Review Article

Emergence of a novel calcium signaling pathway in plants: CBL-CIPK signaling network

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ABSTRACT

In the environment, plants are exposed to plethora of adverse stimuli such as abiotic and biotic stresses. Abiotic stresses including dehydration, salinity and low temperature poses a major threat for crop productivity. Plant responds to these stresses by activating a number of signaling pathways which enable them to defend or adjust against these stresses. To understand the mechanisms by which plants perceive environmental signals and transmit these signals to cellular machinery to activate adaptive responses is of fundamental importance to biology. Calcium plays a pivotal role in plant responses to a number of stimuli including pathogens, abiotic stresses, and hormones. However, the molecular mechanisms underlying calcium functions are poorly understood. It is hypothesized that calcium serves as second messenger and, in many cases, requires intracellular protein sensors to transduce the signal further downstream in the pathways. Recently a novel calcium signaling pathway which consist of calcineurin B-like protein (CBL) calcium sensor and CBL-interacting protein kinase (CIPK) network as a newly emerging signaling system mediating a complex array of environmental stimuli. This review focuses on the overview of functional aspects of CBL and CIPK in plants. In addition, an attempt has also been made to categorize the functions of this CBL-CIPK pair in major signaling pathways in plants. **[Physiol. Mol. Biol. Plants 2008; 14(1&2) : 51-68]** *E-mail : giridhar98@gmail.com*

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Plants are constantly challenged with several abiotic and biotic stresses in the environment and they have developed an intricate machinery to respond and adapt against all these factors. Unlike animals, plants can not move away from the stress and hence activate a number of defense responses through complex signaling network. A large number of stimuli are processed by these signaling networks which include all these biotic and abiotic stresses at a given time. Several signaling mechanisms which consist of perception, transmission and processing of a typical signal operates to generate specificity and cross-talk in networking simultaneously. A large number of second messengers have been implicated in plants such as calcium, NO, ROS (reactive oxygen species, phosphatidic acid, cyclic nucleotides (McCarty and Chory, 2000). Several second messengers are activated by different stresses and often same second messengers are shared by the different stresses and signaling pathways at the same time. This orchestration of signaling components generates a spatial and temporal complex array of simultaneously operating signaling system (Xiong *et al*, 2002; Batistic and Kudla, 2004). Moreover, the combination of second messengers triggers several downstream signaling processes including diverse array of phosphorylation cascades, protein translocation and ultimately gene expression. Despite the fact that plants are sessile organism, they have developed a web of network which involves channeling and integration of signaling pathways and hence provide adaptive and defense responses against adverse environmental stimuli to plants. It is becoming clearer now that signaling pathways in plants are not linear or definite. . Rather, there exist complex networking which consist of specific and cross-talking web or networks which occur simultaneously at a given time point. Like animals, a new concept of "scale-free" signaling network or web is being recognized as the information processing in plants (Hetherington and Woodward, 2003; Hetherington and Brownlee, 2004). Scale-free networks are composed of several connecting points called nodes and several highly interconnecting nodes, called as hubs which are essential components of scale-free signaling. These kind

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of networks are very efficient and flexible which are suited to differentiate and process multiple signals at the same time (Barabasi and Oltvai, 2004). Highly organized and dynamic nature of these scale-free webs enables plants to execute necessary and appropriate strategies to adjust or cope under the environmental stress.

Calcium serves as a central hub in this scale-free network of signal transduction. Several extra cellular signals such as light, hormones, biotic and abiotic stimuli elicit change in calcium levels in the cell (Sanders *et al.*, 1999; Rudd and Franklin-Tong, 2001; Harper 2001; Knight and Knight 2001). It is an important question that how several different stimuli or stress signal leads to changes in the calcium concentration and how specificity in signaling network is being maintained. Based on several studies in animal and plants, it is quite clear now that calcium signal is not only represented as changes in intracellular concentration but spatial and temporal information is also encoded simultaneously (McAinsh and Hetherington, 1998; Allen *et al.*, 2001; Hetherington and Brownlee, 2004). Therefore, a combination of changes in all the parameters by particular stimuli is termed as "calcium signature" and leads to generate specificity in calcium mediated signaling pathway (McAinsh and Hetherington, 1998; Allen *et al.*, 2001; Hetherington and Brownlee, 2004). It is interesting to make a note that in plants, the calcium signature or calcium transient were mainly studied in the signaling pathway in guard cell and in systems where calcium act a second messenger. It is being conceived that calcium is primarily acting as a switch triggering downstream responses (Scarse-Field and Knight, 2003). A very good example of such a signaling network is abiotic stress signaling where multiple abiotic stresses could leads to generation of similar calcium signature but yet trigger a very specific signaling and adaptation response. An additional layer of regulation in calcium mediated signaling is being achieved by calcium-binding proteins which act as sensors of calcium (Snedden and Fromm, 1998; Luan *et al.*, 2002; Sanders *et al.*, 2002; Reddy, 2001; Batistic and Kudla, 2004). Calcium sensors in plants belong to two classes, one as sensor responder and other as sensor relay (Sander *et al.*, 2002). The sensor responder proteins bind calcium and a change in confirmation takes place which modulates their own activity by intra-molecular interactions (Harmon *et al.*, 2001). Calcium dependent protein kinase (CDPK) is the best characterized protein family which have calmodulin like calcium binding domain and Ser/Thr protein kinase domain in a single protein. Upon calcium binding they are directly activated and transduce the signal via phosphorylation cascades and regulate the gene expression (Sander *et al.*, 1999; Hrabak *et al.*, 2001; Cheng *et al.*, 2002; Luan *et al.*, 2002; Harper *et al.*, 2004). In contrary, sensor relay protein have only calcium binding motifs, which upon binding calcium, undergo change in confirmation and activate their interacting target proteins to transmit the calcium signal (Luan *et al.*, 2002). Calmodulin (CaM) and calmodulin-like proteins have been best characterized as calcium sensor relay proteins which do not have enzymatic activity and hence activates or deactivates their interacting proteins (Luan *et al.*, 2002). A new family of calcium sensors from *Arabidopsis* have been identified which are similar to calcineurin B-subunit like and neuronal calcium sensor proteins from animals (Liu and Zhu, 1998; Kudla *et al.*, 1999, Luan *et al.*, 2002). These plant calcium sensors were referred as calcineurin B-like (CBL) proteins (Kudla *et al.*, 1999). Like calmodulin, CBL proteins are calcium sensor relay which upon binding calcium, undergo changes in confirmation and activates their target proteins. CBL proteins interact with a novel SNF1-like protein kinase family called CBL-interacting protein kinase (CIPK) (Shi *et al.*, 1999; Halfter *et al.*, 2001). In plants CBL-CIPK pathway is emerging as a major calcium signaling network. Functional analysis of several CBL and CIPK family members in *Arabidopsis*, rice and other plant species led to implicate this pathway in ABA and abiotic stress response (Luan *et al.*, 2002; Batistic and Kudla, 2004; Gong *et al.*, 2004) and K-nutrition (Li *et al.*, 2006; Xu *et al.*, 2006; Cheong *et al.*, 2007; Pandey *et al.*, 2007). In this review, I am emphasizing on the physiological role of CBL-CIPK pair in multiple signaling pathway such as ABA, osmotic, dehydration, cold, salinity and regulation of potassium (K) uptake and nutrition in plants. An attempt has been made to categorize the role of these CBL-CIPK components in different signaling pathways.

