Review Article

Decoding and Relay of Calcium Signals by CBL-CIPK Module in Plants

MUKESH KUMAR MEENA, ATISH SARDAR[#] and DEBASIS CHATTOPADHYAY*
National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110 067, India
#Present address: Jogesh Chandra Chaudhuri College, 30, Prince Anwar Shah Rd, Badam Talla, Tollygunge, Kolkata, West Bengal 700 033, India

Calcium is an essential macronutrient and a second messenger for signal transduction in plants. Apart from acting as a second messenger, calcium is also required for cytoskeleton, cell division, pollen tube growth and as a co-factor. Cytoplasmic calcium ion ($[Ca^{2+}]_{cyt}$) is maintained at a low level, however, is rapidly elevated using storages in organelles on perception of a stimulus. Ca^{2+}-binding proteins that sense the kinetics and magnitude of elevated $[Ca^{2+}]_{cyt}$ convert the chemical signals to biological signals and define specificity of responses. These proteins are broadly classified into sensor relays and sensor responders. Sensor relay proteins require another interacting protein to transmit the signal; whereas, the sensor responders combine within one protein the relay, amplification and response functions. A significant achievement has been made in the last three decades that identified and characterized various proteins instrumental in decoding Ca^{2+}-signals in plant cells. The latest addition in Ca^{2+}-signaling is Calcineurin B-like proteins (CBLs) and their interacting kinases (CIPKs). It is believed that flexibility of interactions between different CBL and CIPK proteins and their sub-cellular localizations are crucial in sensing and responding to specific signals. In this review, we have laid emphasis on the recent and emerging advancements in understanding of the CBL-CIPK module.

Keywords: Plant; Calcium; Signaling; CBL; CIPK

Introduction

Plants are autotrophs and, therefore, heterotrophic organisms depend directly or indirectly on plants for food. Plants not only regulate concentration of gases in the air but are also the major source of natural medicines for animals, including human beings. This realization has led to studies and careful observations on plant's growth, survival and productivity. Excess or deficit of necessary natural resources creates adverse conditions for plants, especially, the immobile land plants. Natural extremities in the form of various abiotic and biotic stress factors adversely affect growth and productivity of the plants (Zhu, 2016).

To sustain and by adapting adverse conditions, plants have evolved general and specific tolerance and resistance mechanisms. In most of the cases, external signals are detected by the cell membrane-localized receptors, causing transient changes in cellular concentrations of various second messenger chemicals, such as calcium ion (Ca^{2+}), cyclic AMP, inositol triphosphate (IP_3). Biological conversion of a signal mediated by a chemical messenger requires biological sensors, which further transmit the signal to generate specific response. Some of these second messengers also cross-talk regulating each other to convey signals for response. However, the evolutionarily conserved phenomenon of increased cytosolic Ca^{2+}($[Ca^{2+}]_{cyt}$) in response to diverse environmental cues renders it the most important and studied second messenger (Xu *et al*., 2000; Volotovski *et al*., 1998; Edel *et al*., 2017).

A low (nano molar) cytosolic calcium level is maintained to prevent precipitation of molecules having phosphate group. This is tightly regulated by a system of pumps, channels and transporters located on various cellular membranes. High (milli-molar) free Ca^{2+} is stored in endoplasmic reticulum (ER), vacuoles, organelles and cell walls. In cell walls, Ca^{2+} is a part of the cytoskeleton. Within vacuoles and organelles,

*Author for Correspondence: E-mail: debasis@nipgr.ac.in

Ca^{2+} is sequestered by various proteins and biomolecules. Several reports proposed that the cells' sensitivity to various stimuli is dependent on its ability to sequester and utilize Ca^{2+} from internal stores (Lemtiri-Chlieh *et al*., 2003; Catala *et al*., 2003; Qudeimat *et al*., 2008; Zhao *et al*., 2009).

Although Ca^{2+}-sensing mechanisms are evolutionarily conserved in plant kingdom, the mechanisms of maintaining the specificity in signal transmission and response generation are an important subject of study for long. The kinetics and magnitude of Ca^{2+} concentration, i.e. 'Calcium signature' varies with signals and possibly contributes to the specificity of responses (Knight *et al*., 1995). Further specificity and amplification of signals are determined by Ca^{2+}-sensor proteins, which decode, amplify and relay the information encoded by calcium signature. In plants, an extensive and diverse set of proteins that sense and bind Ca^{2+} translate and relay the signals (Luan *et al*., 2002; Batistic and Kudla, 2004; McCormack *et al*., 2005; Kim *et al*., 2009). Some of these proteins have enzymatic activities and thus, can amplify the signals. Some of these do not have enzymatic activity and, therefore, amplify and relay the signals by interacting with some other proteins having enzymatic or transcriptional activities. Plant Ca^{2+} sensor proteins have been classified into sensor relays and sensor responders (Sanders *et al*., 2002). Sensor responder proteins combine within one protein a sensing function and response activities for example, Calcium dependent Protein Kinases (CDPKs). Whereas, sensor relay proteins effectively bind Ca^{2+} ions, undergo Ca^{2+}-induced conformational changes, and interact with specific downstream proteins with amplification and relay functions, usually by phosphorylation and dephosphorylation (Sanders *et al*., 2002; Kushwaha *et al*., 2008). In this review, we will discuss the present information on one such sensor relay module namely, calcineurin B-like proteins and their interacting kinases, CBL-interacting protein kinases (CIPKs).

