localizations are often regulated by CBLs. Combinations of CBLs and CIPKs constitute a signaling network that primarily regulates plant stress responses.

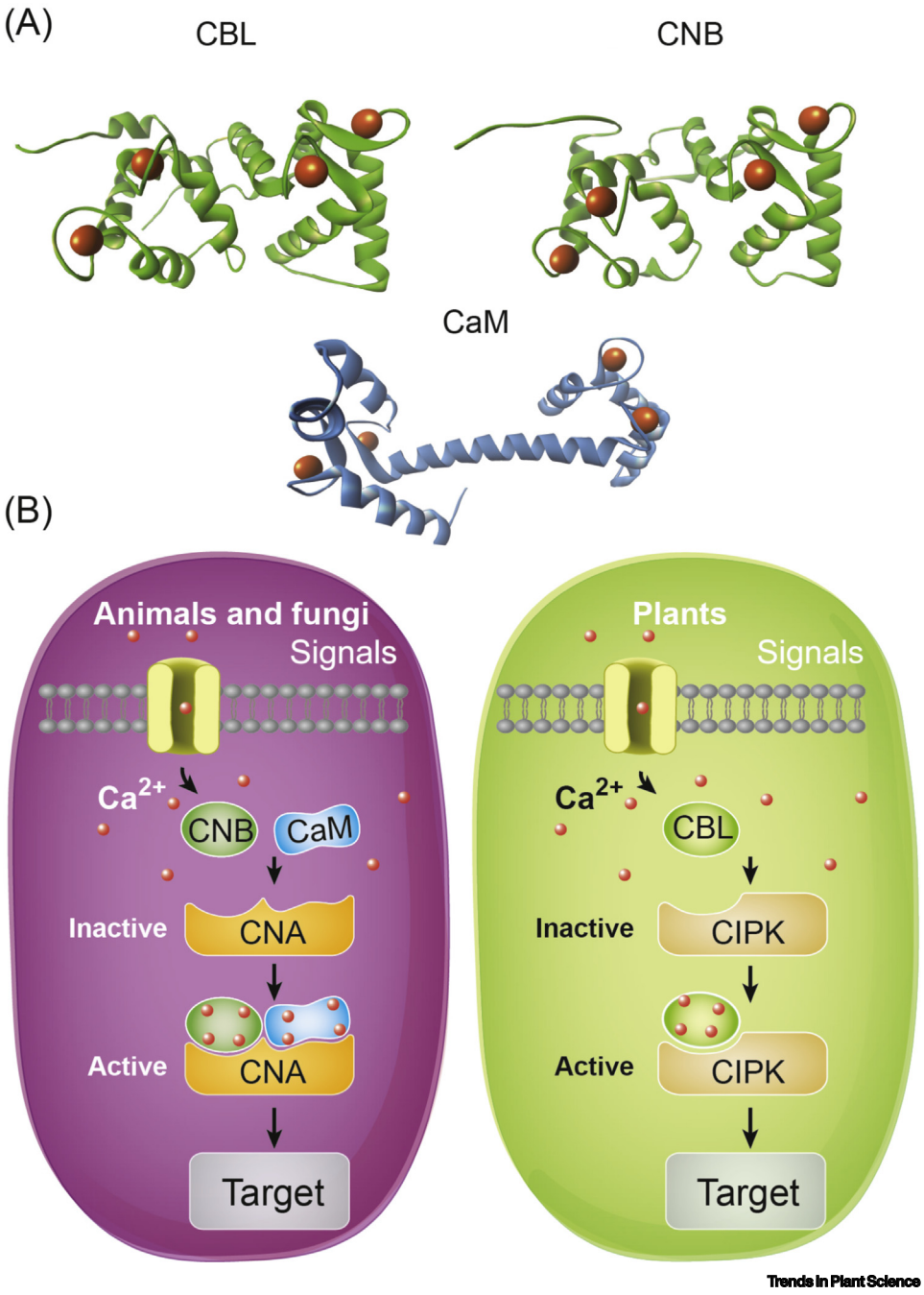
Membrane transport: the process that enables movement of solutes such as ions and small molecules across lipid bilayer-based cell membranes. Membrane transport largely depends on membrane-embedded proteins that can form structures such as channels or carriers.

Second messenger: in response to external stimuli, cells produce small molecules that are required and sufficient to trigger further signal transduction events that ultimately result in cellular responses.

Regulatory Mechanisms of CBL–CIPK Interactions

CBLs Enhance the Kinase Activity of CIPKs

CBL proteins share an overall structural homology consisting of four EF-hand domains responsible for binding Ca^{2+}. In contrast to the relatively compact structure of CBLs, CIPKs contain several functionally distinct domains. All CIPKs have their kinase domain in the N terminal half and several



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regulatory domains in the C terminal region. These regulatory domains include the CBL-binding domain [37], later referred to as the NAF domain/FISL motif that features several conserved hydrophobic amino acids responsible for interaction with CBL proteins [38,39]. Interestingly, the CBL–CIPK interaction is reminiscent of that between calcineurin B and calcineurin A based on the structural analysis (Figure 2) [40]. This structural mimicry implicates similarity in the regulation of the Ca^{2+} sensor-phosphatase in animals and fungi versus Ca^{2+} sensor-kinase pairs in plants. Indeed, further biochemical experiments established that, similar to the regulatory mechanism of calcineurin A by B, CBL–CIPK interaction potentiates the kinase activity of CIPKs in that CBL binding to CIPK releases the N terminal kinase domain from the C terminal autoinhibitory domain (Figure 2) [39,41]. Therefore, dynamic CBL–CIPK complex formation may provide a molecular switch for the kinase activity that further targets downstream proteins in the signaling cascades, which represents a basic paradigm in the CBL–CIPK signaling network (Figure 2).

