Research Paper

CBL-CIPK Paradigm: Role in Calcium and Stress Signaling in Plants

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(Received on 27 October 2005; Accepted on 6 June 2006)

Tolerance or susceptibility to abiotic stresses in plants is a coordinated action of various genes including those encoding calcium binding proteins and other components of stress signal transduction pathways. These components may cross talk with each other, however still little information is available on this aspect. Calcium ions (Ca^{2+}) function as a major secondary-messenger signaling molecule, which plays a fundamental role in plant growth and development under normal as well as stress conditions. In general, stress signals results in cytosolic Ca^{2+} perturbations, which are unique and precisely decoded by Ca^{2+} sensing proteins to relay the signaling cascade. The relatively recently discovered calcium sensor calcineurin B-like proteins (CBLs), and their interacting partners CBL-interacting protein kinases (CIPKs), have emerged as key network that plays an important role in plants in response to calcium and stress signaling. CIPK initiates a phosphorylation cascade and can regulate the expression of major genes involved in imparting stress tolerance. In this review we have focused on the various aspects of calcineurin signaling networks in yeast in relation to salt stress, historical background of plant CBL and CIPK proteins and an up to date coverage of their structure, localization, genomic organization, and expression profiles under stress conditions. Furthermore, hypothetical models depicting the role of CBL-CIPK in stress signaling are described. Future aspects to explore and solve the complexity of this pathway are also covered.

Key Words: ABA, abiotic stress, calcium signaling, calcium sensors, CBL-CIPK, Salt stress, Ser-Thr protein kinase, SOS pathway

Introduction

Plants are constantly challenged with nature's fury in the form of various abiotic and biotic stresses. Abiotic stresses such as cold, salt, dehydration and heat act as mal-factors and lead to aberration of the plant from its normal process of growth and development. All these stresses impede the productivity of the plants and prevent them from reaching their full genetic potential. Indeed, abiotic stress is the primary cause leading to worldwide crop loss and dipping the average yield for most crops by more than 50% [1]. In addition, increased salinity of arable land is expected to have devastating global effects, resulting in up to 50% land loss by the middle of 21st century [2]. In response to these stress factors various genes gets up-regulated which can mitigate the effect of stress and lead to plant adaptation. In nature, stress does not generally come in isolation and many stresses co-exit with each other. Resistant plants seem to reflect the "Darwanian concept" of the survival of the fittest. For their survival, plants activate several signaling pathways, which intern may modulate various metabolic processes that confer stress tolerance.

Calcium is one of the principal candidates for functioning as central node in such a 'signaling web'. Stimulus specific information is encoded in the form of calcium signatures and this contributes to specificity in the signaling pathway. As Ca^{2+} levels are tightly regulated in the cell, any subtle change or perturbation in cytoplasmic Ca^{2+} levels can provide specific signals for expression of genes pertaining to a particular pathway. Furthermore, calcium-binding proteins (calcium sensors) can provide an additional level of regulation in the calcium signaling [3,4]. These sensory proteins recognize and decode the information provided in the calcium signatures, relay the information downstream to initiate a phosphorylation cascade leading to regulation of gene expression [3,5]. Thus, these Ca^{2+} binding proteins strengthen the specificity in the signaling pathway. In plant cells many Ca^{2+} sensors have been identified which include calmodulin (CaM) and calmodulin-related proteins [3,6], calnexin [7], and Ca^{2+}-dependent protein kinases (CDPKs) [4,8-10].

A unique family of calcium sensor protein was identified over the past few years, in *Arabidopsis* by two independent groups [11,12]. Jiang Zhu and colleagues [13] in 1996 commenced a mutant screen for *Arabidopsis* plants, which were over-sensitive to salt stress. As a result of this screen, three genes SOS1, SOS2, and SOS3 (Salt Overly-Sensitive) were identified. Each of the sos mutants exhibited hypersensitivity to sodium and lithium. *SOS3*, also known as *AtCBL4*, was isolated through positional cloning and was found to encode a protein with similarity to regulatory B subunit of calcineurin (protein phosphatase 2B), and neuronal calcium sensors (NCS) from animals, and therefore designated as calcineurin B-like proteins (CBLs) [11,12]. CBLs seem

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to be one of the important components in calcium signaling pathway induced in response to abiotic stresses. While calcineurin has been well characterized in mammalian system and in yeast, the significance and function of this protein in plants is just beginning to understand**.** In this review we have focused on various aspects of calcium signaling network including the role of yeast calcineurin in relation to salt stress. A historical account of discovery of plant CBL and CIPK proteins is given. We have covered structure, localization, genomic organization, expression profiles under stress conditions of CBL/CIPK protein family. Furthermore, some models depicting the role of CBL-CIPK in stress signaling as revealed by the analysis of various SOS mutants has also been covered in this review.

A. Calcineurin

(a) Animal calcineurin: The work on calcineurin (CaN) was started in 1988 with its purification for the first time from bovine brain, wherein high concentrations of CaN are present [14]. CaN is a Ca^{2+}/Calmodulin-activated, serine-threonine protein phosphatase (PP2B) and play a vital function in the control of intracellular calcium signaling [14]. This protein is a heterodimer of a 59-62 kDa catalytic subunit, calcineurin A, (CnA) and a 19 kDa Ca^{2+} binding regulatory subunit Calcineurin B (CnB). These two subunits are highly conserved and essential for the enzyme activity. In animals, CaN transmits the signals to the nucleus through the dephosphorylation of nuclear factor of activated T cell (NFAT) transcription factors, which is necessary for its translocation to the nucleus, thus leading to the activation of T cells [15]. Inhibition of phosphatase activity of CaN by FK506 or Cyclosporine A (CSA) resulted in the re-localization of NFAT to the cytosol and loss of its DNA – binding ability [16]. CaN also expresses in the nervous system [17] and is known to play key role in the induction of long-term potentiation (LTP), long-term depression (LTD) and in the establishment of learning and memory [18,19]. CaN has a very narrow substrate specificity and important substrates are DARP 32 and inhibitor-1 i.e. the two potent inhibitors of phosphatase 1, NFAT, a family of transciption factors involved in the activation of T cells and the IP_3 receptors.

(b) Yeast calcineurin: CaN, in yeast, is an integral component in response to salt stress and mediates NaCl tolerance through the regulation of Na^+ ion and restricting its accumulation in cell par toxic levels. Salt stress is mediated by multiple determinants, which basically restrict Na^+ uptake across plasma membrane and facilitate Na^+ and Cl^+ sequestration into the vacuole. CaN functions to prevent the intracellular buildup of Na^+ ions and augment the events, which results in efflux of this cation across plasma membrane [20]. Exact mechanism by which CaN is activated is still not clear. Calcium in the cytosol is maintained at sub micromolar levels as it can precipitate phosphate, which is the energy currency of the cell in the form of ATP, to calcium phosphate. This stringent regulation of $CaCl_2$ is mainly provided by H^+/Ca^{2+} antiporters in which 3 H^+ are moved out and one Ca^{2+} is sequestered in and Ca^{2+} pumps, which are directly energized by ATP hydrolysis. There is some evidence that G proteins, phospholipase C (PLC) and insitol triphosphate (IP_3) are involved in calcium release [21,22]. Recent reports point to the fact that CaN signaling is required for appresoria formation, which is the infection structure formed by *Magnaporthe grisea* (fungus) [22]. Inhibition of PLC activity using neomycin resulted in pronounced inhibition of appressorium formation [23]. This indicates that PLC activity and subsequent Ca^{2+} release may be required for CaN activation. However, experimental evidences have also proved that NaCl mediated cytosolic Ca^{2+} increase was attenuated by the addition of chelating agents EGTA, BAPTA, cation channel pore blockers and by the use of competitive inhibitors of Ca^{2+} transport. These results indicate that external Ca^{2+} is the source for cytosolic Ca^{2+} transient. Moreover, ENA1, a P type Na^+ ATPase, induced in response to CaN activation was inhibited by the addition of EGTA and FK506, indicating that cytosolic Ca^{2+} transient (released from extra-cellular source) mediates CaN activation resulting in ion homeostasis [24].