Calcineurin B-like Proteins (CBLs) and Its Interacting Protein Kinase (CIPKs)

The CBLs and CIPKs were first reported in a genetic screening of *salt overly sensitive (sos)* mutants in Arabidopsis and were identified as SOS2 and SOS3 proteins (Liu and Zhu, 1998; Kudla *et al*., 1999).

During salinity stress, SOS3 (CBL4) binds to Ca^{2+} and possibly undergoes conformational changes (Nagae *et al*., 2003). This Ca^{2+}-bound CBL4 interacts with SOS2 (CIPK24) to release it from auto-inhibition. The active SOS2 localizes to the plasma membrane phosphorylates and activates SOS1 (Na^+/H^+ antiporter), leading to Na^+ extrusion (Gong *et al*., 2004). Calcineurin B-like proteins (CBLs) are Ca^{2+} 'sensor relay' proteins that show similarity with calcineurin B and neuronal Ca^{2+}-sensor proteins like NCS1 and frequenin (Kudla *et al*., 1999). Arabidopsis genome encodes 10 CBL proteins. Similarly, all the higher land plant genomes encode multiple CBL genes (Meena *et al*., 2015a). All CBL proteins share a rather conserved core region consisting of four EF-hand Ca^{2+} binding domains that are separated by spacer regions, encompassing an absolutely conserved number of amino acids in all CBL proteins (Batistic and Kudla, 2004). Arabidopsis CBL proteins can be divided into two groups based on their N-terminal domain architecture; CBL proteins with a short N-terminal domain of 27-32 amino acids and CBL proteins with an extended N-terminus (41-43 amino acids). Arabidopsis CBL1, CBL4, CBL5, CBL8 and CBL9 belong to the first group, all possessing conserved motifs (MGXC/MGXXC) for lipid modification, except CBL8. N-myristoylation and/or S-acylation at their N-termini enable them to attach and localize to different cellular membranes (Saito *et al*., 2018). The second group of Arabidopsis CBLs comprises of proteins CBL2, CBL3, CBL6, CBL7 and CBL10 that does not contain any discernable lipid modification motif, however, harbor an extended N-terminal domain that is similar to the K^+-channel interacting proteins from the NCS group. Arabidopsis CBL10 possesses a very long N-terminal extension than others, forming a potential single transmembrane domain important for its localization (Kim *et al*., 2007).

Although CBLs bind Ca^{2+}, they do not have enzymatic activity and relay the signals by interacting specifically with a family of serine-threonine kinases known as CBL-interacting protein kinases (CIPKs). CIPKs are very similar in the kinase domain to sucrose non-fermenting1 (SNF1) protein kinases in yeast and AMP-dependent kinases (AMPKs) in animals but in addition, have retained unique C-terminal regulatory domains (Shi *et al*., 1999; Luan, 2009). They belong to the SNF1-related protein kinase

3 (SnRK3) family (Harper *et al*., 2004). CIPKs are composed of a ser/thr kinase domain at the N-termini, a NAF and/or FISL domain and a PPI motif (protein phosphatase type-2C interaction motif) adjacent to and potentially overlapping the NAF domain at the C-termini. It was proposed that the regulation of response-relay is propagated as CIPK/PP2C-mediated phosphorylation/dephosphorylation cycle of the substrates concerned (Lee *et al*., 2007). The NAF domain functions as both an autoinhibitory as well as CBL-binding domain. The auto-inhibition by the NAF domain is relieved upon CBL-binding through predominantly hydrophobic interaction (Albrecht *et al*., 2001; Sánchez-Barrena *et al*., 2005; Akaboshi *et al*., 2008; Luan, 2009). This triggers CIPK auto-phosphorylation and trans-phosphorylation capability of the activation loop within the kinase domain by an unidentified kinase that promotes full activation of the complex (Guo *et al*., 2001). Recent study proposed that phosphorylation of the corresponding CBLs by the interacting CIPK is also required for the whole complex to function (Hashimoto *et al*., 2012). The Arabidopsis genome encodes 26 CIPK proteins and 10 CBLs (Weinl and Kudla, 2009), hence, a CBL can interact with more than one CIPK. CBL-CIPK signaling network consists of two flexible combinable modules in which the CBL proteins provide the sensor relay function and the CIPKs contribute the response activity. The CBL moiety determines the cellular localization of the resulting CBL-CIPK complex and contributes to spatial specificity in target recognition. The CIPK moiety contributes to the final activity and substrate specificity of the complex (Batistic *et al*., 2008; Batistic *et al*., 2010). Preferential complex formation of individual CBLs with the defined subsets of CIPKs and *vice-versa* is proposed to determine specificity of response (Albrecht *et al*., 2001; Kolukisaoglu *et al*., 2004).