Ca^{2+} Enhances CBL–CIPK Interaction and/or Kinase Activity?

A critical regulatory feature in the CBL–CIPK module concerns the role of Ca^{2+} in complex formation. In a canonical Ca^{2+} signaling pathway, it is generally believed that elevated levels of Ca^{2+} upon stimulus of a primary signal trigger Ca^{2+}-binding and conformational changes of the sensor proteins, which in turn, interact with and regulate downstream effector proteins in a Ca^{2+}-dependent fashion. This dogma appears to be amenable to the CBL–CIPK modules because CBL1–CIPK1 interaction showed a requirement for micromolar levels of Ca^{2+} *in vitro* [16]. Crystal structure on the CBL4/SOS3–CIPK24/SOS2 complex further supports this idea in that Ca^{2+} is important to promote the formation of the complex and enhances the kinase activity [40]. However, in the structural analysis of the CBL2–CIPK14 complex, the structure of CBL2 does not change in response to the presence of additional Ca^{2+} [42], bringing up the hypothesis that Ca^{2+} may not be necessarily required for the CBL–CIPK interaction. Indeed, some other studies indicated that interactions between CBLs and CIPKs may occur independently of Ca^{2+} [39,43], although it is assumed that activation of the kinase complex, and particularly subsequent targeting towards downstream substrates, requires both Ca^{2+} and CBL sensors. It should be noted that all these studies were performed using *in vitro* experimental systems in which adequate Ca^{2+} may already be incorporated into the sensor proteins and the mechanism underlying Ca^{2+}-dependent regulation *in vivo* awaits future clarification.

Interaction with and Regulation by Phosphatases

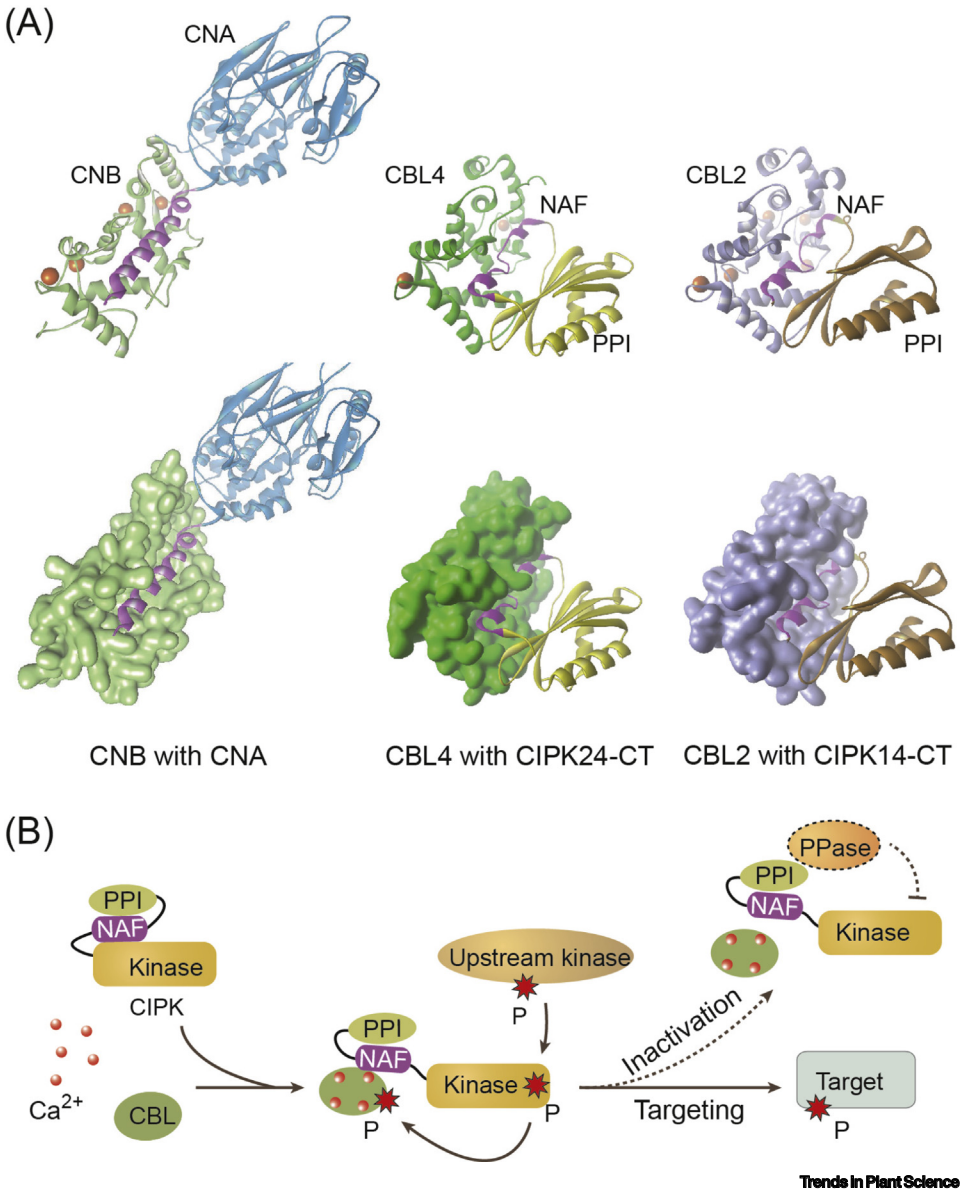
Adjacent to the CBL-interacting domain, CIPK proteins contain another less well-characterized domain for protein phosphatase interaction (PPI). This domain is responsible for association with type-2C protein phosphatases (PP2C) such as ABI1, ABI2 [44], and AIP1 [45]. The functional significance of this domain in the regulation of CIPKs is not well understood. In the structural analysis, the CBL-interacting domain and the PPI domain may overlap, suggesting that CBL and PP2C interaction with CIPK may be mutually exclusive [40]. Such a structural feature may provide a mechanism for preventing simultaneous activation by a CBL and inactivation by a