Calcineurin B-like protein (CBL) identified as novel calcium sensor in plants

CBLs were originally identified in genetic (Liu and Zhu, 1998) and yeast two hybrid screen (Kudla *et al.*, 1999) which showed high similarity with calcineurin B-subunit and neuronal calcium sensors (NCS) from animal and therefore designated as calcineurin B-like proteins (Kudla *et al.*, 1999). Ten members of CBLs were identified in both *Arabidopsis* (AtCBL1-10) and in rice (OsCBL1-10) by genome annotation which shows high degree of similarity in both the species, 33% -93% in Arabidopsis and 44%-94% in rice (Kolukisaoglu *et al.*, 2004). Similar to CaM (calmodulin), NCS, calcineurin B subunit (CNB), CBL are characterized by the common helix-loop-helix structural motif (the EF hands) that act as calcium

binding motif in all these proteins (Luan *et al.*, 2002; Kolukisaoglu *et al.*, 2004). The overall three dimensional structure of CBL proteins is similar to that of CNB and NCS where these polypeptides are folded into two globular domains, which are separated by a short linker region (Nagae *et al.*, 2003). The calcium binding EF-hand domain is loop of 12 amino acids flanked by two alpha-helices, where amino acids at positions 1(X), 3(Y), 5(Z), 7(-Y), 9(-X) and 12(-Z) are critical for binding of calcium ion. The Asp at position 1, Gly at position 6 and Glu at position 12 are the most conserved amino acid in the loop. The Gly at position 6 is required to maintain the structure of the loop, which cannot accommodate any other amino acid at this site. Each pair of EF-hands is considered the basic functional unit. Pairing of EF-hands is thought to stabilize the protein and increase its affinity toward calcium (Seamon and Kreetsinger, 1983). Although each globular domain binds calcium and undergoes conformational changes independently but the two domains act in concert to bind the target proteins. Upon increase of calcium to sub micro-molar or low micro-molar levels, all the CaM/NCS/CNB/CBL are activated and cooperative binding is required for this "on/off" mechanism to function efficiently. All CBLs harbor four EF-hands with varying degrees of conservation as compared to the canonical EF-hand sequences. Only three functional EF-hands exist in CBL since in the first EF-hand in all CBLs, there is a critical amino acid substitution which render this EF-hand non-functional (in terms of calcium binding) very similar to NCS (Burgoyne and Weiss, 2001). Instead in NCS, this domain mediates the interaction with its target protein; a similar situation is being predicted for CBLs which need more experimental work. The number as well spacing of EF hands is invariant in all CBL proteins. This suggests a conserved three-dimensional structure of all the CBLs. Recently, crystal structure of one of the CBL, CBL2 from *Arabidopsis* has been determined (Nagae *et al.*, 2003). This protein forms a compact alpha-helical structure with two pairs of EF-hand motifs. This structure is similar to CNB and NCS in overall folding topology. It is important to make a note that pattern of CBL2 folding differs significantly in local confirmation. Each CBL2 molecule binds with two calcium ions. The calcium ions are coordinated in the first and fourth EF-hand domains, and the second and the third EF-hand motifs are maintained in an open form by internal hydrogen bonding without coordination with calcium ions (Nagae *et al.*, 2003). The other CBL members also share the non-canonical EF-hand structure of CBL2 and the conservation of EF-hand structure might indicate similar calcium-binding properties (Kolukisaoglu *et al.*, 2004). In contrast, there are several CBL proteins with different EF-hand structure which could make them different from other family members with respect to binding affinity to calcium. In the case of CBL6, there is mutation in the 1st EF-hand and hence it cannot bind calcium at this EF-hand as observed for CBL2 (Nagae *et al.*, 2003). At the same time, in the case of CBL1 and CBL9, three EF-hands might bind calcium such as EF-1, EF-3 and EF-4. The conventional EF-4 hand of CBL1 and CBL9 would allow binding of calcium at EF-3 hand unlike in CBL2 (Nagae *et al.*, 2003; Kolukisaoglu *et al.*, 2003). The CBL8 and CBL10 also harbor conventional amino acid composition in EF-hand 4. These CBLs might have higher affinity for calcium than CBL2 and CBL7 where there is a mutation in tyrosine residue. The differential calcium binding of these CBLs may also attribute to their specificity in the different signaling networks.

Several CBLs such as CBL1, CBL4 and CBL9 have been associated with the membrane fractions (Ishitani *et al.*, 2000; Luan *et al.*, 2002). Several signaling processes are initiated across the membrane by calcium fluxes (Rudd and Franklin-Tong, 2001). Like NCS, one of the important features of CBL protein is co-translational modification by fatty acid myristate. Myristoylation occurs at glycine residue next to methionine at the N-terminal target sequence MGXXXS/T, and is removed post-translationally by amino-peptidases (Farazi *et al.*, 2001). Myristoylation is shown to have an important functional role either in protein-protein interaction or in protein-membrane attachment. In fact, myristoylated proteins are weakly associated with the membrane. To enhance the affinity to the lipid environment of the membrane, a palmitoyl group is frequently added post-translationally to the cysteine residue adjacent to the myristoylated glycine, which thereby allows a stable attachment of the protein to the plasma membrane (Bijlmakers and Marsh, 2003).

The N-terminal sequences of CBL1, CBL4, CBL5 and CBL9 harbor conserved myristoylation motifs, and *in vitro* myristoylation assays confirmed myristoylation of these proteins (Ishitani *et al.*, 2000; Kolukisaoglu *et al.*, 2004). In case of CBL4/SOS3, myristoylation has been shown to be important and necessary for salt tolerance (Ishitani *et al.*, 2000) and CBL4/SOS3 recruit CIPK24/SOS2 to the plasma membrane in yeast (Quintero *et al.*, 2002). In recent studies, CBL1 and CBL9 are also found to co-localize with their target protein CIPK23 in the plasma membrane (Xu *et al.*, 2006; Li *et al.*, 2006; Cheong *et al.*, 2007). It is quite possible that these myristoylation and or palmitoylation help these CBL and CIPK pairs to attach to membrane where calcium fluxes occur and/or where target proteins are localized. (Ishitani *et al.*, 2000).

Target of CBLs are protein kinases known as CBL-interacting protein kinase (CIPK)

Unlike CaMs, which interact with diverse array of target proteins, such as kinases, ion transporters, enzymes, transcription factors (Yang and Pooviah, 2003), CBLs interact with a single family of protein kinases known as CBL-interacting protein kinases (CIPKs; Shi *et al.*, 1999; Halfter *et al.*, 2000; Kim *et al.*, 2000; Luan *et al.*, 2002; Kolukisaoglu *et al.*, 2004; Batistic and Kudla, 2004). These kinases show similarity to sucrose non-fermenting (SNF) protein kinases from yeast and AMPK (AMP-activated protein kinases) from animals. CIPKs are the novel plant specific kinases which shows similarity to SnRK3 subgroup of plant kinases (Hrabak *et al.*, 2003) but their mode of action and regulation are quite different from this group of kinases (Luan *et al.*, 2002; Kolukisaoglu *et al*., 2004; Gong *et al.*, 2004).