Evolutionary Analysis of the CBL-CIPK Signaling Network

The availability of a large number of sequenced plant genomes has allowed analysis of the complexity, conservation and evolution of CBL and CIPK signaling network. These efforts have identified 10 CBLs and 31 CIPKs from rice (*Oryza sativa*), 10 CBLs and 27 CIPKs from poplar (*Populus trichocarpa*), 8 CBLs and 43 CIPKs from maize, 6 CBLs and 32 CIPK-type kinases from *Sorghum bicolor*, 7 CBLs and 23 CIPKs from *Brassica napus*, 7 CBLs and 20 CIPKs from *Triticum aestivum*, 19 CBLs and 51 CIPKs from *Brassica rapa*, 8 CBLs and 20 CIPKs from *Vitis vinifera*, 5 CBLs and 15 CIPKs from *Solanum melongena*, 8 CBLs and 26 CIPKs from *Manihot esculenta*, 11 CBLs and 34 CIPKs from *Malusdo mestica*, and so on (Kolukisaoglu *et al*., 2004; Yu *et al*., 2007; Zhang *et al*., 2008; Weinl and Kudla, 2009; Piao *et al*., 2010; Chen *et al*., 2011; Lyzenga *et al*., 2013; Zhang *et al*., 2014;Sun *et al*., 2015; Li *et al*., 2016; Yin *et al*., 2017; Xi *et al*., 2017; Mo *et al*., 2018; Mohanta *et al*., 2015; Niu *et al*., 2018). In addition, the genome sequences for algae and non-vascular plants have also enabled us to address the evolutionary aspects of this signaling network. The presence of CBL-CIPK network in fern (*Selaginella moellendorffii*), in moss (*Physcomitrella patens*) and in some algal species such as *Chlorella* sp. and *Ostreococcus tauri* suggests that Ca^{2+}-signaling through CBL-CIPK pathway is conserved throughout the plant kingdom (Batistic *et al*., 2010). The genome of *Selaginella moellendorffii* encodes four CBL and five CIPK genes; moss *Physcomitrella patens* possesses four CBL and seven CIPK-type proteins and one CBL and one CIPK-type protein was identified in the green alga *Chlorella* sp. and free-living alga, *Ostreococcus tauri* (Batistic *et al*., 2010).

Comparison of gene and peptide sequences of CBL family suggests an evolution of gene structure within the plant family. The single Calcineurin B of yeast is of 175 amino acid (aa) and encoded by 604 bp gene divided in two exons. The protein possesses an N-myristoylation motif. The only CBL gene (Ot16g00900, *OtCBL*) of unicellular green algae *Ostreococcus* is of single exon and the protein is devoid of any conserved N-myristoylation motif. OtCBL and yeast Calcineurin B proteins share only about 30% similarity, suggesting OtCBL might not have derived from yeast Calcineurin B. The single cell algae *Chlorella* CBL gene (FJ901249.1) encodes the first complex gene of size longer than 1.5 kb with five exons and the protein possesses the motif for N-myristoylation. Four CBL genes in bryophyte *Physcomitrella patens* can be divided into two groups, two (Pp1s4_347V6.1, Pp1s107_63V6.1) are of small in size (~650 bp) with single exon and two (Pp1s28_66V6.2, Pp1s64_196V6.1) are larger (~2 kb) and multiexonic. Two of these *P. patens* CBLs

possess N-myristoylation motifs. Therefore, it appears that CBL proteins with and without N-myristoylation sites have different origins and both the types have converged in *P. patens* (Meena *et al*., 2015a).

The single Calcineurin B gene of yeast possesses an N-terminal MGCXXS/T motif to allow lipid modification of the protein by myristoylation and palmitoylation and help to localize in plasma membrane. Similarly, CBLs found in moss and fern also possess this motif (Batistic *et al*., 2008). These findings suggest that the ancient singular CBL protein represented a membrane-anchored signaling module and during evolution, structural diversification of the affected CBL proteins without the ancient lipid modification motif might have resulted in alternative subcellular localizations. This also establishes the early evolution of the CBL-CIPK system at the base of the lineage of plants, which suggests a role of the CBL-CIPK signaling system even in single-celled organisms and the evolution of plant lineage coincided with the evolution of complexity of the CBL-CIPK network. Moreover, the increase in complexity of the CBL-CIPK system with more members of CBLs and CIPKs present in land plants that have enabled the colonization of ecologically diverse and environmentally fluctuating habitats. Although, there are ten or more CBL-encoding genes in Arabidopsis or in other higher plants, sequence analyses suggested that higher plants have nine CBL genes, and the other CBL genes were originated due to genome duplication or local tandem repeats for example, CBL1 and CBL9 are paralogs in Arabidopsis (Meena *et al*., 2015a).