Figure 1. Decoding Cellular Ca^{2+} Signals by the Plant-Specific CBL-CIPK System. (A) Comparison of the crystal structures of CBL protein, CNB, and CaM. CBL protein (PDB ID: 1V1G) is folded into two globular domains connected by a short linker. Each domain is formed by a pair of adjacent EF-hand motifs. The overall structure of CBL is almost identical with that of CNB (PDB ID: 4ORC). CaM (PDB ID: 5A2H) displays a dumbbell-shaped structure consisting of two globular domains. Note that as compared with CBL or CNB, CaM has a much longer central linker helix that could accommodate many diverse target proteins. By contrast, CNB and CBLs have rather specific target proteins. Red spheres denote calcium atoms (Ca) or calcium ions (Ca^{2+}). (B) Paradigm shift from calcineurin in animals and fungi to CBL–CIPK in plants. In animal or fungal cells, following the Ca^{2+} signal elicited by external stress signals, CNB and CaM bind Ca^{2+} and subsequently interact with and activate the calcineurin A protein phosphatase, which in turn, leads to the activation of downstream targets. In plant cells, CBL specifically recognizes and regulates the localization and activity of CIPK, a group of protein kinases. The CBL–CIPK module decodes Ca^{2+} signals in the plant cell by further regulating downstream targets. Abbreviations: CaM, Calmodulin; CBL, calcineurin B-like protein; CIPK, CBL-interacting protein kinase; CNA, calcineurin A; CNB, calcineurin B; PDB, protein data bank.

phosphatase of a CIPK, thereby providing an on–off switch for regulated modification of the downstream substrates. Consistent with this notion, the potassium channel AKT1 can be activated by CBL–CIPK and inhibited by PP2C when coexpressed in *Xenopus* oocytes [45,46]. This reversible regulation of the substrate by a kinase-phosphatase pair has been found to be a universal mechanism for the control of many other processes in plant cells [47–50].

CIPK Activation and Phosphorylation by Other Kinases

Aside from the activation by CBLs, CIPKs can also be activated by phosphorylation of the loop region in the kinase domain. Biochemical analysis identified within the activation loop several serine and threonine residues that could be modified by trans-phosphorylation events, leading



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to enhanced activity of CIPKs independent of Ca^{2+} [51]. It is thus hypothesized that some upstream kinases might exist to activate CIPKs and Geminivirus Rep-Interacting Kinases (GRIKs) may serve as one group of the regulatory kinases that fulfill such a role [52]. It is possible that other types of kinases may also act as such upstream activators in different physiological contexts because CIPKs physically interact with several classes of kinases, including SnrK2s [53], MAPKs [54], and RLKs [55]. Further work is needed to establish the functional relationship between CIPKs and those interacting kinases, thereby unraveling the potential crosstalks between CBL–CIPK network and other plant signaling pathways.

Phosphorylation of CBLs by CIPKs

An additional level of complexity in the regulation of CBL–CIPK modules is presented by the CIPK-mediated phosphorylation of their interacting CBL partners. This mechanism has attracted special interest because it suggests that CBLs also serve as native substrates of CIPKs and the two subunits of the complex may regulate each other through a positive feedback loop. In other words, CBLs bind to CIPKs to activate CIPKs that in turn phosphorylate CBLs to enhance the functionality of CBLs. Several studies show that the C terminal region of CBLs can be directly phosphorylated by CIPKs at some conserved serine/threonine residues [56–59]. This phosphorylation is thought to be functionally important for the specificity and activity of CBL–CIPK complexes to regulate their downstream targets. Indeed, phosphorylation of CBL1 by CIPK23 is required for efficient phosphorylation *in vitro* and activation of AKT1 in *Xenopus* oocytes [58]. Moreover, phosphorylation of CBL10/SCaBP8 by its partner CIPK24/SOS2 enhances the complex formation during salt stress and is required for conferring salt tolerance on arabidopsis plants [56]. Interestingly, phosphorylation of CBLs by CIPKs seems to be more specific than the physical interaction between them. For instance, CIPK9 preferentially phosphorylates two of its CBL partners, CBL2 and CBL3 [59], whereas CIPK24/SOS2 selectively phosphorylates CBL10/SCaBP8 instead of the well-known partner CBL4/SOS3 *in vitro* [56]. This specific phosphorylation pattern may contribute to the fidelity of the signal transduction mediated by a particular CBL–CIPK signaling module. Although CBL phosphorylation by CIPKs may provide a way to control the function of a particular CBL–CIPK module, the underlying mechanism under specific physiological conditions demands further investigation.