In *Arabidopsis,* 25 members in the CIPK family were identified, whereas in rice, a complement of 30 CIPKs are reported (Luan *et al.*, 2002; Kolukisaoglu *et al.*, 2004). CIPK proteins have a N-terminal SNF1-type catalytic kinase domain with typical 11-domain structure and high sequence similarity between these proteins (51-90% identical amino acid) and a highly variable C-terminal regulatory region which is absent in SNF1-related kinases (24%-58% identical at amino acid level; Kolukisaoglu *et al.*, 2004). In all the CIPKs, a conserved activation loop which harbors a threonine residue is located between DFG and APA sequences (conserved subdomain VIII; Hrabak *et al.*, 2003). This activation loop can become the target of phosphorylation by another kinase. Conversion of this threonine to aspartate by mutagenesis experiment showed that binding of CBL to CIPK is not required for the activation of kinase activity (Guo *et al,.* 2001; Gong *et al.*, 2002; Gong *et al.*, 2004). Within this loop another serine residue can also become the target of phosphorylation by other kinases. Hence a possibility of involvement of another family of kinase such as CDPK or MAP (mitogen activated protein) kinases or any unknown kinase in the regulation and fine-tuning is speculated. In plants, trans-phosphorylation and inter-phosphorylation regulates the signaling pathways of CDPKs and MAP kinases. A similar mechanism might also be inherited to some of the family members of CIPKs as well. In the C-terminal regulatory domain of CIPKs, a highly conserved motif termed as NAF/FISL domain which has a consensus sequence XL (or M, I, F,Y) N (or T)**AF**D (or E, Q) I (or L, F, M)**I**S (or A, T, G,L, I) L (or M, F, Y, S, T, G)**S**XG (or F, D, E, S) F (or L,S) D (or N, S, G)**L**S (or A, E, G) G (or N, S, T, P) L (or F)**F**E (or D, G, A) (where X is any amino acid residue and residues in bold are absolutely conserved (Kim *et al.*, 2000; Albrecht *et al.*, 2001; Guo *et al.*, 2001). NAF/FISL domain is responsible for interacting with CBLs, deletion of this domain abolish interaction with CBLs suggesting its absolute requirement. It has been noted that NAF/FISL domain itself is not sufficient to generate the observed specific interaction between CBLs and CIPKs, other regions adjacent to this domain in the CIPKs or nearby region might also be responsible for a higher degree of interaction preferences between them (Kim *et al.*, 2000; Albrecht *et al.*, 2001; Guo *et al.*, 2001). Preferential complex formation between CBLs and CIPKs explain the spatial and temporal specificity generated in calcium signaling cascade (Kim *et al.*, 2000; Albrecht *et al.*, 2001; Guo *et al.*, 2001). Deletion of NAF/FISL motif in the CIPKs makes them constitutively active and CIPKs activation does not require interaction with CBL, but further deletion of the junction region between the NAF/FISL motif and the catalytic kinase domain abolished activation of the protein (Guo *et al.*, 2001). Mutagenesis analysis of CIPK24/SOS2 protein has shown that NAF/FISL motif in the regulatory region of CIPKs protein auto-inhibits the substrate phosphorylation by these kinases. Deletion of this NAF/FISL domain along with mutation of threonin to aspartate in the activation loop leads to super-active kinases (Guo *et al.*, 2001; Gong *et al.*, 2002; Qui *et al.*, 2002; Gong *et al.*, 2004). However it is unclear that activation of CIPKs by Ca^{2+}-CBL and phosphorylation of threonine residue in the activation loop take place *in vivo* or not and whether occurs individually, simultaneously or synergistically. C-terminal of CIPK also has another novel protein phosphatase domain known as PPI-domain which is responsible for interaction with ABI1 and ABI2 (ABA-insensitive; Guo *et al.*, 2002; Ohta *et al.*, 2003). This PPI domain in all CIPKS is highly conserved which consist of 37 amino acid residues and is necessary and sufficient for interaction with ABI1/2. This PPI domain also exists in DNA damage checkpoint kinase, Chk1, in various organisms including humans (Ohta *et al.*, 2003). Mutation in the PPI domain abolishes the interaction between CIPKs and ABI1/2. In ABI2, a protein kinase -interaction (PKI) domain exists in ABI protein phosphatase (Ohta *et al.*, 2003). The mutation in *abi2-1* mutant in PKI domain abolish the interaction with CIPK24/SOS2 and hence causes increased tolerance to salt and ABA insensitivity in plants (Ohta *et al.*, 2003). When interaction preference of ABI1 and ABI2 towards CIPKs was compared, it was found that CIPK20/PKS18 interacted preferentially with ABI1 whereas other kinases like CIPK8/PKS11 and CIPK14/PKS24 interacted mainly with ABI2 (Ohta *et al.*, 2003). This interaction of PPI -domain in CIPKs and PKI-domain in ABI1/2 needs to be investigated in detail for all

the CIPK protein family members. Here, the three major signaling pathways where CBL-CIPK pair is involved to play a key regulatory role are described.

Involvement in ABA signaling, cold, osmotic and dehydration responses.

Although cold, salinity, osmotic and drought stresses are clearly different from each other in their physical nature and each elicits specific plant responses. They also activate some common reactions in plants. The most widely studied common response is the induction of some plant genes by all four stresses (Shinozaki and Yamaguchi-Shinozaki, 1997) Because of this and other overlaps in their responses, these stresses are often considered together in molecular studies. Plant responses to these stresses involve nearly every aspect of plant physiology and metabolism. Consequently, there exists a complex signaling network underlying plant adaptation to these adverse environmental conditions. Most of these abiotic stresses lead to increased production of ABA that is either specific or common to responses towards these stresses. Hence, these processes might be going through abscisic acid (ABA)-dependent and -independent gene regulation (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein *et al.*, 2002; Xiong *et al.*, 2002).

The plant hormone - ABA regulates many aspects of plant development and physiology, including seed maturation and dormancy as well as responses to environmental stress conditions such as drought, salinity and low temperature *(*Fedoroff*,* 2002*;* Finkelstein *et al.*, 2002; Himmelbach *et al*., 2003). Inhibition of seed germination provides a useful bioassay for both forward and reverse genetic analyses that has revealed a number of molecular components in plant ABA signal transduction pathways (Giraudat, 1995). These components range from early signaling intermediates such as G proteins and protein kinases/phosphatases, to late stage transcription factors and RNA metabolic proteins (reviewed by Fedoroff, 2002; Finkelstein *et al.*, 2002). While some components appear to be regulators of multiple ABA responses, few are required for all responses, suggesting that ABA responses in different cell types or at various developmental stages may differ to certain extent.

In adult plants, ABA serves as a critical chemical messenger for stress responses. Under abiotic stress conditions, especially drought and high salt stress, ABA biosynthesis is up-regulated (Xiong and Zhu, 2003). Several studies have shown that ABA accumulation is required for the development of stress tolerance in plants. As a rapid response, stomatal closure is induced by ABA accumulation. This is followed by a slower response to ABA manifested in the induction of stress genes. Consequently, there is a large overlap of ABA- and drought- and salt-responsive gene expression in the vegetative tissues of adult plants (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein *et al.,* 2002; Xiong *et al.*, 2002). Furthermore, characterization of stress gene promoters indicates that many gene promoters contain *cis*-acting elements that are specifically responsible for abiotic stress and ABA. Due to their ABA responsive promoter elements, these genes can be activated by stress-induced increase in ABA levels, as part of an ABA biosynthesis-dependent regulatory pathway. In addition, the stress-responsive elements are responsible for stress responsive expression without the need of ABA biosynthesis, resulting in an ABA-independent regulatory pathway (Shinozaki *et al.*, 1998). However, some apparent ABA-independent pathways may require ABA for full response due to crosstalk between ABA and stress response pathways (Knight and Knight, 2001; Xiong *et al.*, 2002; Kim *et al.*, 2003). It has been proposed that specific crosstalk "nodes" may up- or down-regulate the signaling strength and interaction between the different ABA-dependent and ABA-independent stress response pathways (Knight and Knight, 2001; Kim *et al.*, 2003). Calcium seems to be a prime candidate for such crosstalk nodes as it has been shown to serve as a second messenger for both ABA and stress responses.

The expression pattern of CBLs and CIPKs family member differs in response to abiotic stresses and phytohormone ABA. The functional specificity of CBLs and CIPKs is not only determined by their interaction, biochemical and sub-cellular localization but to a great extent by their spatial, temporal, and environmental responsive expression patterns. All CBL and CIPK family members have not shown abiotic and ABA responsive expression. A few of them such as CBL1 and CBL9 are highly inducible by multiple abiotic stresses such cold, drought, osmotic, salinity and phytohormone ABA (Kudla *et al.*, 1999; Cheong *et al.*, 2003; Pandey *et al.*, 2004), in contrary, CBL2 is responsive to light but not for abiotic stress or phytohormone ABA (Nozawa *et al.*, 2001). In CIPK gene family, CIPK3 transcript is strongly inducible by cold, drought, salinity, wounding and ABA (Kim *et al.*, 2003). The other CIPKs which are responsive to abiotic stress are CIPK9 (Pandey *et al.*, 2007); CIPK11 /PKS5, responsive to ABA, drought and salinity (Fuglsang *et al.*, 2007); CIPK21, weakly inducible by ABA and abiotic stresses (Pandey *et al.*, unpublished). Moreover some of these CBLs and CIPKs are also

regulated spatially and temporally at different developmental stages of plants (Luan *et al.*, 2002; Batistic and Kudla, 2004: Gong et al., 2004). Recently function of two CBLs, CBL1 and CBL9 in abiotic stresses and ABA signaling pathways have been studied in detail. Though CBL1 and CBL9 are very similar (90% identical at amino sequence) but they regulate different signaling pathways (Cheong *et al.*, 2003; Pandey *et al.*, 2004).