A model describing CaN mediated signal transduction pathway in response to salt stress in yeast is shown in Fig. 1. The first response to salt stress is the transient increase in cytosolic Ca^{2+} levels, which may be mediated by both extracellular and intracellular sources to result in the full activation of NaCl responsive genes. The increase in cytosolic Ca^{2+} is sensed by Ca^{2+} sensor proteins such as CnB and CaM, which accordingly change their conformation in Ca^{2+} dependent manner and get activated. Calcium bound CnB and CaM binds CnA and this result in formation of functional CaN holozyme. Activated CaN dephosphorylates the zinc finger transcription factors Crz1p/Tcn1p/Hal8p, which facilitates their translocation to the nucleus [24-27]. These transcription factors then interacts with CaN dependent response elements (CDRE) present in the promoter of the genes which are activated by CaN. Various genes activated by CaN include TRK1, ENA1, VCX1, PMR1 and PMC1 [24-27]. The nature and function of these genes in response to reinstating ionic homeostasis is stated as under (also see Fig. 1):

1) Functional CaN (CnA and CnB) can influence the Na^+ and K^+ uptake system to have higher affinity for K^+, thus limiting Na^+ uptake. This function of CaN relies on a putative high affinity K^+ transporter

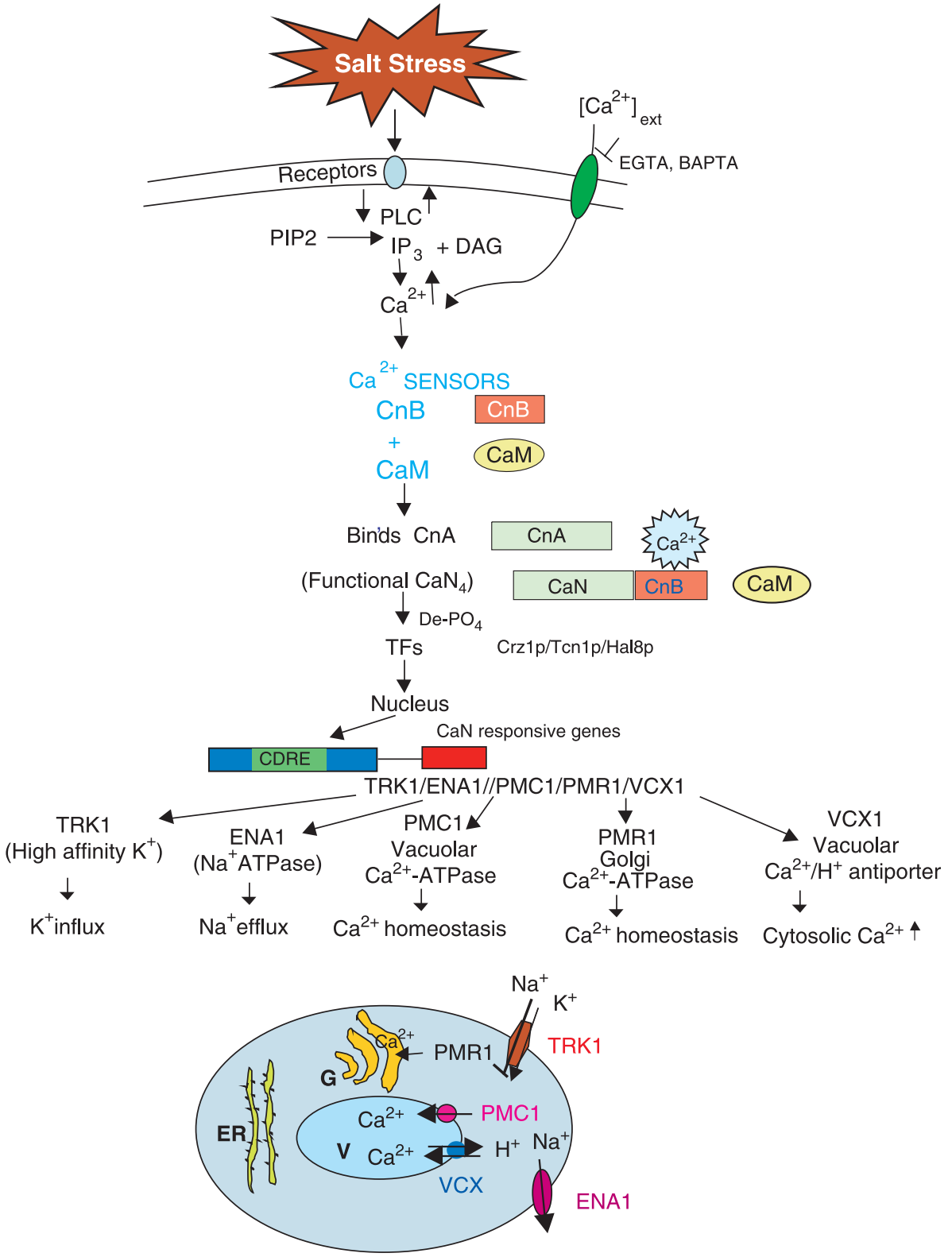

Fig. 1: Calcineurin mediated signal transduction pathway in response to salt stress in yeast. *Stress signal is first perceived at the membrane level by receptors and the signal is then transduced down stream. Ca^{2+} release can be primarily from extracellular source (apoplastic space) as addition of EGTA and BAPTA blocked CaN mediated activity. Ca^{2+} release may also result from activation of PLC, leading to hydrolysis PIP_2 to IP_3 and subsequent release of Ca^{2+} from intracellular Ca^{2+} stores. This change in Ca^{2+} concentration is sensed by Ca^{2+} sensors CnB and CaM, which accordingly change their conformations and bind to CnA. CaN in the form of a holozyme get activated (functional CaN) and dephosphorylates various transcription factors (TFs). These TFs translocate to nucleus and bind to CaN dependent response elements (CDRE), present in the promoter region of CaN responsive genes. These genes include* TRK1, ENA1, PMR1, PMC1 *and* VCX1 *perform several functions, the details of which are mentioned in the text. V represents vacuole; G, Golgi apparatus; ER, endoplasmic reticulum; TRK1, a high affinity K^+ transporter; ENA1, a Na^+ ATPase; PMR1, a Golgi localized Ca^{2+} ATPase; PMC1, a vacuolar Ca^{2+} ATPase; and VCX1, a vacuolar Ca^{2+}/H^+ antiporter.*

i.e. TRK1. CaN regulate the phosphorylation status of TRK1 resulting in an influx of K^+ ions. As there is more influx, balance of K^+ ions takes place resulting in ionic homeostais.

2) CaN is required for the induction of *ENA1* gene. This gene encodes a plasma membrane localized P-type ATPase that is primarily responsible for efflux of Na^+ from *S. cerevisae* cells [20].

3) CaN participate in negative control of vacuolar H^+/Ca^{2+}exchanger (VCX) [28,29]. H^+/Ca^{2+} antiporter transports $3H^+$ out of vacuole and draws one Ca^{2+} into the vacuole. The negative regulation of vacuolar H^+/Ca^{2+}exchanger by CaN implies that Ca^{2+} instead of being sequestered in the vacuole is released out thus further increasing the cytosolic calcium levels. This may result in an enhancement of CaN activity via CnB and CaM.

4) In most of the systems switch ON mechanisms are accompanied by OFF mechanisms. Restoration of the increased cytosolic Ca^{2+} level to the resting phase is important for the cell. PMRI and PMCI are the Ca^{2+} ATPase localized on Golgi and vacuolar membrane, respectively. CaN positively regulates both PMR1 as well as PMC1 and function towards the maintenance of cytosolic Ca^{2+} homeostasis.

Recently, several salt tolerant mutants were generated following ethylmethane sulphonate treatment. One of the mutations was mapped in the PMR1 gene which encodes a Golgi localized P type Ca^{2+} ATPase. In this mutant the levels of cytosolic Ca^{2+} were maintained high in comparison to the wild type under high NaCl stress. The pmr1 mutation resulted in the continuous activation of CaN, which enhanced the expression of *ENA1/PMR2* genes rendering salt tolerance to yeast. It was inferred that Pmr1 acts as a major Ca^{2+}-ATPase under high salt stress [30].

B. Plant CBL and CIPK proteins

The SOS3 gene, which was identified by Jiang Zhu and colleagues, shares significant sequence homology with the regulatory subunit of yeast calcineurin (CnB) and animal neuronal calcium sensors. However, despite this similarity of SOS3 with CnB, it is clear that *Arabidopsis* does not have calcineurin in its data bank [31]. Whereas in yeast, Ca^{2+} sensing subunit i.e. CnB interacts and activates a phosphatase to regulate Na^+ levels, in plants SOS3/AtCBL4 interacts and activates a kinase. The lack of CnA in *Arabidopsis* indicates the divergence of salt stress pathway in two different evolutionary species. Later, 10 isoforms of this gene were discovered in *Arabidopsis* and named as calcineurin B-like proteins (CBLs) based on their significant similarity to animal calcineurin B [12]. It seems that during evolution plants may have lost CnA, and CnB works *via* activating protein kinases. There are number of CnB activating kinases probably to modulate various environmental signals perceived by the plants at the same time. Details on historical background of plant CBL and CIPK proteins are specified in Table 1.