Localization of CBL and CIPK Proteins

Localization studies have revealed that the Arabidopsis CBL proteins harboring a short N-terminal domain (CBL1, CBL4, CBL5 and CBL9) were localized at the plasma membrane due to myristoylation and palmitoylation. CBL proteins harboring an extended N-terminal domain (CBL2, CBL3 and CBL6) are localized at the tonoplast (D'Angelo *et al*., 2006; Cheong *et al*., 2007; Batistic *et al*., 2008; Batistic *et al*., 2010; Schlücking *et al*., 2013; Saito *et al*., 2018; Zhang *et al*., 2017). CBL7 and CBL8, both underwent evolutionary changes of their original N-terminal sequences, are localized in the cytoplasm and in the nucleus and, CBL10, having unique N-terminal region, can be detected in endosomal compartments, as well

as at the tonoplast (Kim *et al*., 2007). Structure-function studies suggest presence of amino acid motif MGXXS/T is essential for myristoylation and cysteine residue at position 3 is essential for S-acylation at the N-termini of CBL1, CBL4, CBL5 and CBL9. These modifications enable them to attach to different cellular membranes (Weinl and Kudla, 2009). On the other hand, CIPKs do not possess recognizable localization signals and most CIPK-GFP fusion proteins exhibit a cytoplasmic and nucleoplasmic localization (D'Angelo *et al*., 2006; Batistic *et al*., 2010; Saito *et al*., 2018). However, CIPKs are targeted to different cellular compartments with the help of their respective interacting CBL proteins as revealed by CBL-CIPK interaction analyses using bimolecular fluorescence complementation (BiFC) in Arabidopsis (D'Angelo *et al*., 2006; Cheong *et al*., 2007; Batistic *et al*., 2008, Waadt *et al*., 2008). For example, AtCIPK1 is targeted to the plasma membrane by AtCBL1 or AtCBL9, whereas AtCBL2-AtCIPK1 complex is exclusively localized to the tonoplast (Cheong *et al*., 2007; Waadt *et al*., 2008; Batistic *et al*., 2008; Schlücking *et al*., 2013; Saito *et al*., 2018; Zhang *et al*., 2017). Although, interaction of AtCBL1 and AtCIPK1 occurred independently of the lipid modification of CBL1, however, AtCBL1 and -5 mutants that couldn't undergo myristoylation or palmitoylation couldn't mediate localization of AtCIPK1 and -11 in the plasma membrane, suggesting, localization of the CBL1-CIPK1/CBL5-CIPK11 complexes depend on the lipid modification status of AtCBL1/CBL5 (Batistic *et al*., 2008; Saito *et al*., 2018). The information on localization of most of the CBLs in other land plants is lacking.

Role of CBLs and CIPKs in Abiotic Stress Signaling Pathways

Potassium, plays important roles in many physiological and biochemical reactions in plant cells. Cytoplasmmaintains a high K^+/Na^+ ratio. Upon decrease in this ratio due to high cellular Na^+ concentration during dehydration and high salinity, cells use K^+ transporters to import K^+; and Na^+ transporters to export Na^+ out of cells. There are three types of K^+ channels in plants; the plant Shaker-like family, TPK proteins (tandem-pore K^+ channels), and plant Kir-like channels (Luan *et al*., 2009). Each K^+ channel has its specific function, although they are all related to K^+ transport. Arabidopsis K^+ transporter (AKT)

belongs to Shaker-like K^+ channel family. The AKT channels contain an ankyrin repeat domain. This domain is phosphorylated or dephosphorylated by certain CIPKs or Protein Phosphatase 2C group of phosphatases, respectively. A function of the CBL-CIPK system in regulating K^+ homeostasis was identified in a genetic screen of the low-K^+-sensitive (lks1) mutant as sensitive to low-K^+ conditions (Xu *et al*., 2006). Subsequently, the LKS1 gene was identified as CIPK23. In addition, absence of either CBL1 or CBL9 did not cause root growth arrest under low-K^+ conditions, however, absence of both the proteins led to root growth arrest similar to that of the mutant plants lacking AKT1 or CIPK23 (Xu *et al*., 2006; Cheong *et al*., 2007), indicating a co-operative/ redundant function of CBL1 and CBL9 in K^+-deficiency. CIPK23 was shown to be targeted to the plasma membrane with the help of CBL1 and CBL9 and activated by interaction with these two CBLs due to release from auto-inhibition (Xu *et al*., 2006; Cheong *et al*., 2007). These complexes then positively regulate the activity of AKT1 by phosphorylation. Among several potassium transporters, CIPK23 exclusively interacts with AKT1 (Lee *et al*., 2007). In addition to the regulation of K^+ uptake in roots, CBL1/CBL9-CIPK23 module was also implicated in stomatal regulation under dehydrating conditions (Cheong *et al*., 2007). The closest homolog of AtCIPK23, AtCIPK9 also interact with AtCBL3 to regulate K^+-homeostasis under low-K^+ stress in Arabidopsis (Liu *et al*., 2013). Similarly, AtCIPK6 and AtCIPK16 were shown to activate AtAKT1 (Luan 2009; Lee *et al*., 2007). However, the interaction affinities among these CBLs, CIPKs, and AKT1 were different. AtAKT1 is not only positively activated by phosphorylation, but is also regulated by dephosphorylation via certain PP2Cs (Lee *et al*., 2007). It was proposed that CBLs also interact with AIP1 (AKT1-interacting PP2C1), which significantly reduces AtAKT1 activity after $[Ca^{2+}]_{cyt}$ attains normal level. Similar negative regulation by PP2C happens following the K^+ high affinity channel (HAK) activation by AtCBL1/AtCBL9-AtCIPK23 complex (Lee *et al*., 2007; Lan *et al*., 2011). Lan *et al*., (2011) showed that PP2Cs can interact with CIPKs via both the protein phosphatase interacting motif (PPI) and the kinase domain. Various evidences suggested that CBL-CIPK-PP2C-AKT1 pathway was under complex regulation and play a vital role in K^+ transport.