Functional Diversity of the CBL–CIPK Network: Unified Themes in the Regulation of Membrane Transport

Plants take up numerous minerals from the soil, usually in the ionic forms. Some of these ions are essential as nutrients (e.g., K^+ and NO_3^-), whereas others in the soil solution could be toxic at high

Figure 2. Structural Features and Working Model of the CBL–CIPK Signaling Modules. (A) Structural comparison of CNB–CNA and CBL–CIPK complexes. Upper panels display stereo diagrams of CNB bound to CNA (left; PDB ID: 1TCO), CBL4 in complex with CIPK24 (middle; PDB ID: 2EHB), and CBL2 in complex with CIPK 14 (right; PDB ID: 2ZFD). Note that only partial structures encompassing the CBL-interacting domains of CIPK24 and CIPK14 are resolved and shown here. The C terminal region of CNA that interacts with CNB or the CBL-interacting domain of CIPK is shown in purple. Calcium ions are depicted as red spheres. Lower panels show the molecular surface models of CNB or CBLs, featured with the hydrophobic crevice that is required for the physical interaction. The recognition mode of CBL and CIPK resembles that observed for the CNB–CNA interaction. (B) Schematic representation of CBL–CIPK-mediated signaling pathway. CIPK consists of an N terminal catalytic domain for kinase activity and a C terminal regulatory region containing the NAF motif for interaction with CBL as well as the PPI motif for interaction with protein phosphatase 2C. At the resting state, the kinase activity of CIPK is minimal due to inhibition of the kinase domain by the C terminal region. When the cellular signaling is initiated, Ca^{2+}-bound CBL interacts with CIPK via the NAF domain and releases the inhibitory effect of the C terminus, leading to activation of the kinase. CIPK, in some cases, may also phosphorylate its interacting CBL(s) as a feedback regulation of the CBL–CIPK complex. The kinase is also proposed to be activated upon phosphorylation by upstream protein kinases in the activation loop in response to other signaling inputs. Activated CBL–CIPK complex phosphorylates and modifies the activity of downstream targets. The module is proposed to be inactivated by protein phosphates that potentially terminate the signaling. Abbreviations: CBL, Calcineurin B-like protein; CIPK, CBL-interacting protein kinase; CNA, calcineurin A; CNB, calcineurin B; CT, C-terminus; NAF, a 24-amino acid domain defined for CBL–CIPK interaction; PDB, protein data bank; PPI, protein phosphatase interaction; PPase, protein phosphatase.

concentrations (e.g. Na^+ and NH_4^+). Extensive studies have established that CBL–CIPK signaling pathways play a central role in plant adaptive responses to fluctuating ionic conditions, particularly in low-nutrient environments and toxic-metal stresses. In this regard, much has been covered in previous reviews [2,6,8,35,60]. Here, we briefly describe several well-known examples and focus on more recent findings on the functional significance of the CBL–CIPK network. Increasing evidence supports a unified theme that CBL–CIPK serves as a major mechanism for the regulation of membrane transport processes (Figure 3).

Membrane Association of CBLs Determines Functional Specificity of the CBL–CIPK Complex

In response to various signals, Ca^{2+} elevation in the cytoplasm can be generated by influx from extracellular spaces (i.e., apoplast) and/or by retrieval from the intracellular compartments (e.g., vacuole and other organelles). Temporally defined Ca^{2+} releases from the spatially distinct stores can be triggered by specific environmental cues and lead to corresponding cellular responses. To decode the specific and spatially restricted Ca^{2+} signals, some Ca^{2+} sensors must be targeted to different membranous compartments in close proximity to the Ca^{2+}-release sites [5]. A critical feature of plant CBL–CIPK modules that allows them to decode such spatially distinct Ca^{2+} signals is their targeting to different cell membranes. In arabidopsis, half of the CBL members (CBL1, 4, 5, 8, 9) contain an N terminal lipid modification motif or a polybasic domain that anchors the proteins to the plasma membrane [61,62] and some other members (CBL2, 3, 6, 10) harbor sequences responsible for association with the vacuolar membrane [62,63]. Through physical interaction, CBLs can then recruit their partner CIPKs to the destined membranes (either plasma membrane or tonoplast), where they target a unique set of substrates. Since the singular CBL isoform from green algae features the conserved N terminal motif that allows dual fatty acyl modification [32], it is attempting to postulate that CBL–CIPK emerges as a membrane-associated signaling module in the primitive plant species. This ancestral membrane localization mechanism retains functional importance during plant evolution and becomes more diversified in higher plants, fulfilling the fundamental roles of CBL–CIPK in the regulation of various membrane transport processes dictated by Ca^{2+} signaling.