Table 1. The historical background of plant CBL and CIPK proteins

S.No.	Year	Major Discoveries / Events	Species	Reference(s)
1	1996-98	Screening of salt overly sensitive mutants and positional cloning of *SOS* genes.	*Arabidopsis*	[11,13,65,76]
2	1998	Stress signaling through calcineurin mediates stress adaptation in plants. In this study a truncated form of catalytic subunit and the regulatory subunit of yeast CaN were co-expressed in transgenic tobacco plants and these lines exhibited NaCl tolerance.	*Arabidopsis*	[77]
3	1999	AtCBL1 could bind Ca^{2+}, interacted with rat CNA and complemented the salt-sensitive phenotype in yeast CNB mutant. *AtCBL1* mRNA strongly increased in response to drought, cold and wounding, in contrast *AtCBL2* and *3* constitutively expressed under all the above stresses.	*Arabidopsis*	[12]
4	1999	Identification of novel kinases associated with CBL like calcium sensors.	*Arabidopsis*	[44]
5	2000	Autophosphorylation of SOS2 proving that it is a functional protein kinase required for salt tolerence	*Arabidopsis*	[53]
6	2000	SOS2 protein kinase physically interacts with and is activated by SOS3.	*Arabidopsis*	[45]
7	2000	Genetic analysis of plant salt tolerance using *Arabidopsis*.	*Arabidopsis*	[78]
8	2000	Each individual member of AtCBL family specifically interacted with only a subset of CIPKs generating specificity.	*Arabidopsis*	[54]
9	2001	The NAF domain of CIPK is a novel and critical domain for interaction with AtCBLs.	*Arabidopsis*	[46]
10	2001	Differential subtraction screening identified the genes that are uniquely stress regulated in salt overly sensitive mutants.	*Arabidopsis*	[79]
11	2001	SOS3 binding motif in SOS2, a 21 amino acid motif (NAF) was found to be an autoinhibitory motif. Removal of this regulatory domain of SOS2 resulted in constitutive activation of protein kinase. Moreover, Thr^{168} to Asp mutation in the activation loop of SOS2 resulted in constitutive expression of SOS2.	*Arabidopsis*	[49]

S.No.	Year	Major Discoveries / Events	Species	Reference(s)
12	2001	AtSR1, a SNF-1 related protein kinase interacted with AtCBL2 and its transcript responded to light.	*Arabidopsis*	[42]
13	2002	SOS2 and SOS3 were shown to regulate SOS1 transport activity. SOS1 was shown to contribute to plasma membrane Na^+/H^+ exchange activity.	*Arabidopsis*	[68]
14	2002	SCaBP5 and its interacting protein kinase PKS3 were found as regulators of ABA responses. Mutants with silenced *SCaBP5* or *PKS3* were hypersensitive to ABA in seed germination, seedling growth and gene expression.	*Arabidopsis*	[64]
15	2002	Three biochemically active SOS2 mutant kinases were bio chemically analyzed. These mutations were SOS2T168D, SOS2T168DΔF, and SOS2T168DΔ308. These mutants preferred Mn^{2+} relative to Mg^{2+}.	*Arabidopsis*	[51]
16	2002	Protein kinase 11 (*PKS11*) and 18 (*PKS18*) was found to express in roots and leaves of mature *Arabidopsis* plants respectively. Enzymes were biochemically characterized. Transgenic plants over-expressing PKS11 were resistant to high concentrations of glucose and those over-expressing PKS18 were hypersensitive to ABA in seed germination and seedling growth. *PKS18* silenced plants were ABA insensitive.	*Arabidopsis*	[50,56]
17	2003	*CIPK3* regulates ABA responses during seed germination and various abiotic stress induced gene expression. Disruption of *CIPK3* altered the expression pattern of a number of stress induced gene markers in response to cold, salt and wounding. This was the first report showing direct involvement of *CBL* and *CIPK* genes in controlling the expression of various stress genes.	*Arabidopsis*	[59]
18	2003	*CBL1* regulates salt, drought and cold responses in *Arabidopsis*. The study was based on T-DNA insertions mutants and complementation of mutant lines.	*Arabidopsis*	[62]
19	2003	Isolation and characterization of a novel rice Ca^{2+} regulated kinase (*OsCK1*) in response to cold, light, cytokinins, sugars and salts.	*Oryza sativa.*	[80]
20	2003	Deletion analysis led to the discovery of a 37 amino acid residue designated as protein phosphatase interaction (PPI) motif of SOS2 that is necessary and sufficient for interaction with ABI12.	*Arabidopsis*	[55]
21	2003	The crystal structure of AtCBL2 was determined at 2.1A resolution.	*Arabidopsis*	[32]
22	2003	Mutation of *CBL1* impairs plant responses to drought and salt stress but not ABA.	*Arabidopsis*	[61]
23	2004	This study identified branches in the SOS pathway. The study demonstrated that the activity of tonoplast Na^+/H^+ exchanger is controlled by SOS2 kinase.	*Arabidopsis*	[81]
24	2004	SOS2 was shown to regulate the H^+/Ca^+ antiporter CAX1. SOS2 interacted with the N terminus of CAX1 and the activation of CAX1 via SOS2 was independent of SOS3.	*Arabidopsis*	[72]
24	2004	Expression of *CBL9* was shown to be inducible by multiple stress signals and ABA in young seedlings. Mutants of *CBL9* showed hypersensitivity to ABA at various stages of growth and development.	*Arabidopsis*	[58]
25	2005	The crystal structure of SOS3 was solved in complex with Ca^{2+} and with Ca^{2+} and Mg^{2+}. It was shown that SOS3 exists in dimeric conformation.	*Arabidopsis*	[36]
26	2005	Expression of *AtCIPK14* was shown to be induced by metabolic sugars and localization study pointed to a vascular specific expression of AtCIPK14.	*Arabidopsis*	[60]
27	2005	The localization and function of OsCBL2 was examined in rice. OsCBL2 was shown to be G.A responsive and localized to tonoplast of aleurone cells mediating the vacuolation of those cells.	*Oryza sativa*	[43]
28	2006	A CIPK from pea that interacts with and phosphorylates pea calcium sensor CBL and is coordinately upregulated with *CBL* in response to abiotic stresses, wounding, calcium and salicylic acid.	*Pisum sativum*	[39]

C. Structure of CBL

The broad structure of the CBL protein is comparable to that of CNB and NCS proteins. The polypeptide chain of CBL is folded into two globular domains, which are connected by a short linker [32]. Most of the Ca^{2+} sensors bind Ca^{2+} using a helix-loop-helix motif termed as the 'EF hand' which binds a single Ca^{2+} molecule with high affinity [33]. The Ca^{2+} sensors utilize the side chain oxygen atoms of the EF hand motif for Ca^{2+} coordination. In 1973 Kretsinger and Nockolds [34] first discovered the EF hand structural motif in the crystal structure of parvalbumin.

Plant CBLs contain four EF hands that differ in their degree of conservation in comparison to the canonical EF hand sequence. The size of the linker region between the EF hand loops is entirely conserved in all reported plant CBL proteins. This conservation appears to be exclusive to this family of calcium sensor proteins. EF1 and EF2 are separated by 22 amino acids, EF2 and EF3 by 25 amino acids and EF3 and EF4 have 32 amino acids inserted between them. This uniqueness in the number as well as spacing of EF hands also holds true for all predicted rice CBL (OsCBL) proteins [35]. The extension or reduction of the N and C terminal regions

of CBL proteins is primarily responsible for their size variations.

The crystal structure of AtCBL2 has recently been solved at 2.1 Å resolution [32]. The polypeptide chain of AtCBL2 is folded into two globular domains i.e. the N and the C terminal domain and forms a compact a helical structure. It is composed of 9 α-helices and 4 short β-strands. A short linker connects these two domains. Each AtCBL2 molecule binds two calcium ions. The first and fourth EF hand of AtCBL2 coordinates two calcium ions, whereas internal hydrogen bonding results in an open conformation of EF2 and EF3 rendering them incapable of calcium binding.

Recently, the structure of SOS3/AtCBL24 was also solved, which exists in a dimeric conformation [36]. The crystal structure of SOS3 dimer in complex with Ca^{2+} was resolved at 2.75 Å resolution and in complex with Ca^{2+} and Mn^{2+} at 3.0 Å resolution respectively. It was shown that Ca^{2+} binding is responsible for SOS3 dimerization by using analytical ultracentrifugation experiments and circular dichroism measurements. SOS3 exists as a two domains structure connected by a short linker as also true for AtCBL2. The two molecules forming a dimer interact through their C terminal ends. The oligomerization state of SOS3 depends on various factors including localized increase in protein concentration, binding of specific ligand with protein or change in cellular milieu of the cell. The electron density mapping at SOS3 metal binding sites revealed that SOS3 can bind four Ca^{2+} ions.

The differential Ca^{2+} binding ability of different Ca^{2+} sensors may also be critical criterion towards the deciphering of the Ca^{2+} signatures and manifesting signal specificity. The first EF hand (EF1), although conserved in all CBLs, has single amino acid substitutions at critical Ca^{2+} binding positions (either X, Y and Z or -Y, -X and -Z). AtCBL protein family can be divided on the basis of the canonical EF hands present in its members. Four AtCBLs namely 6, 7, 8 and 10 harbor a single canonical EF hand. AtCBLs1 and 9 harbors 2 canonical EF hands whereas AtCBL2, 3, 4 and 5 lack any canonical EF hand. Disparity in the sequence of EF hands may result in differential affinity of CBL proteins towards binding Ca^{2+} ions. Whether such differences contributes to differential deciphering of Ca^{2+} signatures in response to various environmental cues remains to be experimentally verified [35]. AtCBL1 and AtCBL9 have EF3 and EF4 as conventional EF hand Ca^{2+} binding motifs. These conventional EF hands would favor Ca^{2+} binding with higher affinity as compared to other AtCBLs. The canonical EF hand sequence can also be identified in the EF binding domain 3 of AtCBL6, EF2 of AtCBL7 and EF4 of AtCBL8 and AtCBL10 [35].