Mutant based approaches have begun to shed a great deal of light on the individual members of CBL and CIPK gene family. Over-expression of CBL1 protein in transgenic Arabidopsis plants altered the stress response pathways. Although drought-induced gene expression was enhanced, gene induction by cold was inhibited. In addition, CBL1-overexpressing plants showed enhanced tolerance to salt and drought but reduced tolerance to freezing (Cheong *et al.*, 2003). By contrast, *cbl1* null mutant plants showed enhanced cold induction and reduced induction of stress genes under drought. The mutant plants displayed less tolerance to salt and drought but enhanced tolerance to freezing (Albrecht *et al.*, 2003; Cheong *et al.*, 2003). Based on over-expression and genetic analysis of mutants, it has been proposed that CBL1 functions as a positive regulator of salt and drought responses and a negative regulator of cold responses in plants. Furthermore, these findings clearly suggest that CBL1 serves as an upstream regulator of stress gene expression and as a rate- limiting factor in multiple abiotic stress pathways. Also it is apparent that CBL1 appears to represent an integrative node in plant responses to abiotic stimuli. Interestingly, despite the observed effects of *cbl1* mutants on several abiotic stress responses, these mutants did not display any significant responsiveness to ABA and this suggest that CBL1 function upstream or independent of any modulation of stress signaling by ABA (Albrecht *et al.*, 2003; Cheong *et al.*, 2003; Batistic and Kudla, 2004).

The function of CBL9 was ascertained by reverse genetic approach where CBL9 gene function was disrupted in *Arabidopsis thaliana* plants. In these plants response to ABA was drastically altered (Pandey *et al.,* 2004). The mutant plants became hypersensitive to ABA in the early developmental stages, including seed germination and post-germination seedling growth. In addition, seed germination in the mutant also showed increased sensitivity to inhibition by osmotic stress conditions produced by high concentration of salt and mannitol. Further analyses indicated that increased stress sensitivity in the mutant may be a result of both ABA hypersensitivity and increased accumulation of ABA under the stress conditions. Also, the expression of ABA and drought-induced genes appears to be hyper-induced in *cbl9* mutant. The *cbl9* mutant plants showed enhanced expression of genes involved in ABA signaling, such as ABA-INSENSITIVE 4 and 5. Overall, these finding suggest that CBL9 concurrently functions as a negative regulator of calcium-induced ABA signaling and ABA biosynthesis pathways.

CBL1 and CBL9 interact with a subset of CIPKs (Albrecht *et al.*, 2001; Kolukisaoglu *et al.*, 2004; Y.H. Cheong, G.K. Pandey and S. Luan, unpublished). The functional analysis of CIPK1, one of the kinase strongly interacting with both CBL1 and CBL9 in the yeast two-hybrid system (Albrecht *et al.*, 2001; Kolukisaoglu *et al.*, 2004) reveal the integration and channeling of the diverse stress responses (D'Angelo *et al.*, 2006). Comparison of the expression patterns indicate that these three proteins; CBL1, CBL9 and CIPK1 execute their functions in the same tissues. Physical interaction of CIPK1 with CBL1 and CBL9 targets the kinase to the plasma membrane (D'Angelo *et al.*, 2006). Moreover similar to the loss of CBL9 gene function, mutation of either CIPK1 or CBL1 renders plants hypersensitive to osmotic stress. However, in contrast to the *cbl1* mutant, loss of the CIPK1 gene function does not affect salt sensitivity, but instead interferes with ABA responsiveness, a phenotype that it shares with the *cbl9* mutant. Also the *cbl1cipk1* double mutant analysis further suggest that the protein kinase CIPK1 regulates distinct stress-response pathways and represents a cross-talk node that integrates ABA-dependent and ABA-independent aspects of abiotic stress signaling (D'Angelo *et al.*, 2006).

The CBL1-interacting kinase, CIPK1 contributes specifically in channeling the plant's drought and osmotic stress responses, while others, not yet characterized kinases, could convey the channeling of cold stress responses. The opposite functions of CBL1 and CBL9 in drought stress responses and the fact that both calcium sensors target an overlapping set of CIPKs point to an antagonistic function of the two CBLs due to competition for interaction with their effector kinases. Further support for this assumption is provided by the observation that loss of CBL1 function leads to ''ABA-induced-like'' expression pattern of stress marker genes. In this competition hypothesis, CBL1 could, for example, function as a positive regulator of drought-induced gene expression by interacting with an unknown kinase (CIPKy) that also interacts with CBL9. CBL1 and CBL9 would compete for the same CIPK target(s). Disruption of CBL9 would lead to more effective CBL1 function due to removal of competition from CBL9. In agreement with this

hypothesis, CIPK1 could, on one hand, function in ABA signaling (by interacting with CBL9) and, on the other hand, mediate ABA-independent signals (by interaction with CBL1). The final signal output within a specific cell would consequently be determined by the actual amount of CBL1/CIPK1 complexes in relation to the number of CBL9/CIPK1 complexes. These kind of studies provides evidences for alternative protein complex formation as a mechanism integrating signals and generating output specificity in plant abiotic stress responses. The competitive complex formation model may help explain the scale-free network-like properties of information processing in calcium-decoding signaling systems.

Based on the ABA and abiotic stress inducibility of another CIPKs family member, CIPK3, the functional analysis was carried out by reverse genetic approach. *CIPK3*, a Ser/Thr protein kinase that associates with a calcineurin B-like calcium sensor, regulates ABA response during seed germination and ABA- and stress-induced gene expression in Arabidopsis. The expression of the *CIPK3* itself is responsive to ABA and stress conditions, including cold, high salt, wounding, and drought. Disruption of *CIPK3* altered the expression pattern of a number of stress gene markers in response to ABA, cold, and high salt. However, drought-induced gene expression was not altered in the *cipk3* mutant plants, suggesting that *CIPK3* regulates selective pathways in response to abiotic stress and ABA. These results identify CIPK3 as a molecular link between stress- and ABA-induced calcium signaling and gene expression in plant cells. Because the cold signaling pathway is largely independent of endogenous ABA production, *CIPK3* represents a cross-talk "node" between the ABA-dependent and ABA independent pathways in stress responses. Single mutant analysis of CBL9 (Pandey *et al.*, 2004) and CIPK3 (Kim *et al.*, 2003) implicated the role of these proteins in ABA and osmotic stress signaling pathways. In an interesting genetic study by Pandey *et al.* (2008) it has been identified that CBL9-CIPK3 pair function in the same pathway that negatively regulates ABA response during seed germination in Arabidopsis. By yeast two-hybrid and immuno-precipitation analysis, the physical interaction between CBL9 and CIPK3 been determined. The phenotypic analysis of *cbl9cipk3* double mutant revealed that the function of CBL9 and CIPK3 is not cumulative. Together with transgenic evaluation of *cbl9* mutant transformed with constitutively active CIPK3 kinase can rescue the ABA and osmotic stress hypersensitivity, indicating that CIPK3 functions downstream of CBL9 that regulates the activity of CIPK3 by forming a signaling complex (Pandey *et al.*, 2008).

In a parallel study by Guo *et al.* (2003) the function of SCaBP5/CBL1- PKS3/CIPK15 pair in ABA signaling pathway were determined. RNA silencing of both SCaBP5 and PKS3 leads to ABA hypersensitive phenotype in the silenced transgenic lines. Because CBL1/SCaBP5 and CBL9 share very high level of homology (almost 90% identical at amino acid level), the use of coding region for RNA interference might have silenced both CBL1/SCaBP5 and CBL9 and hence leads to this hypersensitive phenotype. Based on the single knockout mutant analysis of CBL1 (Cheong *et al.*, 2003; Albrect *et al.*, 2003) and CBL9 (Pandey *et al.*, 2004), it seems to be clear that CBL1 function in regulating dehydration, osmotic stress and CBL9 involves in regulating ABA specific pathway and they antagonize each others function (Cheong *et al.*, 2003; Pandey *et al.,* 2004). The complexity of CBL1 and CBL9 function is further investigated by creating *cbl1cbl9* double mutant which shows synergistic regulation of ABA mediated stomatal responses and low-K sensitivity/uptake by interacting with CIPK23 (Cheong *et al.*, 2007).