It was shown that CIPK6 from various plants was able to improve salinity tolerance (Tripathi *et al*., 2009; Chen *et al*., 2013; He *et al*., 2013). Arabidopsis CIPK6 interacts with AtAKT2 at the ER membrane. With the help of CIPK6-interacting protein CBL4, the complex of AtAKT2-CIPK6 migrates to plasma membrane from the ER membrane in a kinase-independent manner to regulate AtAKT2 activity (Held *et al*., 2011). Subsequently, overexpression of several CIPKs from various plants were reported to impart tolerance to different abiotic stresses (Huang *et al*., 2011; Deng *et al*., 2013; Chen *et al*., 2014; Hu *et al*., 2016; Lv*et al*., 2014; Liu *et al*., 2015; Wang *et al*., 2018).

Nitrate is the major form of nitrogen and is essential for plant growth and development (Bouguyon *et al*., 2012). Four main transporters are known to be responsible for nitrate acquisition from the soil; CHL1 (AtNRT1.1), AtNRT1.2, AtNRT2.1, and AtNRT2.2. Experimental evidences suggest that AtCIPK23 negatively regulates nitrate response in low-nitrate conditions (Ho *et al*., 2009). AtCIPK8 was rapidly induced by nitrate in a differential transcriptomics study and is involved in regulating the primary nitrate response through CHL1. Arabidopsis mutant lacking functional CIPK8 indicated that AtCIPK8 is involved in long-term nitrate-regulated root growth and positively regulates the primary nitrate response (Hu *et al*., 2009). CIPK7, -12 and -14 were shown to interact with ubiquitin ligase ATL31 to phosphorylate it in Ca^{2+}dependentmanner to regulate Carbon/ Nitrogen-nutrient response (Yasuda *et al*., 2017).

High level of soil Magnesium (Mg^{2+}) is toxic to plants. Absence of both CBL2 and CBL3 proteins in the Arabidopsis mutant *cbl2cbl3* caused severe growth retardation of the plant in presence of high level of Mg^{2+} (Tang *et al*., 2015). Surprisingly, the Arabidopsis mutant retained lower Mg^{2+} level than the wild type plants. CIPK3, -9, -23 and -26 were reported to interact with CBL2 and CBL3 in the vacuole membrane and the quadruple CIPK mutant displayed similar toxicity to high Mg^{2+} concentration. This observation suggested that CBL2 and -3 interact with CIPK3, -9, -23 and -26 for vacuole sequestration of Mg^{2+} (Tang *et al*., 2015). Lesion in CIPK23 gene also renders the plant sensitive to iron deficiency (Tian *et al*., 2016). *Poplar euphratica* CBL1 was reported to interact with CIPK24, -25 and -26 to regulate Na^+/

K$^+$ homeostasis in the plant (Zhang *et al*., 2013).

The molecular basis of biological functions of CIPKs has been worked out by solving crystal structures of the proteins. The kinase activity of CIPKs relies on Ca^{2+}-dependent interaction of corresponding CBLs with the NAF domain of CIPKs to relieve from auto-inhibition, phosphorylation of activation loop and the interacting CBLs. The structure analysis of CIPK23 and -24 revealed differential abilities of CIPKs in displaying basal kinase activity. While CIPK23 remains intrinsically inactive and requires external stimuli for activation, CIPK24/SOS2 structure allows basal catalytic activity even in absence of stimulus (Chaves-Sanjuan *et al*., 2014).