D. Localization of various CBLs

Proteins often undergo post-translational modifications and these modifications can determine the sub-cellular localization of proteins. Some structural parameters of CBLs suggest that these Ca^{2+} sensors could change their cellular localization and help the protein to perform different functions [3]. Some CBLs can undergo co-translational modification by the addition of myristate group to the N-terminal target sequence (MGXXXS/T) at the glycine residue [37]. This myristoylation plays an important role in the anchoring of the protein to the membrane and also for protein-protein interaction. To augment the affinity of the protein to the membrane, which is mostly composed of lipids, a palmitoyl group is frequently added post-translationally to the cysteine residue adjacent to the myristoylated glycine. This enhances the stability of protein-membrane interaction.

Four members of AtCBL protein family i.e. CBL1, CBL4, CBL5 and CBL9 harbor conserved myristoylation motifs in their N- terminal sequences. Myristoylation has been shown to be essential for the function of AtCBL4/SOS3 in salinity tolerance. Treating young *Arabidopsis* seedling with the myristoylation inhibitor 2-hydroxy myristic acid (HMA) mimicked the phenotype of the *sos3-1* (mutant) plants, which have reduced salt tolerance [38]. Localization studies by using immunoflorescent techniques and confocal microscopy, has revealed that *Pisum sativum* CBL (PsCBL), a homologue of AtCBL3 is localized in the cytosol [39]. This observation is consistent with the in silico prediction that the AtCBL proteins, which lack myristoyl group, seem to be localized in the cytosol.

E. Genomic organization of *AtCBLs*

Genes encoding *CBLs* and *CIPKs* from plants other than *Arabidopsis* and rice plants have also been reported in the NCBI database, although no detailed work has been done. These include *Medicago truncatula* (9 CBLs and 11 CIPKs), *Triticum aestivum* (11 CBLs and 29 CIPKs), *Hordeum Vulgare* (9 CBLs and 14 CIPKs), *Glycine max* (7 CBLs and 13 CIPKs), *Pinus* sp. (2 CBLs and 7 CIPKs), and the moss *Physcomitrella patens* (4 CBLs and 3 CIPKs). Genes encoding CBLs or CIPKs have not been identified outside the plant kingdom by computer analysis indicating that the function of these genes is restricted to plants [35]. *AtCBLs* are encoded by small gene family [12]. However, *PsCBL* (one isoform of pea CBL gene family) is present in a single copy in the pea genome [39].

The coding regions of *AtCBL* genes contain six or seven introns. The N terminal coding region of *AtCBL10* harbors an additional intron, lacking any counterpart in

other *AtCBLs*. The position of four introns is entirely conserved in all the 10 *AtCBL* genes however, the other three introns lack in either one or two members of the *CBL* gene family. This may be attributed to the event of intron loss during evolution. *AtCBL7* gene, which is located in tandem orientation with *AtCBL3* in *Arabidopsis* genome, contains an intron in a position specific for only this locus [35].

5'-untranslated region (UTR) of *AtCBL1*, *2*, *3* and *4* harbor introns. *AtCBL1* and *9* contain one intron and *AtCBL2-4* harbor 2 introns in their 5' UTR region [35]. The regulatory function and significance of this unusual intron composition still awaits experimental validation. Analyses of the *Arabidopsis* genome have revealed that segmental as well as tandem duplications of chromosomal regions during evolution has contributed significantly in shaping the current structure of this genome [40,41]. *CBL* genes are by and large concentrated on chromosomes IV and V. Chromosomes II and III of *Arabidopsis* genome lack any CBL loci and *AtCBL8* represents the only exceptional *CBL* gene located on chromosome I [35].

F. Expression profile of *CBLs* in stress conditions

To evaluate the role of these genes under stress conditions, the induction of mRNA under various stress conditions was investigated. mRNA levels of *AtCBL1* strongly increased in response to wounding, drought and cold treatments. However, the levels were constitutive under heat shock or mechanical touch [12]. These studies also observed that the expression of *AtCBL2* and *AtCBL3* did not respond to these stimuli. It was later shown that the transcript of *AtCBL2* increased upon illumination of leaves and seems to follow a light regulatory pathway [42]. It is noteworthy to mention here that PsCBL showing 90% identity with AtCBL3 strongly upregulated under various stresses including cold (40^0C, 9 h), salt (150 mM, 12 h), wounding (3 h), salicylic acid (4 h) and exogenously provided $CaCl_2$ (5 h) [39]. The difference in the two studies seem to be the fact that previous studies concentrated on early time points (before 2 hours) whereas PsCBL upregulated strongly at later time points. We propose that *PsCBL* and most likely *AtCBL3* are involved in the maintenance of stress response, initiated by other AtCBLs. *AtCBL4*/*SOS3* expression was up regulated in response to salt (NaCl) stress and functions in maintaining ionic homeostasis. *AtCBL9* transcript was highly induced in response to abscisic acid treatments. Very recently, it has been found that the transcript of *Oryza sativa* CBL (OsCBL2) is strongly upregulated in response to GA for up to 48h of incubation. However, no upregulation was seen in response to ABA treatments [43].

G. CBL-interacting protein kinases (CIPKs):The effectors of calcium signaling

To transduce and amplify the decoded Ca^{2+} signal, protein sensors often interact with downstream target molecules. These downstream molecules are therefore also termed as effectors of Ca^{2+} signaling. In context to CBLs, a family of novel serine-threonine protein kinases was recognized as a cellular target for AtCBL Ca^{2+} sensor proteins by using yeast two hybrid screens [44,45]. In silico analysis as well as yeast two-hybrid interactions discovered the presence of 25 *CIPK* genes in the *Arabidopsis* and 30 *CIPKs* in rice genome [35,46,47]. In contrast to *CBLs*, which are mainly concentrated on chromosome IV and V, the 25 *CIPK* genes are dispersed among all five *Arabidopsis* chromosomes. Moreover, in contrast to the *CBL* gene family members in which all the 10 *AtCBLs* harbor introns, only eight of 25 *AtCIPK* genes harbor multiple intron sequences and the others are intron less. The intron containing members of *CIPKs* include *1*, *3*, *8*, *9*, *17*, *21*, *23*, *24*, and *16*. As in CBLs, the phase and location of these introns, if present, is fairly conserved [35].

The pattern of introns, as observed for *Arabidopsis CIPKs* also holds true for rice CIPKs. In silico analyses have reveled that rice *CIPK* gene family can also be divided into intron-less (22 *OsCIPKs*) and intron-harboring (eight *OsCIPKs*) members. *OSCIPKs* harbor 11 to 13 introns, which are all conserved in phase and position. *PsCIPK* gene (67% identical with AtCIPK12) is an intron-less representative of the *CIPK* gene family [39]. Similar to AtCIPK1 [44], *PsCIPK* is also present in single copy in the pea genome [39].

H. Domains of CIPKs and its interaction with CBLs

Sequence analysis of CIPKs (CBL interacting protein kinases) revealed a two-domain structure: the N-terminal catalytic and C-terminal regulatory domain (Fig. 2). The N-terminal part of these proteins comprises of the catalytic region with a characteristic 11-domain structure. This domain is structurally most similar to the SNF1 (Sucrose non fermenting) kinase from yeast and AMPK (AMP-activated protein kinase) from animals [3,46]. Due to this structural similarity, the CIPKs have also been assigned to the SnRK3 subgroup of plant SNF-like kinases [48]. However, CIPKs are functionally distinct from SNF kinases and exhibit different modes of regulation [35].

The catalytic domain of CIPK contains a typical activation loop between the conserved amino acid residues DFG and APE (Fig. 2). The mutation of a conserved Thr (threonine) residue in the activation loop with aspartate (D) resulted in a constitutively hyperactive enzyme, the activity of which becomes independent of

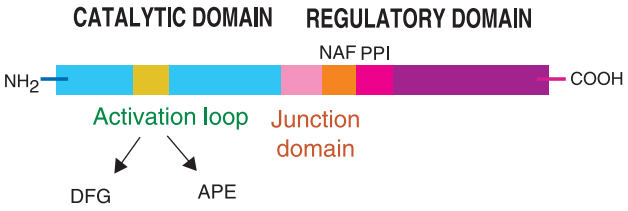

Fig. 2: Schematic representation of the domains of CBL-interacting protein kinases (CIPKs). *CIPK comprises of a two-domain structure, N terminal catalytic (shown in* blue*) and C terminal regulatory (shown in* pink*). Catalytic domain harbors an activation loop between the conserved amino acids DFG and APE (shown by diagonal lines). Regulatory domain is unique and harbors NAF motif (shown with check box), which acts as an autoinhibitory domain and is also responsible for the interaction of CIPK with CBL. PPI domain is adjacent to NAF motif and responsible for interaction with phosphatases. Catalytic and regulatory domains are connected by a junction domain which is also responsible for kinase activation (shown by red colour).*

CBL [49]. The mutation of Thr residue with aspartate (T-D) partially mimics the phosphorylation by an upstream kinase(s) [49,50]. Interestingly, SOS2/AtCIPK24 could also be activated *in vitro* by the mutation of serine-156 or tyrosine-175 residue in the activation loop to aspartate [51]. These results indicate that some putative kinase(s) regulate the activity of CIPKs by phosphorylating important residues in the activation loop. However, the identity of kinases, phosphorylating CIPK still eludes. Transphosphorylation by yet unknown kinases is important for the activation of calcium dependent protein kinases (CDPKs) [52]. It has been speculated that the inter-phosphorylation events between the CDPKs and CIPKs could be an important parameter in the regulation of these kinases. Moreover, MAPKs and even other kinases may also phosphorylate CIPKs leading to their activation [see 47].