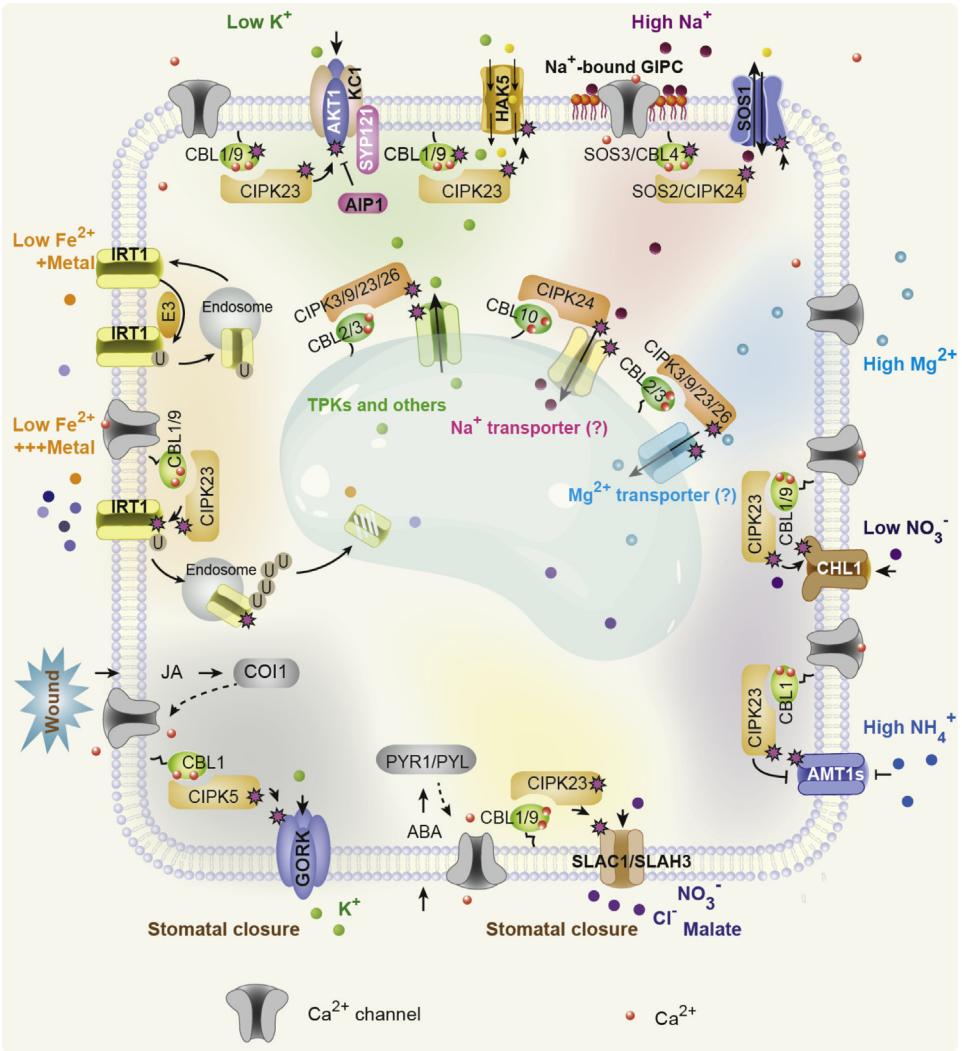
Action at the Plasma Membrane: From Mineral Uptake and Sensing to Stomatal Movement

In light of a possible functional redundancy of CBL1 and CBL9, which share high sequence homology, *cbl1 cbl9* double mutant was created and found to be more tolerant to drought stress than the wild type and more sensitive to low-K starvation [64,65]. Genetic screening identified a single *cipk* mutant, *cipk23*, phenocopied the *cbl1 cbl9* double mutant in both drought and low-K conditions, indicating that CIPK23 should be a common downstream component that mediates the function of CBL1 and CBL9 in stomatal regulation and K^+ uptake processes [64]. It is hypothesized that CBL1 and CBL9 can perceive the Ca^{2+} signal triggered by low K^+ in the environment [66]. This pair of calcium sensors interact with CIPK23 and recruit the CBL–CIPK23 complex to the plasma membrane where the CIPK23 kinase phosphorylates and activates the voltage-gated K^+ channel AKT1 that is required for root K^+ absorption and low-K tolerance in plants [65,67–69]. Although AKT1 activity is also modulated by other factors such as the AtKC1 subunit and the syntaxin SYP121 that may assemble into the channel complex, the CBL1/9–CIPK23-mediated activation of AKT1 can happen without the other factors [70,71]. The native AKT1 channel complex in plant cells has yet to be resolved by biochemical approaches.

For most plant species, nitrate (NO_3^-) and ammonium (NH_4^+) in the soil serve as two major nitrogen sources. The plasma membrane-localized CBL1/9–CIPK23 modules play a critical role in the regulation of NO_3^- uptake and sensing by controlling the activity and conformation switch of CHL1 (NTR1.1), a transporter and a sensor for NO_3^- [72]. Interestingly, the same CBL–CIPK

complexes are also involved in the inhibition of NH_4^+ uptake mediated by AMT1-type transporters, preventing toxic accumulation of cytoplasmic NH_4^+ in plant cells [73]. It is believed that NO_3^- facilitates K^+ transport as a counter anion, whereas $NH4^+$ competes against K^+ uptake in plant cells. Thus, this multifunctional CBL–CIPK signaling pathway in the plasma membrane provides a key regulatory mechanism for synergistic interplay of potassium-nitrogen nutrition in plants.

A more complex role of the CBL1/9–CIPK23 pathway in plant nutrition came from its involvement in iron acquisition. Because both *cipk23* and *cbl1 cbl9* mutants are hypersensitive to iron deficiency, it was hypothesized that the Ca^{2+} signal evoked by iron starvation could induce the conformation changes of CBL1 and CBL9, which in turn leads to CIPK23 activation [74]. CIPK23 physically interacts with the ferrous (Fe^{2+}) transporter IRT1, but CIPK23-mediated phosphorylation of IRT1, unlike other cases discussed earlier, appears to facilitate recruitment of an E3 ligase to IRT1 for efficient endosomal sorting and subsequent degradation [75]. As IRT1 also transports



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zinc, manganese, cobalt, and cadmium, concentrations of which are often dominant in iron-deficient soils, CIPK23-facilitated IRT1 degradation may prevent plants from accumulating highly reactive metals from the soil and optimize iron uptake.