To understand the mechanistic regulation of CBL-CIPK mediated ABA, osmotic and dehydration signaling response, the search for their targets/substrate need a greater attention. Till now there have been not many reports which demonstrated the target of CBL-CIPK pair in these pathways. However, complete linear pathway for the involvement of CBL-CIPK pair in salinity, SOS3/CBL4-SOS2/CIPK24-SOS1 (Zhu, 2003) and in regulating low-K^+ uptake and sensitivity, CBL1 or CBL9-CIPK23-AKT1 (Xu *et al.*, 2006; Li *et al.*, 2006; Cheong *et al.*, 2007) have been demonstrated.

Recently an AP2-domain/ERBP containing transcription factor, AtERF7 was shown to interact physically with PKS3/CIPK15 (Song *et al.*, 2005). PKS3/CIPK15 phosphorylate AtERF7 *in vitro* and the genetic analysis of RNA silenced lines also implicated the mutant of AtERF7 in ABA hypersensitive responses in seed germination and seedling growth (Song *et al.*, 2005). AtERF7 along with two other proteins, AtSIN3 and HDA19 shown to act as repressor of ABA mediated gene expression (Song *et al.*, 2005). Similarly in a microarray analysis of *cipk3* vs wild type Arabidopsis plants, several genes were found consistently up or down regulated in *cipk3* mutant (J.J. Grant, G.K. Pandey, S. Luan, unpublished). One of the important examples was an AP2 –domain containing transcription factor which was down-regulated more than two fold in *cipk3* mutant under ABA treatment (J.J. Grant, G.K. Pandey, S. Luan, unpublished). Detailed reverse genetic analysis of this AP2-domain containing mutant, *abr1*, showed similar

ABA sensitive phenotype as *cipk3* mutant (Pandey *et al.*, 2005). Although a direct interaction between CIPK3 and ABR1 was not established but it is speculated that they might be working in the similar pathway (Pandey *et al.*, 2005).

Based on *cbl9* mutant analysis, the higher level of ABI4 and ABI5 transcripts were accumulated under osmotic stress condition, suggesting a possibility of some CBL9-CIPKx pair directly or indirectly interacts with some other protein and hence regulate these key regulators such ABI4 and ABI5 which act as negative regulators of ABA signaling. Similarly Guo *et al.* (2003) have also suggested that SCaBP5/CBL1-PKS3/CIPK15 along with protein phosphatase ABI1/2 may also regulate targets/substrate proteins which might ultimately regulate ABA signaling pathway by modulating gene expression. However not many endogenous targets/ substrates of this CBL-CIPK pair have been identified which regulate the ABA signaling and hence the complete mechanistic pathway needs to be ascertained in future studies.

Regulation of Salt Signaling/ tolerance

Agricultural productivity is severely affected by soil salinity because salt levels that are harmful to plant growth affect large terrestrial areas of the world. Soil salinity represents an increasingly prominent problem in agriculture. Since it is quite difficult to physically remove salt from the soil, improving crop tolerance to high salt becomes a critical task for breeders and biotechnologists. Understanding salt tolerance mechanisms in plants is a prerequisite for achieving this goal. Salt injury to plants is caused by ionic toxicity that is specific to a particular ion (such as Na^+), and osmotic stress that can also be induced by other conditions such as drought or low temperature (Hasegawa *et al.*, 2000; Apse and Blumwald, 2002). Most important mechanisms for plant salt tolerance include "osmotic adjustment" and "salt compartmentalization" (Hasegawa *et al.*, 2000; Apse and Blumwald, 2002). The osmotic adjustment is achieved by the accumulation of compatible solutes in the cell to balance the osmotic gradient across the plasma membrane so that the cellular water status is maintained. The processes of salt compartmentalization generally include export of salt to the exterior of the cell, translocation of salt to physiologically quiescent tissues (e.g., old leaves), and sequestration/storage in organelles (such as vacuoles). Because NaCl is the most abundant salt encountered by plants under salinity stress conditions, transport systems that mediate the accumulation, exclusion, translocation, and organelle sequestration of Na^+ and Cl^- are particularly important for plant salt tolerance (Niu *et al.*, 1993;Flowers *et al.*, 2000; Munns *et al.*, 2006). Uptake of Na^+ from the soil may be mediated by non-selective cation channels (Amtmann *et al.*, 1999). Once Na^+ enters the cytosol, it can potentially be excluded (back to the soil) by Na^+/H^+ antiporter located in the plasma membrane, or sequestered into the vacuole by Na^+/H^+ exchangers (NHX proteins) located in the tonoplast (Apse and Blumwald, 2002; Yokoi *et al.*, 2002; Qiu *et al.*, 2003). In addition, Na^+ must be translocated and re-distributed to different parts of the plant through the vascular system. Salt re-distribution plays a critical role in salt detoxification as demonstrated by studies on two Na^+ transporters AtHKT1 and SOS1 in *Arabidopsis* (Apse *et al.*, 1999; Shi *et al.*, 2002; Apse *et al.*, 2003; Berthomieu *et al.*, 2003; Qiu *et al.*, 2004; Rus *et al.*, 2004). A critical role of HKT-type transporters in salt tolerance was also recently demonstrated in rice (Ren *et al.*, 2005).

Ca^{2+}-dependent pathways play critical roles in salt tolerance (Luan *et al.*, 2002; Zhu, 2003). Earlier work implicated Ca^{2+} as second messenger in abiotic stress such as salt stress responses (Knight, 2000; Sanders *et al.*, 2002). It is believed that Ca^{2+} transmits the stress signal further downstream in the pathway by binding to protein sensors.

In a genetic screen designed to identify components of the mechanisms controlling salt tolerance in Arabidopsis, several *SOS* (Salt overly sensitive) genes were identified. The SOS3-SOS2-SOS1 regulated pathway is studied intensively in salt tolerance pathway in Arabidopsis (Zhu, 2003). One of these genes, *SOS3/ CBL4*, encodes a novel EF-hand Ca^{2+} sensor (Liu and Zhu, 1998). The *SOS2/CIPK24* gene encodes a novel Ser/ Thr protein kinase that also functions in salt tolerance in Arabidopsis (Liu *et al.*, 2000). SOS3 has been shown to interact physically with SOS2 in yeast two-hybrid assays as well as *in vitro* (Halfter *et al.*, 2000). Moreover, SOS3 activates SOS2 kinase activity in a Ca^{2+} dependent manner and *sos3/sos2* double-mutant analysis also indicates that *SOS3* and *SOS2* function in the same pathway (Halfter *et al.*, 2000).

The first target of the SOS3-SOS2 regulatory pathway to be identified is the plasma membrane Na^+/H^+exchanger (antiporter) encoded by the *SOS1* gene. Molecular genetic analysis of *Arabidopsis sos* mutants have led to the identification of a plasma membrane Na^+/H^+exchanger, SOS1, which plays a crucial role in sodium extrusion from root epidermal cells under salinity. The *SOS1* transcript level is up-regulated under salt stress. The *sos1* mutant plants were hypersensitive to salt stress and accumulate Na^+ in shoot than wild type plants. The

expression of SOS1 transcript is also up-regulated under stress and its expression is ubiquitous but stronger in epidermal cell surrounding the root tip as well as parenchyma cells bordering the xylem (Shi *et al.*, 2002). SOS1 functions as Na^+/H^+antiporter on the plasma membrane and plays a crucial role in efflux of Na^+ ion from root cell and long distant transport of Na^+ from root to shoot (Shi *et al.*, 2002). Arabidopsis plants over-expressing SOS1 shows enhanced tolerance to salt stress since they have less Na^+ in the xylem transpiration stream, ability to grow flower and bolt in sodium containing soil as compared to the wild type (Shi *et al.*, 2003).