The regulatory C-terminal domain of CIPKs was considered novel as it was found to be uniquely present in this subgroup of kinases and is absent in other SNF-1 related kinases [44]. Within the C-terminal regulatory domain, a stretch of 21 amino acids, designated as the NAF/FISL motif has been shown to be required and sufficient for interaction with AtCBLs. NAF domain acts as an auto-inhibitory domain and binds to the catalytic domain and thus blocks access of the substrate to the catalytic site. The enzyme in the normal state shows low autophosphorylation activity. CBL interacts with CIPK via FISL/NAF motif and relieves the catalytic domain of autoinhibition thus making the enzyme active. The deletion of NAF motif makes CIPK constitutively active and independent of CBL. Part of CIPKs is similar to Chk1 (check point kinase 1) and this similarity is interesting as *sos2* mutants show cell cycle defect at the root meristem in the presence of Na^+ stress [53].

Every CBL does not interact with each of the 25 CIPKs and hence interaction specificity is maintained. Complex formation of CBLs and CIPKs is rather differential and also preferential, generating the specificity required in the Ca^{2+} signaling pathway [46,49,54]. It is speculated that the regions surrounding NAF domain is responsible for maintaining high degree of observed specificity [46,54]. Deletion of the junction region between the NAF motif and the catalytic domain eliminated the activation of the resultant protein [49].

Deletion analysis led to the identification of a novel protein domain of 37 amino acid residues that is necessary and sufficient for interaction with abscisic acid-insensitive 2 (ABI2), phosphatase [55]. This domain was designated as the protein phosphatase interaction (PPI) motif of AtCIPK24. PPI motif is conserved in protein kinases of the SOS2 family and in the DNA damage repair and replication block checkpoint kinases chk1. The mutation of Arg-340 and Phe-341, which are highly conserved in the PPI motif with alanine, abolished the interaction of the SOS2 with the ABI2 signifying that these two conserved residues in the protein kinase are important for interaction with the ABI phosphatases. A mutation in ABI2 (*abi 2-1*) that affect the interaction resulted in an increased tolerance to salt shock and abscisic acid insensitivity in plants [55].

The kinase activity of AtCIPK1 has been demonstrated *in vitro* using both GST-CIPK1 fusion protein and the purified CIPK1 after cleavage. CIPK1 showed autophosphorylation activity, and the divalent cation Mn^{2+} was shown to be a much more effective co-factor than Mg^{2+}. CIPK could autophosphorylate on its serine and threonine residues but not on a tyrosine residue, suggesting that CIPK is a serine/threonine protein kinase with Mn^{2+} as a preferred cofactor [44]. The kinase activity of AtCIPK1 was also determined in terms of its substrate phosphorylation. The substrates tested included casein, myelin basic protein, histone HI, and histone IIIS. Neither of these protein substrates showed phosphorylation to any significant extent when compared to autophosphorylation. The interaction of AtCIPK1 with AtCBL1 was investigated in a “pull down” assay. AtCBL1 interacted with AtCIPK1 in a Ca^{2+} dependent manner [44].

Regarding cofactor preference, in broad sense, for various CIPKs whose activity profile has been analyzed in detail, Mn^{2+} is a preferred cofactor over Mg^{2+} [44,50, 51, 56, 57]. Optimum activity of these enzymes is observed at around 2.5 mM Mn^{2+} whereas 5 mM or higher concentrations of Mg^{2+} are required for reaching an optimum activity. These enzymes work maximally at an optimum pH range between 7.0 and 7.5 and temperature optimum of 30°C. These kinases do not

show any significant activity against the commonly used protein substrates such as myelin basic protein (MBP), histone H1 and casein.

I. CBL/CIPK signaling, a complex web, but still specific

In CBL/CIPK network 10 sensor proteins and 25 effector kinases interacting in various permutations and combinations provide a mode to specifically decode and relay the diverse information in the form of various environmental cues. In spite of the complexity in CBL/CIPK network with large number of Ca^{2+} sensors and CIPKs, each stress signal is decoded accurately and leads to a precise response. In brief, several mechanisms like differential tissue distribution, stage specific expression, stress specific expression, differential interaction of CBLs and CIPKs, cofactor dependence of the kinases and the substrate preference of each single CIPK, pH, temperature dependence, kinetic properties, localization, Ca^{2+} binding affinity of different CBLs, various promoter elements of these genes will further contribute to fine tuning of the signals along with stringency in reactions. The explanation for the complexity and specificity for this pathway is described below:

(i) ***Tissue distribution, stage specific gene expression and sub-cellular localization:*** The expression of various CBLs and CIPKs corresponds to a particular developmental stage or are restricted to a particular tissue performing specific roles. Sub-cellular localization of a protein may be a critical factor in deciding the function of each calcium sensor and its downstream signaling kinases. For instance, *AtCBL1* gene showed high expression level in roots and stem whereas the expression level was low in leaves and undetectable in flowers. *AtCBL2* showed preferential expression in roots. In contrast, *AtCBL3* showed a ubiquitous expression in all the tissues of the plant [12]. AtCBL9 showed a ubiquitous expression in all the developmental stages of the plant. Promoter-Gus activity revealed that *AtCBL9* is highly expressed in the radicles of germinated seedlings [58].

In accordance, *CIPK* genes also exhibited differential expression. The expression of *CIPK9*/*PKS6* was detectable in various parts of plant tissues including leaves, stem, flowers and siliques but was undetectable in roots of *Arabidopsis* [57]. However, the transcript in young seedlings was relatively less abundant in comparison to mature plant tissue. In contrast, *PKS11* showed preferential expression in the roots of *Arabidopsis* plants [56]. The expression pattern of *PKS18*/*CIPK20* was analyzed in different tissues of mature *Arabidopsis* plant. *PKS18* was expressed in leaves of adult plants and the levels were beyond detection in other examined tissues of the plant [50]. Taken together it can be concluded that different CBLs and CIPKs exhibit differential expression in different tissues under normal growth conditions.

Expression level of CBL and CIPKs may be influenced by the development stage of the plant. For example, the mRNA levels of *AtCIPK3* were high in germinating seeds and young seedlings whereas the levels were barely detectable in all organs of the older plants. Consequently, loss of *CIPK3* function leads to altered phenotypes, particularly during seed germination and in young seedlings [59].

As mentioned earlier, CBLs show specific expression pattern in response to particular stress stimuli. This differential expression of CBLs in response to specific stress stimuli is important for maintaining the specificity in the signaling pathway. CIPKs also exhibit stress specific expression pattern. For example, expression of *AtCIPK3* was induced strongly in response to cold followed by drought, high salt, wounding and ABA [59]. In contrast *CIPK20/PKS18* mRNA did not accumulate significantly by any of the stress treatments [50]. *CIPK8*/*PKS11* also did not show any significant induction by any of the stress treatments. However, transgenic plants over-expressing PKS11 were resistant to high levels of glucose suggesting the possible function of this gene in sugar signaling in plants [56]. Very recently, it has been reported that mRNA levels of *AtCIPK14* increased in response to metabolic sugars such as sucrose, glucose and fructose. Certain A/T-rich elements were found within the AtCIPK14 promoter, similar to promoter elements of sugar responsive genes. Involvement of *AtCIPK14* in the regulation of sugar transporter in vascular tissue has been hypothesized [60]. The expression profiles suggest that the expression pattern of different *CBLs* and *CIPKs* provide flexibility in the signaling network and allows the components to act in accordance with the changing environmental conditions and developmental needs of the plant.

The localization of a Ca^{2+} sensor protein to a specific compartment of the plant cell plays an important role in decoding the spatially distinct Ca^{2+} signatures. In silico analysis indicates that some structural features specify sub cellular localization for these proteins. As mentioned earlier CBLs harboring the myristoylation sites i.e. AtCBL1, 4, 5 and 9 have been localized predominantly at the plasma membrane [11,46,54]. Moreover, other CBLs lacking this myristoylation motif may be primarily cytosolic. This pattern of localization of CBLs and their interacting kinases, allows specific decoding of Ca^{2+} signatures, which are differentiated spatially within a given cell.

CIPKs however do not harbor any decipherable localization signal or any target motif [35]. Therefore, the localization of CIPKs could exclusively be dependent

on their respective interaction partner, which would thus serve the dual role as a calcium sensor and as an anchoring protein, regulating the localization and activity of the interacting CIPK at different locations within the cell.