Crosstalk Between the CBL–CIPK Network and the ABA Signaling Pathway

Abscisic acid plays roles in plant growth, development, seed germination, and responses to abiotic stresses. Reverse genetics analyses revealed crucial functions of distinct CBL proteins and CIPKs in ABA signaling. Expression of almost all the CBLs and CIPKs were induced by ABA and abiotic stresses like cold, high salt, wounding and drought. The AtCBL9-AtCIPK3 complex negatively regulates the ABA response during seed germination (Pandey *et al*., 2008). The AtCBL1-AtCIPK1complex was shown to be involved in ABA-dependent stress responses, while the AtCBL9-AtCIPK1 complex was involved in ABA-independent stress responses, inspite of CB1 and -9 being paralogous genes (D'Angelo *et al*., 2006). Interaction of AtCIPK26 with the RING-type E3 ligase and KEG protein led to degradation of KEG by the ubiquitin-proteasome system (Lyzenga *et al*., 2013). KEG was known to target ABA-insensitive 5 (ABI5) for degradation to negatively regulate ABA signaling (Stone *et al*., 2006; Liu and Stone 2010). AtCIPK26 interacted with ABI1, ABI2, and ABI5 in the ABA signaling pathway and played a positive role in ABA signaling in seed germination (Lyzenga *et al*., 2013). AtCIPK15, which interacts with ABI2, was propose to serve as a global negative regulator of ABA responses during seed germination, seedling growth, stomatal closure, and gene expression (Guo *et al*., 2002). High expression of C-terminal peptide of Calreticulin (CRT) improved salinity tolerance of Arabidopsis in CIPK6-dependent manner. CIPK6 expression was required in CRT-expressing lines for

higher expression of Dehydration Response Element Binding 1 (DREB1) protein, known as a component of ABA signaling pathway (Tsou *et al*., 2012).

However, negative regulation of ABA-signaling by CBL-CIPK network has also been reported. Early events of abiotic stress response involve ABA-mediated stomatal closure. CBL2 and -3 in association with CIPK9 and -17 and PAT-10, a protein S-acyl transferase10, are localized at the tonoplasts (vacuolar membranes) negatively regulate ABA signaling during stomatal movement by positively mediating vacuolar accumulation of potassium ions (Song *et al*., 2018). In contrast, CBL1 and -9 interaction with CIPK23 results in phosphorylation and positive regulation of slow anion channel associated 1 SLAC1, a guard cell anion channel activated by ABA. ABA-insensitive 1 (ABI1), a phosphatase, which is inactivated in response to ABA signaling inhibits SLAC1. ABI, a phosphatase that inactivates CIPK23, is inactivated in response to ABA and results in CBL1/9-CIPK23-mediated activation of SLAC1 (Maierhofer *et al*., 2014). Wheat CBL1 also interacts with wheat CIPK23 (TaCIPK23), and overexpression of TaCIPK23 resulted in enhanced ABA sensitivity and stomatal closure (Cui *et al*., 2018). Phosphorylation of Abscisic acid repressor 1 (ABR1) by CBL9-CIPK3 module has been proposed to regulate ABA-dependent physiological processes of adult plants in addition to seed germination (Sanyal *et al*., 2017).

Role of CBL–CIPK Network in Plant Immunity

Plants lack adaptive immunity and depend on innate immune response. Plants recognize pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) present at the apoplastic regions using pattern recognition receptors. This induces a cascade of immune response called as PAMP-triggered immunity (PTI), culminating in the generation of reactive oxygen species (ROS). Many pathogen secrets some proteins called effector proteins that go inside plant cells to inactivate PTI. In response, plants induce a second line of defense by expressing resistance (R) proteins to inactivate effector proteins. This is called as effector-triggered immunity (ETI) and involves higher surge of ROS production and callose deposition (Jones and Dangl, 2006).The involvement of CBL-CIPK network in plant immunity has been comparatively less investigated. Two rice CIPK genes*,*

OsCIPK14 and OsCIPK15 were first reported to show rapid induction by microbe associated molecular patterns (MAMPs). OsCIPK14 and -15 were shown to be involved in various MAMP-induced defense responses including defense-related gene expression, phytoalexin biosynthesis and hypersensitive cell death. MAMP-induced reactive oxygen species production and cell browning were also suppressed in OsCIPK14/15-RNAi transgenic cell lines (Kurusu *et al.*, 2010). Rice grassy stunt virus p5 protein was shown to interact with OsCIPK5 and -25 to presumably cause potassium deficiency in the infected plants (Xiong *et al.*, 2017). In *N. benthamiana*, NbCBL10 and NbCIPK6 are required for programmed cell death triggered by virus, oomycete, bacterial and nematode effectors. Recently, SlCBL10 and SlCIPK6, identified in a virus-induced gene silencing screen in *N. benthamiana*, were shown to regulate Pto/AvrPto-elicited programmed cell death (de la Torre *et al.*, 2013). SlCIPK6 interacts with SlCBL10 and its *in vitro* kinase activity is enhanced in the presence of SlCBL10 and Ca^{2+}, suggesting that tomato SlCBL10 and SlCIPK6 constitute a Ca^{2+}-regulated signaling module. Overexpression of SlCIPK6 in *N. benthamiana* leaves causes accumulation of reactive oxygen species (ROS) by activating respiratory burst homolog RbohB. The yeast two-hybrid and co-localization studies with fusion proteins reveal SlCBL10 and SlCIPK6 interact with NbRbohB at the plasma membrane. In addition, SlCBL10 and SlCIPK6 contribute to ROS generated during effector-triggered immunity in *N. benthamiana* and tomato, suggesting mechanistic link between Ca^{2+}, CBL, CIPK and ROS signaling in plant immunity (de la Torre *et al.*, 2013). This was supported by another study that reported that Arabidopsis CIPK6 acts as a negative regulator of MAMP-triggered and effector-triggered immunity. CIPK6 was shown to regulate plant immunity in a salicylic acid (SA)-dependent pathway. Absence of CIPK6 expression enhanced ROS production and MAPK activation, and suppressed bacterial growth in Arabidopsis (Sardar *et al.*, 2017). Cassava CIPK23 interacts with CBL1/9 of the same plant and it was shown by virus induced gene silencing that this module acts as a positive regulator of defense response against *Xanthomona saxonopodis* pv. *manihotis* (Yan *et al.*, 2018). Wheat CBL4 (TaCBL4) and CIPK5 (TaCIPK5) positively regulate resistance against fungus *Puccinia*