Identification of multiple targets of CIPK23 raises an intriguing question: how does the same CBL–CIPK module distinguish specific stimuli and accordingly regulate different downstream effectors? It also opens a new area of research aiming to understand the universal and versatile role of Ca^{2+} signaling in plant mineral nutrition.

In addition to regulation of nutrient acquisition, the CBL–CIPK network also controls ion fluxes that contribute to physiological processes in specific cell types, such as membrane transports in guard cells that govern stomatal movements. During abscisic acid (ABA) signaling, CBL1/9–CIPK23 complexes phosphorylate and activate the guard cell anion channel SLAC1 [48]. In the wounding response, jasmonic acid-induced stomatal closure requires phosphorylation and activation of the outward K^+ channel GORK by the CBL1–CIPK5 complex [50]. These studies establish the function of CBL–CIPK in plant hormone signaling that involves Ca^{2+} second messenger in the regulation of fast cellular responses, which often entail ionic transport across the membrane.

Targets at the Vacuolar Membrane for pH Regulation and Magnesium Storage

Compared with the pioneering studies on CBL–CIPK complexes at the plasma membrane, the physiological roles of tonoplast-localized CBL–CIPK modules have not been investigated until more recently. In search for the function of CBL2 and CBL3 that share over 90% amino acid sequence identity, genetic analysis revealed their functional redundancy in the control of mineral nutrition. The *cbl2 cbl3* double mutant, but not *cbl2* or *cbl3* single mutants, showed dramatic growth defects with typical symptoms of nutrient imbalance [63]. Further analysis indicated that *cbl2 cbl3* double mutant, with a similar phenotype as the mutant lacking tonoplast V-ATPase, is considerably affected in vacuolar H^+-ATPase activity, resulting in impaired transport of multiple mineral nutrients across the tonoplast [63]. In addition to regulation of V-ATPase activity and thus vacuolar pH, CBL2 and CBL3 also regulate transport processes that appear to be independent of the H^+ gradient established across the tonoplast. One such process is vacuolar Mg^{2+}

Figure 3. Regulation of Membrane Transport Processes in Plant Cells by the CBL–CIPK Signaling Network.
Many ionic stress conditions in the environment, such as low K^+ availability or high Na^+ levels in soil, would trigger a rapid elevation of cytosolic Ca^{2+} concentration, which is interpreted as a 'signal'. Distinct Ca^{2+} signals could be perceived and decoded by different CBL–CIPK complexes targeting to various transport proteins depending on subcellular locations. In the plasma membrane, multiple membrane transport processes are regulated by the CBL–CIPK network: the CBL1/9–CIPK23 complexes phosphorylate and activate the K^+ channel AKT1 and the K^+ transporter HAK5 for enhanced K^+ uptake; CBL4/SOS3–CIPK24/SOS2 complex stimulates the Na^+/H^+ exchanger SOS1 to extrude excessive Na^+ out of the cell. Under fluctuating NO_3^- concentrations, CBL9–CIPK23 complexes phosphorylate the nitrate transporter CHL1 and modify its transport affinity and sensing capacity to NO_3^-. In response to high NH_4^+, CBL1–CIPK23 inhibits the activity of AMT1-type NH_4^+ transporters to avoid over-accumulation of NH_4^+. Under low Fe^{2+} but excess of other heavy metals, CIPK23-mediated phosphorylation of IRT1 is required for its subsequent ubiquitination and degradation. In the guard cell, CBL1–CIPK5 and CBL1/9–CIPK23 complexes regulate K^+ and anion effluxes through activation of the outward K^+ channel GORK and the anion channel SLAC1, respectively. Low K and high Na initiate additional CBL–CIPK signaling pathways in the vacuolar membrane: CBL2/3–CIPK3/9/23/26 complexes activate K^+ remobilization from the vacuole store through TPK-type K^+ channels and possibly other K^+ transport proteins; CBL10–CIPK24 complex targets an unidentified Na^+ transporter for Na^+ partitioning into the vacuole. In the tonoplast, Mg^{2+} sequestration is also positively regulated by an array of vacuolar CBL2/3–CIPK3/9/23/26 modules. Small spheres with various colors denote different ions. Asterisks indicate phosphorylation of the protein. The dashed lines and question marks denote uncertain pathways or unknown components that remain to be identified. Abbreviations: ABA, Abscisic acid; AIP1, AKT1 interacting protein phosphatase 1; AKT1, arabidopsis K^+ transporter 1; CBL, calcineurin B-like protein; CIPK, CBL-interacting protein kinase; COI1, coronatine insensitive 1; GIPC, glycosyl inositol phosphorylceramide; GORK, gated outwardly rectifying K^+ channel; HAK5, high-affinity K^+ transporter 5; IRT1, iron-regulated transporter 1; JA, jasmonic acid; KC1, K^+-rectifying channel 1; PYL, PYR1-like; PYR1, pyrabactin resistance 1; SLAC1, slow anion channel-associated 1; SLAH3, SLAC1 homolog 3; SOS, salt overly sensitive; SYP121, syntaxin of plants 121; TPK, two-pore K^+ channel.

sequestration, rendering *cbl2 cbl3* double mutant hypersensitive to high levels of Mg^{2+}, a phenotype that is absent in the V-ATPase mutant [76]. Further studies identified a quartet of CIPKs that interact with CBL2 and CBL3 to form eight different CBL–CIPK complexes that are recruited to the tonoplast and redundantly regulate Mg^{2+} translocation into the vacuole [76]. Mg^{2+} influx into the plant vacuole from cytosol is probably mediated by vacuolar Mg^{2+} transport protein(s), the molecular identity of which remains elusive.