(ii) ***Preferential interaction of CBLs with CIPKs:*** Most CBLs interact with a subset of four to eight CIPKs and other CBLs interact with only a few protein kinases. This differential interaction is one of the bases for generating temporal and spatial specificity in the signaling pathway. For example AtCBL1 interacted strongly only with a subset of six AtCIPKs namely AtCIPK1, 7, 8, 17, 18 and 24. AtCBL2 and AtCBL3 formed complex with AtCIPK4, 7, 12 and 13 [35,46]. AtCBL5 showed interaction with AtCIPK2 and AtCIPK11. AtCBL9 strongly interacted with a subset of six kinases i.e. AtCIPK1, 8, 18, 20, 23 and 24. AtCIPK1 preferentially interacted with AtCBL1, 2 and 3. AtCIPK 6 interacted preferentially with AtCBL2 [46]. The structural basis for preferential CBL/CIPK complex formation still lacks clarity. Different full-length CIPK proteins exhibit preferential interaction affinity with defined set of CBLs. Variations in the structure of CBL proteins and the high variability in the regulatory domain of the CIPK especially the regions in close proximity to NAF domain suggest that both interacting partners are responsible for generating interaction specificity for a given protein pair [see 47].

J. Mutant analysis of the CBL/CIPK

Mutant analysis leads to the deciphering of the precise function and relevance of the gene. Reverse genetics approaches have recently become a major tool to unravel the function of an increasing number of members of both CBL and CIPK protein families. The expression of *AtCIPK3* was strongly induced in response to cold, high salt, drought, wounding and ABA. Analysis of *Atcipk3* mutant established a function of this kinase in regulating ABA responses during seed germination and in regulating stress-induced gene expression [59]. Disruption of *Atcipk3* resulted in the alteration in the expression pattern of a number of stress-induced genes, which also serves as markers in response to ABA, cold and high salt. Accordingly, mutant plants exhibited delayed induction of stress marker genes including *RD29A*, *KIN1*, *KIN2*, especially in response to cold, salt and ABA [59]. However drought induced gene expression was not altered in the *cipk3* mutant plants. This was the first report, which identified *CBL* and *CIPK* as master switches controlling the major stress genes. These observations may suggest that *CIPK3* functions in an early response phase of gene expression. Since the pathway induced by drought was not altered in the mutant plants, this suggests that *CIPK3* regulates selective pathways. Cold induced pathway has been observed to be largely ABA independent although drought and salt induces ABA synthesis. However, there is no clear line of demarcation between ABA dependent and independent pathways and these pathways often cross talk with each other. *CIPK3* was identified as a cross talk node between ABA dependent and ABA independent pathways as disruption of *Atcipk3* affected both the pathways [59].

The function of *AtCBL1* was investigated separately, by two independent studies by analyzing the T-DNA-induced insertion mutants as well as plants over-expressing AtCBL1 [61,62]. When CBL1 protein was over-expressed in transgenic plants it resulted in alteration in the stress response pathway. Although the expression of the genes in response to drought was enhanced, the gene induction by cold was inhibited. Moreover, CBL1 over-expressing plants showed enhanced tolerance to salt and drought. However, the freeze tolerance was drastically reduced. In contrast, *cbl1* mutant plants showed enhanced induction of stress genes in response to cold stress and reduced induction in response to drought. *CBL1* therefore seems to function as a positive regulator of salt and drought mediated responses and a negative regulator of cold response in stress signaling pathway. Mutation of *CBL1* leads to a drastically distorted regulation of stress-responsive marker genes like *RD29A* and the ''master transcription factors'' including *CBF*/*DREB* [61]. *CBL1* does not seem to follow an ABA dependent pathway, as these mutants were not altered in their responsiveness to ABA [61,62].

Expression of CBL9 (a calcium sensor sharing high identity with CBL1) was inducible by multiple stress signals like salt, cold and drought stress and by ABA [58]. The disruption of *CBL9* led to severe alteration in plants response to ABA and the mutant plants were rendered ABA hypersensitive. Enhanced expression of the genes involved in ABA signaling such as *ABA-INSENSITIVE 4* and *5* was seen in *cbl9* mutant plants. Osmotic stress and salt stress exerted their inhibitory effect on the mutant seed germination through the production of ABA. During the normal process of seed germination in monocots, calcium serves as a second messenger for the synthesis of gibberellic acid (GA) that promotes the synthesis of a amylase in the aleurone cells [63]. This stimulates the breakdown of amylose to simple sugars, which are then used up by the developing seed. ABA antagonizes GA function, thereby inhibiting the germination process. *AtCBL9* seems to acts as a negative regulator for the ABA biosynthesis and thus promotes the seed germination. In addition, seed germination in the mutant also showed increased sensitivity to inhibition by osmotic stress condition produced by high

concentrations of salt and mannitol. This observation was also attributed to ABA accumulation in the *cbl9* mutant plants under stress conditions [58].

Overall, these findings suggest that *CBL9* functions as a negative regulator of calcium-induced ABA signaling and regulates the biosynthesis of ABA. Two more genes of this pathway were specifically found to be ABA responsive. The Ca^{2+} binding protein SCaBP5, and its interacting protein kinase PKS3 were found to regulate ABA responses [64]. *Arabidopsis* mutants with silenced *ScaBP5* or *PKS3* were hypersensitive to ABA in seed germination, seedling growth, stomatal closure and gene expression. Thus *SCaBP5* and *PKS3* were found to serve as negative regulators of the ABA signaling pathway, controlling ABA sensitivity. Very recently, it has been found that the transcript of *Oryza sativa* CBL (OsCBL2) strongly upregulated in response to GA, however, no upregulation was seen in response to ABA treatments. Moreover, OsCBL2 was shown to be localized to the tonoplast of aleurone cells indicating their function in a GA mediated signaling pathway leading to vacuole function of the aleurone cells [43].

K. SOS pathway in response to salt stress

As mentioned earlier, the *SOS* genes (*SOS1*, *SOS2* and SOS3) were isolated through positional cloning and their isolation and characterization resulted in the discovery of a novel pathway and unrevealed the mechanism involved in plants response to ionic stress. This pathway also unrevealed the significance of Ca^{2+} signal to reinstate cellular ion homeostasis [65, 66]. *SOS3* gene encodes a Ca^{2+} binding protein with N-terminal myristoylation motif and 4 Ca^{2+} binding EF hands. SOS3 senses the change in cytosolic Ca^{2+} concentration and transduces the signal downstream. A loss of function mutation that reduces the Ca^{2+} binding capacity of SOS3 (*sos 3-1*) renders the mutant hypersensitive to salt [38]. This defect can be partially rescued by addition of high levels of Ca^{2+} in the growth medium [11]. Compared to other Ca^{2+} sensors like calmodulin and caltractin, SOS3 binds Ca^{2+} with a relatively low affinity. This difference in the affinity may be an important factor in distinguishing and decoding various Ca^{2+} sensors.

The genetic screenings for salt tolerant genes also lead to the isolation of *SOS2* locus in *Arabidopsis*. *Atsos2* mutant exhibits hypersensitivity to NaCl stress. SOS2, similar to other CIPKs, encodes a novel serine/threonine protein kinase with an N catalytic and C terminal regulatory domain. Under normal conditions, the kinase is under autoinhibition via its FISL/NAF motif. FISL-motif in the regulatory domain of SOS2 is necessary and sufficient for interaction with SOS3. Interaction of SOS3 with SOS2 via the FISL motif relieves the autoinhibition of SOS2 and the kinase becomes active. Deletion of FISL motif results in the constitutive activation of SOS2 [49] and makes SOS2 independent of SOS3. SOS3 activates SOS2 protein kinase activity in a calcium dependent manner [45].

The analysis of *sos3*/*sos2* double mutant had no additive effects of mutant plant towards salt sensitivity, indicating that *SOS3* and *SOS2* function in the same pathway [45]. Over-expression of active forms of SOS2 under the control of CaMV355 promoter rescued the salt-sensitive phenotype of both *sos3* and *sos2* further supporting that SOS3 and SOS2 function in the same Ca^{2+} signaling pathway during salt stress [66]. The transcript level of SOS2 was up regulated in response to salt stress in the root [53].

The first target of the SOS3-SOS2 pathway was identified by genetic analysis of the *sos1* mutant of *Arabidopsis*. *sos1* mutant was hypersensitive to salt and showed impaired osmotic/ionic balance. Genetic analysis confirmed that SOS3, SOS2 and SOS1 function in a common pathway of salt tolerance [65]. *SOS1* gene was cloned and predicted to encode a 127-kDa protein with 12 trans-membrane domains in the N-terminal part and a long hydrophilic cytoplasmic tail in the C-terminal part [67]. The trans-membrane region of SOS1 had significant sequence similarity to the plasma membrane Na^+/H^+ antiporter isolated from bacteria and fungi [67].