SOS1 gene expression during salt stress is partially controlled by *SOS3* and *SOS2* (Shi *et al.*, 2000), and activation of SOS1 Na^+/H^+ antiport activity requires SOS3 and SOS2 (Qiu *et al.*, 2002). Recent studies using yeast have provided additional evidence for the interaction between SOS3 and SOS2 and their role in regulation of SOS1. Sodium efflux by SOS1 under salinity is regulated by SOS3-SOS2 complex. Co-expression of SOS1, 2, and 3 dramatically enhanced the salt tolerance of a yeast mutant in which all endogenous Na^+ transporters had been removed (Quintero *et al.*, 2002). The SOS3-SOS2 kinase complex phosphorylates and activates SOS1 expressed in yeast, enhancing Na^+ exclusion and increasing NaCl tolerance (Quintero *et al.*, 2002). Expression of a constitutively activated SOS2 mutant also increased salt tolerance in yeast expressing SOS1, implying that SOS2 kinase activity is partially sufficient for SOS1 activation. These results provided functional evidence that the SOS proteins function in the same signaling pathway that mediates ion homeostasis and salt tolerance in Arabidopsis (Zhu, 2002). In addition, recent studies demonstrating that over-expression of SOS1 or of constitutively active SOS2 mutant kinases could overcome the salt hypersensitivity of *sos2* and *sos3* mutant and improves the salt tolerance of Arabidopsis (Guo *et al.*, 2004; Shi *et al.*, 2003) and hence suggest that co-overexpression of SOS1, SOS2, and SOS3 may dramatically increase salt tolerance in plants.

It is important to make a note that the *SOS1* up-regulation under salt stress is also impaired in *sos2* and *sos3* mutants. And hence, the SOS3–SOS2 signaling pathway positively regulates salt-stress induced *SOS1* gene expression and/or transcript stability as well as *SOS1* transporter activity (Shi *et al.*, 2003). In addition to increasing cytosolic calcium, salt-stress induced ABA accumulation also appears to regulate the SOS pathway through the ABA insensitive 2 (ABI2) protein phosphatase 2C. ABI2 interacts with the protein phosphatase interaction (PPI) motif of SOS2. This interaction is abolished by the *abi2-1* mutation, which enhances tolerance of seedlings to salt stress (150 mM NaCl) and causes ABA insensitivity. It is quite possible that the wild-type ABI2 might negatively regulate salt tolerance either by inactivating SOS2, or the SOS2 regulated Na^+/H antiporters such as SOS1 or NHX1 (Ohta *et al.*, 2003).

In addition to CBL4/SOS3-CIPK24/SOS2 (Zhu, 2003) other members of this pathway are CBL1 (Cheong *et al.*, 2003; Albrect *et al.*, 2003) and its interacting protein kinase, CIPK1 (D'Angelo *et al.*, 2006), and CBL9 (Pandey *et al.*, 2004) which are involved in osmotic component of salt stress regulation. Only CBL4/SOS3-CIPK24/SOS2 pair seems to regulate the Na^+/H^+ antiporter (SOS1) at the plasma membrane in exclusion of excess Na^+ ions under salt stress. Recently another member of CBL family, CBL10 was found to be involved in salt stress regulation (Kim *et al.*, 2007; Quan *et al.*, 2007). Unlike other CBLs, CBL10 expresses only in the green tissues and not in the roots. *Cbl10* mutant plants exhibited significant growth defects and showed hypersensitive cell death in leaf tissues under high salt conditions. Interestingly, the Na^+ content of the *cbl10* mutant, unlike other salt sensitive mutants identified thus far, was significantly lower than in the wild type under either normal or high salt conditions, suggesting that CBL10 mediates a novel Ca^{2+}-signaling pathway for salt tolerance (Kim *et al.*, 2007). CBL10 protein physically interacted with the salt-tolerance factor CIPK24/SOS2 and the CBL10-CIPK24/SOS2 complex is associated with the vacuolar compartments that are responsible for salt storage and detoxification in plant cells (Kim *et al.*, 2007). Hence CBL10 and CIPK24/SOS2 may constitute an alternate salt tolerance pathway besides SOS pathway that regulates the sequestration/compartmentalization of Na^+ in plant cells (Kim *et al.*, 2007).

In a parallel study, Quan *et al.* (2007) have also shown the function of CBL10/SCaBP8 mainly in the shoots response to salt toxicity. Compared to *sos3* mutant, the salt sensitivity in *scabp8/cbl10* mutant is more prominent in shoot tissues than root. In contrary to Kim *et al.* (2007), they have shown that CBL10/SCaBP8 recruit SOS2 to the plasma membrane, enhance SOS2 activity in a calcium-dependent manner, and activate SOS1 in yeast. In addition, *sos3scabp8* and *sos2 scabp8* display a phenotype similar to *sos2*, which is more sensitive to salt than either *sos3* or *scabp8* alone. Over-expression of SCaBP8 in *sos3* partially rescues the *sos3* salt-sensitive phenotype (Quan *et al.*, 2007). However, over-expression of SOS3 fails to complement *scabp8*. It is quite

interesting that CBL10/SCaBP8 might be involved in regulating salt homeostasis by regulating the Na^+ sequestration/compartmentalization in vacuole (Kim *et al.*, 2007) and extrusion of Na^+ ions out from the cell by regulating SOS1 (Na^+ /H^+ -antiporter) at plasma membrane (Quan *et al.,* 2007).

In most land plants Na^+ is not a favorable ion to be present in soil since higher ratio of K^+/Na^+ in the cytosol is important for the normal growth processes. Na^+ competes with uptake of K^+ through K^+-Na^+ co-transporter, at the same time Na^+ also block the specific K^+-transporter of root cell under salinity (Zhu *et al.*, 2003). This result in excessive toxic level of Na^+ ion in the cell and insufficient level of K^+ ion which is essential for enzymatic processes and osmotic adjustment. Under salt stress the Na^+ ion from soil enter into the root cell through cation channel or transporter (selective and non-selective) or into the root xylem stream via apoplastic system depending upon the plant species (Chinnusamy *et al.*, 2005). In plants including Arabidopsis, Eucalyptus and wheat; HKT (High affinity potassium transporter; Rubio *et al.*, 1995; Gorham *et al.*, 1997) act as low affinity Na^+ transporter under salinity. Therefore HKT and HKT homologs might be responsible for influx of Na^+ ion into the cell under salt stress. However in rice the apoplastic pathways are responsible for significant influx of Na^+ into the cell (Yadav *et al.* 1996; Garcia *et al.*, 1997). Inhibition of Na^+ influx into the root cell or xylem stream seems to be the criteria for maintaining the optimal K^+/Na^+ ratio under saline condition. In a suppression screen for salt hypersensitivity and potassium deficient phenotype of *sos3* mutant, a mutation in HKT1 gene was identified and hence the *hkt1* mutation can suppress the *sos3* phenotype (Rus *et al.*, 2001; 2004).

Regulation of K^+-nutrition and transport

Plant growth requires a number of mineral nutrients and potassium (K^+) is the most abundant inorganic cation in plants constituting up to 5-10% of a plant's dry weight (Leigh and Jones, 1984). Potassium performs vital functions in metabolism, growth, and stress adaptation. On one hand, potassium functions in the cell by directly interacting with proteins resulting in enzyme activation, stabilization of protein synthesis, and neutralization of negative charges on proteins (Kochian and Lucas, 1988; Marschner, 1995; Maathuis and Sanders, 1996). K^+ is a major driving force for osmotic regulation, for example, in stomatal movement, light-driven and seismonastic movements of organs or phloem transport (Marschner, 1995; Perrenoud, 1990). In cell expansion and growth, accumulation of K^+ (together with anions) in plant vacuoles creates the necessary osmotic potential for rapid cell enlargement. K^+ movement also provides a charge separation across the membrane that is essential for the movement of other ions. In chloroplasts, energy production through H^+-ATPases depends on overall H^+/K^+ exchange (Tester and Blatt, 1989; Wu *et al.*, 1991).