Maintenance of Na^+/K^+ Homeostasis Involves Dual CBL–CIPK Pathways at Both the Plasma Membrane and Tonoplast

During evolution, K^+ has been selected over Na^+ as a major monovalent cation to fulfill numerous physiological functions in plant cells. To maintain K^+/Na^+ homeostasis, plants have developed a plethora of mechanisms to prevent Na^+ accumulation but favor K^+ uptake and translocation in different tissues. Another cation, Ca^{2+}, has been utilized as a central signaling agent that controls these processes by fine-tuning the activities of multiple Na^+ and K^+ transport proteins [35]. Parallel to the studies that established the CBL–CIPK network, genetic analysis of the SOS pathway in arabidopsis documented the first example in which a CBL–CIPK signaling module is functionally connected with Na^+ exclusion process (reviewed in [60]). Salt stress elicits a transit elevation of cytosolic Ca^{2+} level in plant cells, which was interpreted as a signal for salinity responses [77]. This long-sought Ca^{2+}-associated salt sensing mechanism was recently demonstrated to involve direct binding of Na^+ to glycosyl phosphoryl ceramide sphingolipids in the plasma membrane for salt-induced depolarization of cell-surface potential, which is thought to be required for the activation of some Ca^{2+} influx channels [78]. Earlier genetic studies have established one of the downstream components as the Ca^{2+} sensor CBL4/SOS3 that presumably perceives the salt stress-triggered Ca^{2+} signal and leads to activation of the CIPK24/SOS2 kinase and its targeting to the plasma membrane [28,43,79]. The CBL4–CIPK24 complex phosphorylates the C terminus of SOS1, a Na^+/H^+ antiporter, leading to the removal of SOS1 autoinhibition [80]. Consequently, the activated Na^+ transporter promotes Na^+ extrusion across the plasma membrane, thus maintaining a lower Na^+ level in the cytosol during salt stress. Another CBL-type Ca^{2+} sensor, CBL10, appears to function in plant salt tolerance through interaction with CIPK24 as well [81–83]. But unlike CBL4/SOS3, that plays a major role in roots, CBL10 is predominantly expressed and functional in the above-ground tissues of plants [81–83]. Contrary to most other salt hypersensitive mutants, *cbl10* mutant plants unusually accumulate less Na^+ in the leaves [81,84], bringing up the hypothesis that CBL10 may mediate an alternative pathway that regulates Na^+ compartmentalization into plant vacuoles. Consistently, CBL10 protein was found to deliver the CBL10–CIPK24 complex to the vacuolar membrane [81,83]. Recent genetic analysis further supported the hypothesis that CBL4/SOS3 and CBL10 initiate two independent pathways required for plant salt tolerance [85], namely, Na^+ exclusion at the plasma membrane and Na^+ sequestration into the vacuole, respectively.

Similar to the regulation of Na^+ tolerance, plant responses to low-K stress also involve dual CBL–CIPK pathways emanating from the plasma membrane and the vacuolar membrane. Most natural soils contain sub-millimolar levels of K^+, and K^+ entry into root cells is facilitated by specific K^+ channels and transporters. In particular, the shaker-type K^+ channel AKT1 and the K^+/H^+ symporter HAK5 function as two major players in K^+ uptake, as demonstrated in arabidopsis [68,86,87]. Interestingly, both AKT1 and HAK5 are regulated by CBL–CIPK in a Ca^{2+}-dependent manner. In response to low-K^+ status, CIPK23 is recruited to the plasma membrane by CBL1 and CBL9 and the functional CBL1/9–CIPK23 complexes are necessary and sufficient for phosphorylation and activation of AKT1 or HAK5 for enhanced K^+ uptake [65,67,88]. Besides the plasma membrane CBL–CIPK pathway for activation of K^+ uptake, a vacuolar CBL–CIPK network has been recently established as a primary mechanism for plant K^+ starvation

response [99]. In search for factors controlling vacuolar K^+ remobilization, *cbl2cbl3* mutant was identified to require much higher levels of K^+ for germination and optimal growth. The phenotypes under different K^+ regimes differ from those found in mutants with impaired K^+ uptake but are reminiscent of defects in vacuolar K^+ remobilization. Four CIPK members are shown to functionally associate with CBL2 and CBL3 that mediate K^+ efflux from the vacuolar lumen to the cytosol. Among several tonoplast K^+-permeable channels potentially facilitating K^+ release from the vacuole, TPK-type K^+ channels appear to serve as one of the targets for the CBL–CIPK network, because several TPK members in arabidopsis can be directly activated by various vacuolar CBL–CIPK modules in a Ca^{2+}-dependent manner [99]. Plant vacuole serves as a large reservoir for K^+ nutrients and vacuolar K^+-pool is often utilized as a flexible store for cellular K^+ homeostasis [89,90]. Under K^+ deficiency, maintaining K^+ homeostasis operates not only at the cellular level but also at the whole-plant level. Effective translocation of K^+ from source to sink tissues requires coordinated action of both plasma membrane and tonoplast K^+ transporters in a number of different cell types. The dual CBL–CIPK pathways control the activity of both plasma membrane and vacuolar transporters, fulfilling a major strategy in K^+ mobilization and utilization especially in response to low-K environments [99].