The Na^+/H^+ antiport activity of SOS1 was studied in the highly purified plasma membrane vesicles isolated from the wild type and sos1 mutant plants. The results clearly revealed that wild type plants treated with 250 mM NaCl could retain the plasma membrane Na^+/H^+ exchange activity whereas 80% reduction was seen in similarly treated sos1 plants. Addition of SOS2 protein *in vitro* lead to two fold increase Na^+/H^+ exchange activity of the salt challenged wild type plant. Conversely, addition of SOS2 failed to stimulate the exchange activity in *sos1* mutant plants. Direct evidence demonstrating that the antiport activity of SOS1 is regulated by SOS2 kinase, came from the experiments in which plasma membrane Na^+/H^+ antiport activity was compared in the vesicles isolated from *sos2* and *sos3* mutant plants. Vesicles of sos2 and sos3 plants exhibited reduced Na^+/H^+ antiport activity in comparison to wild type. The antiport activity could be restored by the *in vitro* addition of activated SOS2 protein kinase [68]. The SOS3-SOS2 kinase complex was found to phosphorylate SOS1 directly [69]. Myristoylation is required to recruit SOS2 to the plasma membrane where it can phosphorylate and activate SOS1. Recently SOS pathway has been functionally reconstituted in yeast further demonstrating that a salt stress induced Ca^{2+} signal is transduced by the SOS3-SOS2 kinase complex to activate SOS1 and re-establish cellular ion homeostasis [69].

The SOS pathway is depicted in Fig. 3. Salt stress, is perceived by an unknown sensor and this elicits a change in cytoplasmic calcium signal. This change in cytoplasmic calcium signal is sensed by a mytistoylated calcium sensor SOS3 and translates the signal down stream. SOS3 interacts with and activates a serine/threonine protein kinase SOS2. SOS3 interacts with SOS2 via the FISL motif and relieves SOS2 of its autoinhibition. SOS3 recruits SOS2 to the plasma membrane where SOS2 phosphorylates and activates a plasma membrane Na^+/H^+ antiporter i.e. SOS1. The excess Na^+ ions are removed out of the cell and cellular ion homeostasis is maintained. SOS pathway, also seem to have other branches. In *Arabidopsis*, Na^+ entry into root cells during salt stress appears to be mediated by AtHKTI, a low affinity Na^+ transporter [70]. The *Athktl* mutation suppresses the *sos3* mutation [71] this suggests that the SOS3-SOS2 kinase complex may prevent Na^+ influx by down regulating *HKTI* gene expression or inactivating the HKTI protein during salt stress [66]. SOS3 and SOS2 may serve as negative regulators of AtHKT1 activity under salt stress.

SOS2 is also shown to interact with vacuolar Na^+/H^+ antiporter (Fig. 3) and SOS3-SOS2 complex may also activate the vacuolar Na^+/H^+ antiport activity [68].

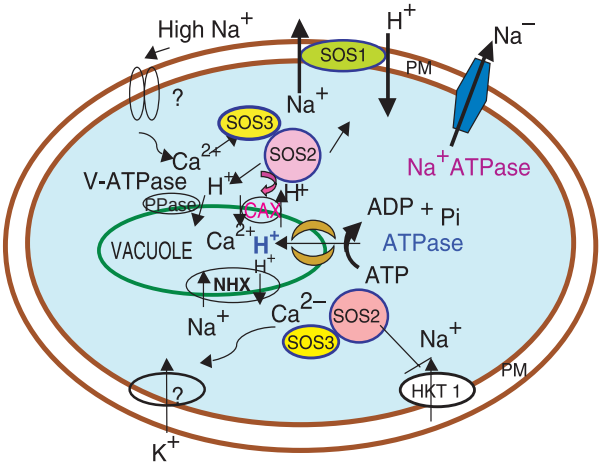

Fig. 3: Regulation of ion homeostasis by SOS and related pathways in relation to salt stress adaptation: *Salt stress is perceived by an unknown receptor (?) present at the plasma membrane of the cell. The salt stress induced cytosolic calcium perturbation is sensed by SOS3, which accordingly changes its conformation and interacts with SOS2. This interaction relieves SOS2 of its auto-inhibition and results in activation of the enzyme. Activated SOS2 in turn phosphorylates SOS1, a Na^+/H^+ antiporter resulting in efflux of Na^+ ions. SOS3-SOS2 complex have other branches mediating control of other pathways. This complex inhibits HKT1 activity thus restricting Na^+ entry into the cytosol. SOS2 also interacts and activates NHX (vacuolar Na^+/H^+ exchanger) further contributing to Na^+ ion homeostasis. CAX1 (H^+/Ca^+ antiporter) has been identified as an additional target for SOS2 activity. This activity reinstates cytosolic Ca^{2+} homeostasis. Further, Na^+ ATPases have been identified in bryophytes, which may be an additional Na^+ efflux pathway maintaining Na^+ concentration par toxic level.*

Systematic studies were undertaken to determine if SOS pathway can regulate the activity of tonoplast Na^+/H^+ exchanger. Tonoplast Na^+/H^+ could be inhibited by 5-(N-methyl-N-isobutyl) amiloride and also by Na^+/H^+ antibodies. The comparative analysis revealed that the Na^+/H^+ exchange activity was significantly lower in sos2 mutants than in the wild type. *In vitro* addition of activated SOS2 protein increased tonoplast Na^+/H^+ exchange activity in the vesicles isolated from *sos2* but did not mediate any effect on the activity of vesicles isolated from wild type, *sos1* or *sos3*. SOS2 has also been shown to regulate the H^+/Ca^+ antiporter CAX1. SOS2 interacted with the N terminus of CAX1 and the activation of CAX1 via SOS2 was independent of SOS3 [72]. This reflects that the components of SOS pathway may cross talk and interact with other branching components to maintain cellular ion homeostasis. Recently, it has been reported that in *Physcomitrella patens*, a moss, two P type Na^+ ATPases, which are structurally related to fungal ENA type ATPases exist. Moses are in the same lineage as the higher plants during the origin of green plants from the unicellular common ancestor. Major signaling pathways including those based on calcium are conserved in mosses and are similar to higher plants [73]. It was also found that *Physcomitrella patens* harbors a plant type SOS1 Na^+/H^+ antiporter. It has been hypothesized that early land plants were tolerant to Na^+ because of their harsh hygrophytic environment [74].

Though, CaN do not exist in *Arabidopsis*, expression of STO gene (salt tolerance related gene) from *Arabidopsis* could complement salt sensitive phenotype exhibited by calcineurin mutant. This suggests the involvement of STO, like SOS3 in regulating internal Na^+/K^+ ratio. Recently, a protein from *Arabidopsis*, namely H-protein promoter binding factor-1 (HPPBF-1), which can bind to STO protein, was isolated. The expression of HPPBF-1 gene was up regulated in response to salt stress and the protein was nuclear localized. Over expression of STO in *Arabidopsis* conferred increased salt tolerance to transgenic plants [75]. Therefore, although calcineurin does not exist in plants there are several genes which function in a similar manner imparting salt stress tolerance to plants. However, the detailed functioning of these genes in response to salt stress still lacks clarity.

L. Models to explain stress signaling through CBL/CIPK network

Taken together, *CBL-CIPK* system responds to various environmental and developmental cues quite precisely due to preferential interactions between different CBL-CIPK members and subcellular localizations. They also function as positive and negative regulators of various

stress genes involved in stress signaling pathways. We explain two models for the functioning of CBL/CIPK pathways. The first is a generic model explaining the overview of CBL/CIPK pathway (Fig. 4). The other model is based on the experimental evidences available from *Arabidopsis*, rice and pea (Fig. 5).

In the generic model (Fig. 4), the transmission of a signal involves the interaction of extracellular ligand (in form of a stress signal) with the transmembrane protein. The ligand binding on the extracellular side of the transmebrane influences the activity of the receptor domain localized on the cytoplasmic side. This generic process is termed as signal transduction as the signal gets transduced across the membrane. The first outcome of a stress signal is the increase in cytosolic Ca^{2+} levels, which may be mediated by the action of PLC. Various second messengers like Ca^{2+}, Reactive Oxygen Species (ROS), InsP and ABA can be produced which further amplify the signal. The localized increase in Ca^{2+} concentration is sensed by Ca^{2+} sensor CBLs. Activated CBLs interact with specific CIPK partners in various permutations. These CIPKs may phosphorylate transcription factors, which in turn can control the activity of some of the major genes involved in mediating stress tolerance. Kinases like CIPK3 may influence the cold responsive genes like RD29A, KIN1 and KIN2 leading to cold stress tolerance [59]. Other CBLs for example AtCBL1and their interacting kinases may control other stress responsive genes both positively and negatively and lead to tolerance against dehydration, osmotic stress, wounding and ABA accumulation [62]. In case of a salt stress, CBL4/SOS3 bind Ca^{2+} and activates SOS2 in turn phosphorylate SOS1 leading to ionic homeostasis. These pathways can often cross talk with each other as many stress pathways also share the major genes involved in stress tolerance.