K^+ deficiency is of great agricultural importance (Laegreid *et al.*, 1999). This fact was recognized early in plant physiological research that led to an extensive description of K^+ starvation symptoms at the physiological level (Marschner, 1995). It is well established that K^+ starvation leads to growth arrest due to the lack of the major osmoticum, impaired nitrogen balance due to inhibition of protein synthesis, and reduced levels of sugars due to inhibition of photosynthesis, and impaired long distant transport (Marschner, 1995)**.** Low potassium content in the soil therefore severely limits crop yield and farming practice often requires large amount of organic and/or chemical fertilizers to overcome this condition. Use of fertilizers imposes an undesirable economical and environmental problem that can be solved by genetic engineering of low-K^+ tolerant crops based on the molecular understanding of K^+ nutrition in plants. One important aspect of plant adaptation to low K^+ stress is cellular and tissue homeostasis of K^+, which involves transport of K^+ across various membranes in various tissues (Amtmann *et al.*, 2004). K^+ transport mechanisms have been studied extensively at the molecular level and many of the transporters have been cloned and functionally analyzed (Maser *et al.*, 2001; Very and Sentenac, 2003; Cherel, 2004; Ashley *et al.*, 2006). There are typically two mechanisms for K^+ acquisition, one is high affinity and the other is low affinity uptake from the roots (Kochian and Lucas, 1988; Epstein *et al.*, 1963; Epstein, 1966; Siddiqi and Glass, 1983). Some of the transporters such as the voltage-gated channels are generally considered as low-affinity transporters although studies also show that these channels can perform high-affinity uptake (Lagarde *et al.*, 1996; Hirsch *et al.*, 1998; Spalding *et al.*, 1999; Dennison *et al.*, 2001; Gierth *et al.*, 2005). In addition, a single transporter can mediate both high- and low-affinity transport (Dennison *et al.*, 2001, Fu and Luan, 1998; Bruggemann *et al.*, 1999). Most of the soil solutions contain less than 1mM K^+, thus the high affinity uptake plays a critical role in potassium nutrition in plants (Adam, 1971; Glass, 1989; Ashley *et al.,* 2006). Under potassium deficiency, high affinity uptake is even more important for plant survival. Studies have shown that such high-affinity uptake may be inducible and tightly regulated under low potassium conditions (Hirsch *et al.*, 1998, Dennison *et al.*, 2001; Gierth *et al.*, 2005). However, it is unknown how plants detect or sense external K^+

concentration and what are the signaling mechanisms that integrate physiological, biochemical, and molecular responses into a concerted adaptive response.

A recent study (Shin and Schachtman, 2004) identifies reactive oxygen species (ROS) as a critical signaling molecule for plant response to potassium deficiency conditions. Interestingly, ROS in root cells has been shown to elicit changes in cellular calcium and is required for root hair growth and mineral uptake (Foreman *et al.*, 2004). Together, these studies suggest that ROS signal is produced under low potassium condition and ROS-induced calcium changes may be a crucial for downstream responses. However, it is not known how low-K^+-induced calcium signal is perceived by the cell. In addition to low-K^+ response, calcium plays multiple roles in many other cellular pathways including signaling processes in response to pathogen, abiotic stresses, and developmental signals (Sander *et al.*, 1999; Rudd and Franklin-Tong, 2001; Reddy, 2001). Calcium is also involved in the regulation of K^+/Na^+ homeostasis and ionic selectivity under saline condition and exogenous Ca^{2+} can improve the salt tolerance (LaHaye and Epstein, 1969; Lauchli, 1990; Liu and Zhu, 1997).

In a forward genetic screen Wu and colleagues (Xu *et al.*, 2006) isolated the *lks1* (low-K^+-sensitive) mutant for plant sensitivity to low potassium as defined by leaf chlorosis phenotype in the *lks1* mutant. *LKS1* was shown to encode the CBL (calcineurin B-like protein) interacting protein kinase CIPK23, a critical component for K+ uptake in plants. Similarly, in a reverse genetic screen by Luan and colleagues (Cheong *et al.*, 2007) identified loss-of-function alleles of CIPK23 as exhibiting severe growth impairment on media with low concentrations of potassium. This phenotype correlates with a reduced efficiency of K^+ uptake into the roots.

Anticipating that the loss of protein kinase activity may directly affect K^+ uptake by the regulation of K^+-transport, the phenotypes of several candidate mutants of potassium transporters isolated by reverse genetic approach in Arabidopsis was tested. Of these, only the *akt1* K^+ channel mutant, where the mutation disrupt the expression of functional voltage gated activated channel AKT1 (Hirsch *et al.*, 1998) was found to exhibit similar (although less pronounced) defects in plant growth and K^+ uptake phenotype. Most remarkably, both CBL1 and CBL9 were identified as interacting partners of CIPK23, and only mutants lacking both CBL1 and CBL9 exhibited sensitivity to low K^+ and CBL1 and CBL9 were identified as the upstream regulators of CIPK23 (Xu *et al.*, 2006; Cheong *et al.*, 2007).

Although the *cbl1* and *cbl9* single mutants did not show the typical phenotype under low-K conditions, the *cbl1cbl9* double mutants showed the same phenotype as the *cipk23*/*lks1* or *akt1* mutants, indicating that CBL1 and CBL9 have overlapping functions. In addition, overexpression of CBL1 or CBL9 significantly increased K^+ content in both roots and shoots under low-K^+ conditions, while the disruption of CBL1 and CBL9 expression in the *cbl1cbl9* double mutant decreased K^+ content, particularly in shoots (Xu *et al.*, 2006).

Using a combination of biochemical and electrophysiological approaches the protein kinase CIPK23 was shown to interact with, and phosphorylate, a voltage-gated inward K^+ channel (AKT1) required for K^+ acquisition in *Arabidopsis* (Li *et al.*, 2006; Xu *et al.*, 2006). In the *Xenopus* oocyte system, these interacting calcium sensors (CBL1 and CBL9) together with target kinase CIPK23, but not either component alone, activated the AKT1 channel in a Ca^{2+}-dependent manner, connecting the Ca^{2+} signal to enhanced K^+ uptake through activation of a K^+ -channel. Disruption of both *CBL1* and *CBL9* or *CIPK23* gene in *Arabidopsis* reduced the AKT1 activity in the mutant roots, confirming that the Ca^{2+}-CBL-CIPK pathway functions to orchestrate transporting activities *in planta* according to external K^+ availability (Li *et al.*, 2006; Xu *et al.*, 2006).

In an attempt to understand the function of all CBL and CIPK members, Luan group performed systematic reverse genetic approach. Besides CIPK23 (Xu *et al.*, 2006; Li *et al.*, 2006; Choeng *et al.*, 2007), *CIPK9* transcript was highly inducible under low-K condition transcriptional profiling. The two independent alleles of CIPK9 T-DNA insertion, *cipk9-1* and *cipk9-2* showed hypersensitive and impaired growth under K-deficient conditions (Pandey *et al.*, 2007). The hypersensitive and growth impairment phenotype in *cipk9* mutant alleles was a specific response as this was not caused by depletion of other ions in the growth media. In contrary to CIPK23 loss of function mutant (Xu *et al.*, 2006; Choeng *et al.*, 2007), the total root and shoot K^+ content was unaffected in both the alleles of CIPK9 and hence the K-uptake by roots was not affected (Pandey *et al.*, 2007). Unlike CIPK23, CIPK9 was not interacting with K-transporter such as AKT1 in yeast two-hybrid assays and raises the question which processes other than K^+ acquisition are important for plant growth under K^+-deficient conditions. One possibility is that CIPK9, as CIPK23, interacts with some other K^+-channel, but that unlike AKT1 this channel does not reside in the root plasma membrane (Pandey *et al.*, 2007; Amtmann and Armengaud, 2007). Another possibility is that CIPK9 regulate/target aspects of plant

adaptation to low K^+ that are not linked to K^+-transport. Although cellular and tissue K^+-homeostasis can protect metabolically active cells from serious K^+-deficiency for a limited period of time, it is clear that a plant which experiences long-term K^+-deficiency will have to re-priorities its growth, development and metabolism to achieve maximal seed production with limited resources (Pandey *et al.*, 2007; Amtmann and Armengaud, 2007). Based on the studies of Wu and colleagues (Xu *et al.*, 2006) and Luan group (Li *et al.*, 2006; Cheong *et al.*, 2007), a comprehensive CBL-CIPK signaling pathway for low-K response in