striiformis f. sp. *tritici* in a ROS dependent manner. Silencing of both these genes resulted in enhanced susceptibility to the fungus (Liu *et al.*, 2018).

Emerging Functions of CBL-CIPK Network

Recently, roles of CBLs/CIPKs in diverse pathways other than abiotic stresses are being investigated. Arabidopsis CIPK14 mutation resulted in enhanced glucose sensitivity of the plant (Yan *et al.*, 2014). CIPK15 regulates stress sensor SnRK1A (Snf1-related protein kinase 1) and links oxygen (O_2)-deficiency signals to the SnRK1-dependent sugar-sensing cascade to regulate sugar and energy production and enable rice growth under floodwater (Lee *et al.*, 2009). CIPK15 is a regulator of Amy3D and critical in directing the O_2-deficiency signal to the sugar signaling cascade in rice. Hexokinase plays a role in glucose dependent suppression of OsCIPK15 (Lee *et al.*, 2009; Yim*et al.*, 2012). The expression of CBL1 is induced by glucose in Arabidopsis. Loss-of-function mutant cbl1 exhibited hypersensitivity to glucose and expressions of several sugar-responsive genes were altered. CBL1 protein physically interacted with AKINb1, the regulatory b subunit of the SnRK1 complex which has a central role in sugar signaling (Li *et al.*, 2013). The Respiratory burst oxidase homologues (Rbohs), a plant NADPH oxidases play an indispensable role in a wide array of cellular and developmental processes. *Arabidopsis thaliana* RbohF(AtRbohF)-mediated production of reactive oxygen species (ROS) is involved in biotic and abiotic stress responses. CIPK26 specifically interacted with the N-terminal domain of Arabidopsis RBOHF in yeast two-hybrid system and with the full-length RBOHF protein in plant cells. In addition, CIPK26 was able to phosphorylate RBOHF *in vitro*. Co-expression of either CBL1 or CBL9 with CIPK26 strongly enhances ROS production by RBOHF in heterologous expression system (Drerup *et al.*, 2013; Kimura *et al.*, 2013). Tomato CIPK6 targets a universal stress protein SlRd2, associated with oxidative stress, for phosphorylation and this interaction resulted in reduced ROS generation (Gutierrez-Beltran *et al.*, 2017). Two studies reported cross-talks between auxin and Ca^{2+} signaling through CIPKs. Overexpression of CIPK6 of Arabidopsis and chickpea (*Cicer arietinum*) and CIPK25 of chickpea in tobacco (*N. tabaccum*) and Arabidopsis led to enhanced auxin transport and root biomass of the

transgenic lines (Tripathi *et al*., 2009; Meena *et al*., 2015b).Recently, Arabidopsis CIPK25 is shown to contribute in root meristem development by balancing auxin and cytokinin signaling (Meena *et al*., 2018). CBL-CIPK complexes play important role in plant reproductive development. CBL2/3-CIPK12 complex is required for polarized pollen tube growth (Steinhorst *et al*., 2015). CBL1 and CBL9 were shown to function in pollen tube germination and growth. Apple MdCBL5 was shown to control calcium signal and influence pollen tube growth (Gu *et al*., 2015). Overexpression of wheat CIPK2 (TaCIPK2) in tobacco decreased stomatal aperture and increased drought tolerance (Wang *et al*., 2016). As more advancement are being made in this field more information on involvement of CBL-CIPK network in various physiological process in plants such as, glucose signaling, stomatal movement, plant immunity, nutrient deficiency are being unraveled.

More evidences are being reported, where a member of CBL family interacts with other proteins in addition to a CIPK family protein. Arabidopsis CBL3 was shown to inhibit the 5'-Methyl-thioadenosine nucleosidase in a calcium-dependent manner (Oh *et al*., 2008, Ok *et al*., 2015). CBL7 and -10 were shown to interact a member of TOC (Translocon of the outer membrane of the chloroplast) complex and inhibit GTPase activity of the complex (Cho *et al*., 2016).