Regarding the second model (Fig. 5), certain CBLs seem to be sensors for more than one stress, for example, CBL1, which in turn interacts with more than one CIPK. CBL can act as both positive and negative regulator of stress pathway. Whereas, CBL1 functions as a positive regulator for salt and drought responses, it serves as a negative regulator for cold responsive genes [62]. *PsCBL* like CBL1, is also strongly up regulated by many factors including cold, salt and wound. However, the induction of *PsCBL* by stress was found to be a late response and may be involved in maintenance of stress responses [39]. Other CBLs, like AtCBL4/SOS3 have so far been shown to respond to a single exogenous stress factor like salinity. AtCBL9 is induced by hormones like ABA, whereas

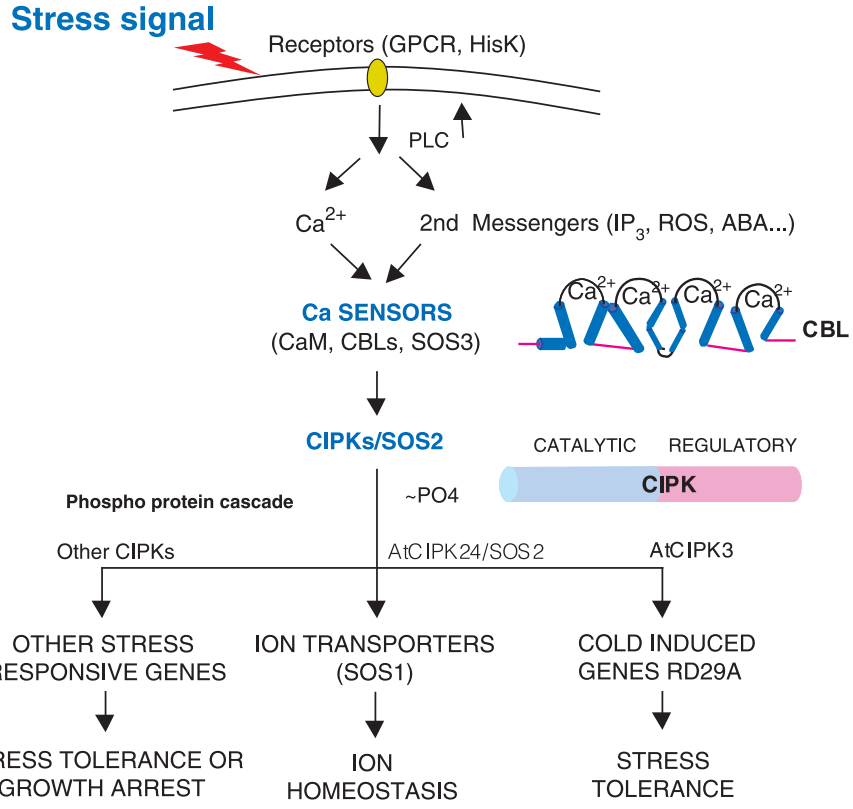

Fig. 4: A generic signal transduction pathway mediated via CBL/CIPK signaling network in response to various stress signals in plants. *Details are furnished in the text. Briefly, the first response to a stress signal is the change in cytosolic Ca^{2+} level, which may be mediated via PLC pathway. This change is perceived by Ca^{2+} sensors (like CBL,), which interact and activate their respective CIPK partner(s), which in turn may phosphorylate the downstream signaling components leading to activation of major stress responsive genes. The schematic structures of CBL and CIPK are also shown.*

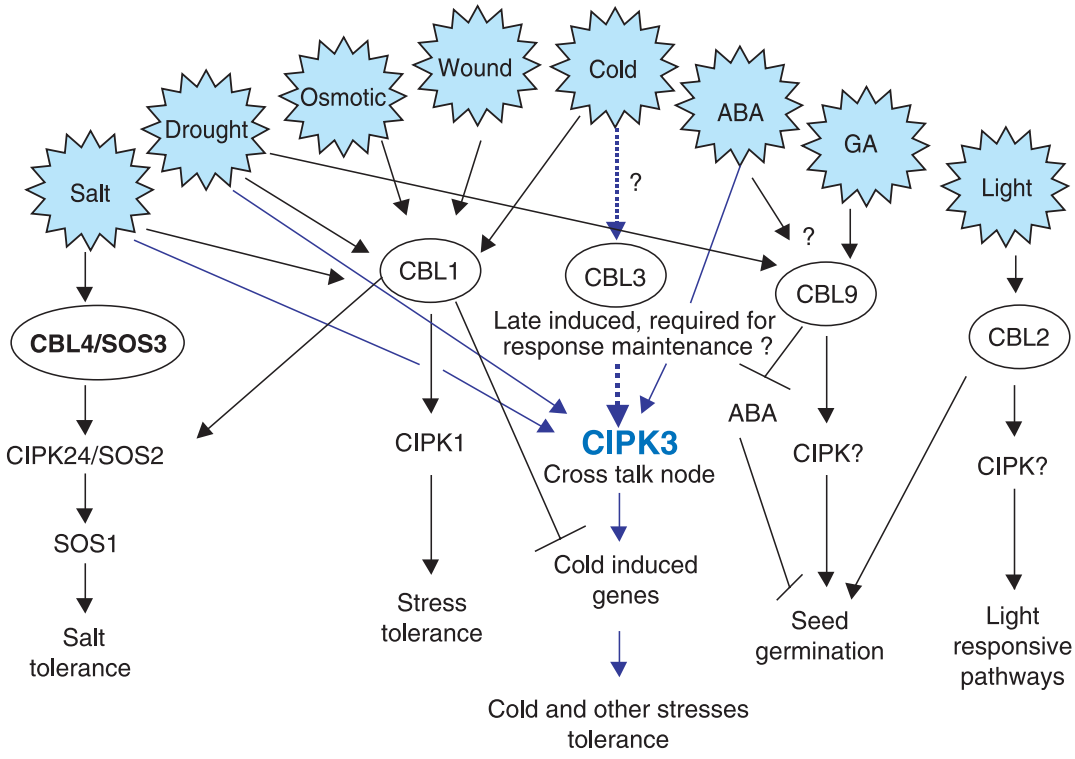

Fig. 5: Involvement and cross-talk between CBLs and CIPKs in modulating stress, hormonal and light responses in plants *(updated from Batistic and Kudla, [51]). The information is compiled from data obtained in Arabidopsis, rice and pea (our un published data). The details are explained in the text. Briefly, CBL1 serves as a positive regulator for salt and dehydration and a negative regulator for cold stress. AtCBL9 has been shown to be a negative regulator of ABA and promotes pathway leading to seed germination. AtCBL2 is light responsive and OsCBL2 is GA responsive and also may serve as important component mediating seed germination. These genes interact with their respective CIPKs and regulate genes involved in stress signal transduction pathway. AtCIPK3 has been identified as a cross talk node mediating both ABA dependent as well as independent pathways. Pathway followed by AtCIPK3 is shown with* blue *arrows.*

AtCBL2 by light [42] and OsCBL2 by gibberellic acid (GA) [43]. CBL9 serves as a negative regulator of ABA responses during seed germination thus antagonizing the inhibition caused by ABA hormone and promoting seed germination [47,58]. Recently OsCBL2 is also involved in vacuolation of aleurone cells mediating seed germination [43]. These Ca^{2+} sensors like CBLs, in turn find their partners either specifically or interact with more than one partner in order to regulate gene expression/ protein activity conferring stress tolerance. Some of these interactions like CBL9/CBL2 with its interacting partners, is yet unknown, regulate developmental responses like seed germination or photomorphogenesis. AtCIPK3 has been shown as cross talk node mediating cold and ABA response pathways [59].

M. Future Prospects

From the time of its discovery in 1997, CBL/CIPK pathway has been well explored in *Arabidopsis* and today there is some clarity on the aspects generating specificity in spite of the huge complexity in this signaling network. This has been possible due to the efforts of the groups of Prof. Luan at University of California, Berkeley and Dr Zhu's work at University of California, Riverside. Some aspects, which have been neglected and relatively need more focus, are: (i) the promoter analysis of various CBLs and CIPKs. As these proteins show high identity at the amino acid level but still behave differentially so the work on UTR regions and the promoter regions requires far more emphasis. Computer analysis should also look for some common regulatory elements in strongly interacting CBL/CIPK partners. (ii) In continuation with the above-mentioned point, 5'UTR region of these genes and the unusual introns present in this region need to be experimentally verified. (iii) More emphasis should be laid on solving the crystal structures of these proteins. Till date no CIPK has been crystallized. CIPK expression in low amount has also been a hindrance to this task. (iv) the facts about physiological substrates of CIPK have also eluded us. Search should focus on the isolation and characterization of various physiological substrates and also check if CBL can be a substrate for any of CIPK. PsCIPK could phosphorylate PsCBL and this activity was significantly reduced by the addition of anti CIPK antibodies [39]. (v) As it is clearly proven that CBLs and CIPKs have role in stress tolerance, therefore stacking of strongly interacting genes and their influence on stress tolerance can be performed in transgenic studies. (vi) CBL/CIPK network has mainly been confined around these two genes families and the

upstream and down stream genes of this pathway have been neglected. Research should also focus on the receptors of this gene family. As CaN in animals can dephosphorylate and activate NFAT and other transcription factors it is tempting to speculate that CIPK may also phosphorylate some transcription factors which in turn can control expression of major genes in the pathway. Moreover, work should not only be confined to model systems like *Arabidopsis* but should also include other economically more useful plants.

Acknowledgement

We thank Department of Biotechnology, Government of India grant for partial support and Council of Scientific and Industrial Research, New Delhi for fellowship to SM. We apologies if some references could not been cited due to space constraint.

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