Arabidopsis have been uncovered. However, these studies raise a number of important questions regarding the mechanisms underlying the regulation of AKT1 channel by the CBL-CIPK complexes. As both AKT1 and CIPKs are multi-domain proteins, identifying the domains that mediate the AKT1-CIPK interaction is critical for understanding the structural basis for specificity of the interaction. Because the AKT1 activity is not abolished completely in *cbl1cbl9* double or *cipk23* mutant, it is quite possible that multiple CBL-CIPK complexes may be regulating the AKT1 channels activity. Therefore it is logical to hypothesize that a more complicated network involving more CBLs and CIPKs may cooperatively regulating AKT1 activity. *In planta* analysis of this large network is difficult task, therefore Luan and colleagues took advantage of the *Xenopus* oocytes model systems (Li *et al*., 2006) to investigate the mechanistic action of CBL-CIPK pair in the regulation of AKT1 potassium channel (Lee *et al.*, 2007). In a comprehensive yeast two hybrid study, two more CIPKs, CIPK6 and CIPK16 were also found to interact with AKT1 in addition to CIPK23. Similarly, two more CBLs, CBL2 and 3 in addition to CBL1 and CBL9 which were already reported in earlier studies (Li *et al.*, 2006; Xu *et al.*, 2006) were found to interact with all three CIPKs such as CIPK6, CIPK16 and CIPK23 (Lee *et al.*, 2007). The interaction assays above identified 4 CBLs and 3 CIPKs that in turn interacted with AKT1, forming a multivalent interacting network. Such a molecular network may be functionally relevant in the regulation of AKT1 activity. Based on the AKT1 channel activity measurement in *Xenopus* oocytes differential activation of AKT1 channel was observed by these four different CBLs and three CIPKs for example interaction of CBL1-CIPK23 with AKT1 produces the strongest channel activity whereas interaction combination of CBL2, 3 or 9-CIPK6 or CIPK16 with AKT1 produces weaker channel activity (Lee *et al*., 2007) which indicates the degree of overlap and complexity in the cellular regulation by CBL-CIPK signaling network. A key factor that determines the specificity of AKT1 activation by CBL-CIPK complexes is the physical interaction of CIPKs and AKT1. The ankyrin repeat domain in the AKT1 protein appears to interact with the kinase domain of the CIPKs thereby determining which specific CIPKs can adhere on to the AKT1 channel protein. The lipid modification of CBLs by myristoylation / palmitoylation also responsible for targeting CBL-CIPK to the membrane but may not be as important as CIPK-AKT1 interaction. Remarkably, this study by Lee *et al.* (2007) open up a new dimension and found a specific PP2C-type protein phosphatase, AIP1 that interact and inactivates AKT1 provides another side of the regulation, namely, inactivation of the channel by dephosphorylation. Overall this study opens up new avenues to further understand the mechanism of ion channel regulation by signaling pathways in plants.

CBL-CIPK module in other plants

An extensive analysis of CBL-CIPK network has been taken place in Arabidopsis because of genome annotation, availability and ease of getting loss of function mutants and large size of public data sets. Mutation in considerable number of genes in both CBL and CIPK gene family leads to appearance of a phenotype under certain phytohormone and abiotic stress conditions. Although a large number of genes in this CBL-CIPK family does not yield a significant phenotype under several conditions tested. Similarly genome annotation in rice have lead to identification of a multigene family for CBLs and CIPKs, 10 CBL and 30 CIPKs members existed (Kolukisaoglu *et al*., 2004) and indicates a similar complexity of this signaling network in rice. A detail analysis of the genomic evolution suggests that the extant number of gene family members largely results from segmental duplications. A phylogenetic comparison of protein sequences and intron positions indicates an early diversification of separate branches within both gene families (Kolukisaoglu *et al.*, 2004). Recently in a functional study by Pardo and colleagues (Martínez-Atienza *et al.*, 2007) have discovered the conservation of SOS pathway in rice. In their study they have identified a rice plasma membrane Na+/H+ exchanger that is the functional homologue of the *Arabidopsis thaliana* SOS1 (Na^+/H^+ antiporter) protein. The rice transporter, denoted by OsSOS1, demonstrated a capacity for Na^+/H^+ exchange in plasma membrane vesicles of yeast cells and reduced their net cellular Na^+ content. The *Arabidopsis* protein kinase complex SOS2-SOS3, which positively controls the activity of AtSOS1, phosphorylated OsSOS1 and stimulated its activity *in vivo* and *in vitro*. Moreover, OsSOS1 suppressed the salt sensitivity of a *sos1-1* mutant of *Arabidopsis*. Putative

rice homologues of the *Arabidopsis* protein kinase SOS2 and its Ca^{2+}-dependent activator SOS3 were also identified. OsCIPK24 and OsCBL4 acted in coordination to activate OsSOS1 in yeast cells, and they could be exchanged with their *Arabidopsis* counterpart to form heterologous protein kinase modules that activated both OsSOS1 and AtSOS1 and that suppressed the salt sensitivity of *sos2/cipk24* and *sos3/cbl4* mutants of *Arabidopsis*.

When CIPK gene family members (OsCIPK1-OsCIPK30) were analyzed for their expression pattern to various abiotic stresses, several gene members, at least 20 OsCIPK genes were found to be differentially responsive to at least one or more stress such as drought, salinity, cold, and phtyohormone ABA (Xiang *et al.*, 2007). Most of the genes induced by drought or salt stress were also induced by ABA treatment but not by cold. Transgenic plants overexpressing the transgenes OsCIPK3, OsCIPK12, and OsCIPK15 in wild type plants showed significantly improved tolerance to cold, drought, and salt stress, respectively. The differentially induced expression of OsCIPK genes by different stresses and the examples of improved stress tolerance of the OsCIPK transgenic rice suggest that rice CIPK genes also have diverse roles in different stress responses. In addition to Arabidopsis and rice, CBL-CIPK signaling components have been identified in leguminous crop pea, *Pisum sativum,* (Mahajan *et al.*, 2006). A PsCBL and PsCIPK were cloned and found to interact with each other. Similar to Arabidopsis CIPKs, the PsCIPK protein was autophosphorylated at threonine residue(s). Remarkably, in their phosphorylation analysis they have demonstrated that PsCBL is phosphorylated at its threonine residue(s) by PsCIPK. Both PsCBL and PsCIPK genes were up-regulated by exposure of plants to salinity, cold and wounding.

FUTURE PERSPECTIVES

The CBL-CIPK signaling network is novel and unique Ca^{2+}- mediated signaling pathway known to exist only in plant. This CBL-CIPK signaling network regulates plant responses to phytohormone ABA, abiotic stresses and potassium nutrition. In *Arabidopsis,* significant progress has been made by functional characterization of this CBL-CIPK networking. Multiple approaches are being used to investigate the functions of these proteins including site-directed mutagenesis or deletion of key residues or domains, reverse genetics (knockouts) to identify null mutations, overexpression, protein interaction screens to identify potential substrates, biochemical analyses to characterize kinetic properties, and expression and localization studies to clarify where and when various family members are expressed. There exist multiple challenges ahead such as, to characterize and identify multiple family members in CBL as well as CIPK gene family and closely related genes that may have similar functions. For instance, loss of function of most of the genes usually does not yield an obvious phenotype, indicating either that the correct conditions to identify a phenotype were not tested or there is functional redundancy for these multi gene family. In some cases, double, triple, or higher order mutants need to be made to uncover a phenotype. Mutant combinations could be made at random, or specific mutants could be combined based on the closeness or distance of a particular gene with other family member which might have similar or redundant functions. A detail identification of physiological substrate for these CBL-CIPK pair is major question to understand the mechanistic detail of a given signaling pathway. In addition the main challenge for future research remains the elucidation of the interconnections, synergistic and antagonist functions of the diverse signaling components of plants. The inter connection and cross talk of CBL-CIPK network with other signaling pathways such as CDPKs, two-component systems and MAPK cascades required detail investigation. Ultimately, utilizing the tools of transgenic to enhance abiotic stress tolerance by employing CBL-CIPK signaling components in agriculturally important crops need to be undertaken.

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