Future Perspectives

CBL-CIPK signaling network is a lately discovered Ca^{2+}-signaling component (Fig. 1). Uniqueness of this pathway is its flexibility. There are multiple CBLs and CIPKs encoded by various plant genomes, with CIPKs more in number than the CBLs. From their numbers, it can be understood that a CBL can interact with multiple CIPKs. Additionally, multiple reports
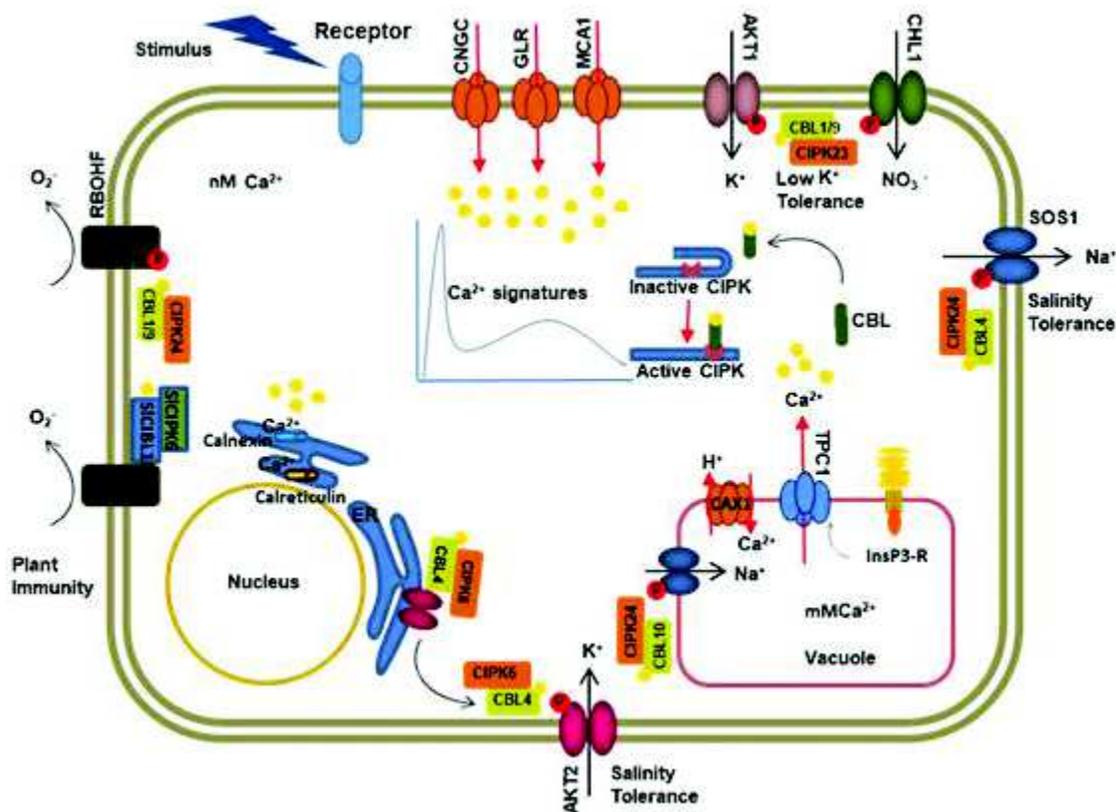
Fig. 1: A schematic diagram showing mode of action of CBL-CIPK complex. A model showing change in $[Ca^{2+}]_{cyt}$ concentration through inflow from external and internal reservoirs upon receiving a signal. Cyclic nucleotides and Inositol-3-phosphate-mediated activation of channels and calcium-binding proteins reservoirs like Calnexin and Calreticulin play important role in supply of Ca^{2+}. Calcium signatures are sensed by calcium-biding sensor protein CBL (also other calcium-sensors). Calcium-bound CBLs interact and activate the relay proteins CIPKs. CBL-CIPK complexes interact, phosphorylate and activate various substrates to induce response

described above have shown that a single CIPK also can interact with multiple CBLs, depending on context. Different sub-cellular localizations and the flexibility of interactions have made this protein family an appropriate signaling component that can sense and relay the numerous temporally and spatially defined changes in Ca^{2+} concentrations in distinct organelle resulting from various external and internal stimuli. Another layer of flexibility could be resulting from differential occupancy of Ca^{2+}-binding sites in CBL (Weinl and Kudla, 2009). It is believed that different permutation and combinations of CBL-CIPK complexes have the potential to sense different calcium signatures. The lacunae in this field and as a whole in plant science is most of the information gathered so far are on Arabidopsis. Even in Arabidopsis, how different signals or different Calcium-signatures are perceived by different CBLs and how the specificity of targeting a particular CIPK

for response is determined is not clear. Information on targets/substrates of most of the CIPKs is not available to have a clear understanding of the signaling pathways. Several other plants with larger genome sizes such as, Poplar, soybean encode more CBLs and CIPKs. Studies with those proteins would reveal novel and applicable information. As this pathway was discovered in the course of studying salt-induced signaling, most of the research with these molecules was concentrated on abiotic stress responses. Biological roles of these molecules in reproductive development, biotic stress, glucose and other responses are the emerging areas in this field.

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