CBLs and CIPKs Also Regulate Nonmembrane Events

Although CBL–CIPK systems are predominantly associated with regulation of membrane transport events, studies also reveal nonmembrane targets for CIPKs. For instance, CIPK11 can phosphorylate and regulate the function of ABI5 and FIT1, transcription factors involved in ABA signaling [91] and iron-starvation response [92], respectively. Such phosphorylation events happen in the nuclear compartment and are probably independent of CBL proteins. Besides, the tomato CIPK6 can complex with an ATP-binding protein in the cytoplasm to regulate the production of reactive oxygen species, a process where CBLs also seem to be absent [93]. Likewise, some CBLs are found to function with protein partners other than CIPKs. For example, CBL3 physically interacts with 5′-methylthioadenosine nucleosidases and inhibits their activity in a Ca^{2+}-dependent manner [94,95]. Taken together, despite the commonly accepted paradigm that CBLs and CIPKs form obligate partners, CIPKs may sometimes phosphorylate substrates in a CBL-independent manner and CBLs may also regulate targets other than CIPKs. These noncanonical regulations may not only add complexity to the CBL–CIPK signaling network but also provide means for crosstalks between different signaling pathways.

Concluding Remarks and Future Perspectives

During the past two decades, considerable progress has been made in the understanding of the physiological roles and regulatory mechanisms of the CBL–CIPK network, a critical and unique Ca^{2+}-decoding system in plant cells. Some guiding principles emerging from the previous studies include: (i) one CBL can partner with multiple CIPKs and one CIPK can interact with several CBLs. (ii) The functionality of CIPKs largely depends on their interacting CBLs that recruit the complexes either to the plasma membrane or to the tonoplast, where they often regulate multiple target proteins and biological processes. (iii) One particular CIPK can have multiple downstream targets and one target protein may be regulated by multiple CIPKs. The kinase–substrate pairing contributes, at least in part, to the specificity of signal-response coupling. (iv) One signal can trigger multiple spatially distinct CBL–CIPK pathways to enable multifaceted signaling outputs that are highly coordinated for an appropriate overall response. One such example is the activation of both plasma membrane and tonoplast CBL–CIPK pathways for low-K and high-Na responses. Future work will be directed to uncovering the mechanism behind the signaling coordination and functional interplay between different CBL–CIPK modules in the plasma membrane and the tonoplast. In illustrating these points, a handful of CBL–CIPK-mediated pathways have been elegantly reconstituted in heterologous systems, providing solid evidence that the target proteins, coupled

Outstanding Questions

What is the Ca^{2+} binding affinity for each CBL protein? Do CBL-type Ca^{2+} sensors respond to Ca^{2+} elevations or basal levels of Ca^{2+}? Do different CBLs perceive different ranges of Ca^{2+} concentration to initiate specific signaling pathways?

How does a CBL–CIPK complex assemble in response to a specific signal? Does formation of CBL–CIPK complex require Ca^{2+}? Are CBL–CIPK modules modified by other unidentified components or cofactors for full activation?

Do CBLs constantly associate with the cell membrane or is their localization dynamically regulated by stress conditions and thus calcium signals?

What is the mechanism that regulates CBL–CIPK complex stability and signaling strength? Is the abundance of CBLs and CIPKs regulated at transcriptional, translational, or post-translational level by external stimuli? Does phosphorylation of CBLs by CIPKs contribute to the stability of the complex and the activity towards their targets?

Are there more effective ways to identify new functional CBL–CIPK complexes in unknown physiological processes? How about new approaches to identifying an entire repertoire of targets for different CBL–CIPK modules at a higher resolution?

How does CBL–CIPK-mediated Ca^{2+} signaling network crosstalk with other signaling pathways in plants?

with the Ca^{2+} sensors and regulatory kinases in the signaling pathways, are necessary and sufficient for generating robust biological activities [45,65,67,88,96]. However, regarding mechanistic processes of CBL–CIPK function, a number of questions remain to be answered (see Outstanding Questions). It has become increasingly evident from studies on CBL–CIPK and other mechanisms that plant signal transduction is often fulfilled by networks crosslinking linear pathways [2,4,7,97,98]. Therefore, revealing the molecular links between different CBL–CIPK pathways will formulate a functional CBL–CIPK network. Studies will also be necessary to link the CBL–CIPK system with other signaling mechanisms to delineate an integrated overall signaling network in a cell. The next wave of research, while generating in-depth insights on the basic biological processes, will also transform the basic knowledge into biotechnological applications aiming at engineering stress-tolerant crops, potentially by manipulating the Ca^{2+} signaling network powered by CBL–CIPK modules.

Acknowledgments

The authors wish to thank the past and present members in the Luan laboratory for their contribution to establishing the CBL–CIPK network. We also apologize to colleagues whose work could not be cited due to space limit of the review. Work on the subject in the authors' laboratory is supported by the National Science Foundation (MCB-1714795) and Innovative Genomics Institute (IGI) of University of California.

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