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

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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-exist 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
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 Ca2+/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 Ca2+ 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 transcription factors involved in the activation of T cells and the IP3 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 CaCl2 is mainly provided by H+/Ca2+ antiporters in which 3 H+ are moved out and one Ca2+ is sequestered in and Ca2+ pumps, which are directly energized by ATP hydrolysis. There is some evidence that G proteins, phospholipase C (PLC) and insitol trisphosphate (IP3) are involved in calcium release [21,22]. Recent reports point to the fact that CaN signaling is required for appresorium 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 Ca2+ release may be required for CaN activation. However, experimental evidences have also proved that NaCl mediated cytosolic Ca2+ increase was attenuated by the addition of chelating agents EGTA, BAPTA, cation channel pore blockers and by the use of competitive inhibitors of Ca2+ transport. These results indicate that external Ca2+ is the source for cytosolic Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ is sensed by Ca2+ sensor proteins such as CnB and CaM, which accordingly change their conformation in Ca2+ dependent manner and get activated. Calcium bound CnB and CaM binds CaN and this result in formation of functional CaN holozyme. Activated CaN dephosphorylates the zinc finger transcription factors CrzlIp/TcnIp/Hal18p, 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 homeostasis.

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. cerevisiae cells [20].

3) CaN participate in negative control of vacuolar H⁺/Ca²⁺exchanger (VCX) [28,29]. H⁺/Ca²⁺ antiporter transports 3H⁺ out of vacuole and draws one Ca²⁺ into the vacuole. The negative regulation of vacuolar H⁺/Ca²⁺exchanger by CaN implies that Ca²⁺ 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 CaN and CaM.

4) In most of the systems switch ON mechanisms are accompanied by OFF mechanisms. Restoration of the increased cytosolic Ca²⁺ level to the resting phase is important for the cell. PMRI and PMCI are the Ca²⁺ ATPase localized on Golgi and vacuolar membrane, respectively. CaN positively regulates both PMRI as well as PMC1 and function towards the maintenance of cytosolic Ca²⁺ 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 Ca²⁺ ATPase. In this mutant the levels of cytosolic Ca²⁺ 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 Ca²⁺-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, Ca²⁺ 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>
<thead>
<tr>
<th>S.No.</th>
<th>Year</th>
<th>Major Discoveries / Events</th>
<th>Species</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1996-98</td>
<td>Screening of salt overly sensitive mutants and positional cloning of SOS genes.</td>
<td>Arabidopsis</td>
<td>[11,13,65,76]</td>
</tr>
<tr>
<td>2</td>
<td>1998</td>
<td>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.</td>
<td>Arabidopsis</td>
<td>[77]</td>
</tr>
<tr>
<td>3</td>
<td>1999</td>
<td>AtCBL1 could bind Ca²⁺, 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.</td>
<td>Arabidopsis</td>
<td>[12]</td>
</tr>
<tr>
<td>4</td>
<td>1999</td>
<td>Identification of novel kinases associated with CBL like calcium sensors.</td>
<td>Arabidopsis</td>
<td>[44]</td>
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<tr>
<td>5</td>
<td>2000</td>
<td>Autophosphorylation of SOS2 proving that it is a functional protein kinase required for salt tolerance</td>
<td>Arabidopsis</td>
<td>[53]</td>
</tr>
<tr>
<td>6</td>
<td>2000</td>
<td>SOS2 protein kinase physically interacts with and is activated by SOS3.</td>
<td>Arabidopsis</td>
<td>[45]</td>
</tr>
<tr>
<td>7</td>
<td>2000</td>
<td>Genetic analysis of plant salt tolerance using Arabidopsis.</td>
<td>Arabidopsis</td>
<td>[78]</td>
</tr>
<tr>
<td>8</td>
<td>2000</td>
<td>Each individual member of AtCBL family specifically interacted with only a subset of CIPKs generating specificity.</td>
<td>Arabidopsis</td>
<td>[54]</td>
</tr>
<tr>
<td>9</td>
<td>2001</td>
<td>The NAF domain of CIPK is a novel and critical domain for interaction with AtCBLs.</td>
<td>Arabidopsis</td>
<td>[46]</td>
</tr>
<tr>
<td>10</td>
<td>2001</td>
<td>Differential subtraction screening identified the genes that are uniquely stress regulated in salt overly sensitive mutants.</td>
<td>Arabidopsis</td>
<td>[79]</td>
</tr>
<tr>
<td>11</td>
<td>2001</td>
<td>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, Thr¹⁶⁸ to Asp mutation in the activation loop of SOS2 resulted in constitutive expression of SOS2.</td>
<td>Arabidopsis</td>
<td>[49]</td>
</tr>
</tbody>
</table>
### 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, SOS2T168DAF, and SOS2T168DA308. These mutants preferred Mn²⁺ relative to Mg²⁺. | 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 Ca²⁺ 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 | 2003 | 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⁺/Ca²⁺ antiporter CAX1. SOS2 interacted with the N terminus of CAX1 and the activation of CAX1 via SOS2 was independent of SOS3. | Arabidopsis | [72]
25 | 2005 | 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]
26 | 2005 | The crystal structure of SOS3 was solved in complex with Ca²⁺ and with Ca²⁺ and Mg²⁺. It was shown that SOS3 exists in dimeric conformation. | Arabidopsis | [36]
27 | 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]
28 | 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]
29 | 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 Ca²⁺ sensors bind Ca²⁺ using a helix-loop-helix motif termed as the ‘EF hand’ which binds a single Ca²⁺ molecule with high affinity [33]. The Ca²⁺ sensors utilize the side chain oxygen atoms of the EF hand motif for Ca²⁺ 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 Ca2+ was resolved at 2.75 Å resolution and in complex with Ca2+ and Mn2+ at 3.0 Å resolution respectively. It was shown that Ca2+ 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 Ca2+ ions.

The differential Ca2+ binding ability of different Ca2+ sensors may also be critical criterion towards the deciphering of the Ca2+ signatures and manifesting signal specificity. The first EF hand (EF1), although conserved in all CBLs, has single amino acid substitutions at critical Ca2+ 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 members of AtCBL protein family i.e. CBL1, CBL4, CBL5 and CBL9 harbor a single canonical EF hand. 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 Ca2+ ions. Whether such differences contributes to differential deciphering of Ca2+ signatures in response to various environmental cues remains to be experimentally verified [35]. AtCBL1 and AtCBL9 have EF3 and EF4 as conventional EF hand Ca2+ binding motifs. These conventional EF hands would favor Ca2+ 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 Ca2+ 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 (MGXXS/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 immunofluorescent 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°C, 9 h), salt (150 mM, 12 h), wounding (3 h), salicylic acid (4 h) and exogenously provided CaCl2 (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 Ca2+ signal, protein sensors often interact with downstream target molecules. These downstream molecules are therefore also termed as effectors of Ca2+ signaling. In context to CBLs, a family of novel serine-threonine protein kinases was recognized as a cellular target for AtCBL Ca2+ 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 revealed that rice CIPK family gene can also be divided into intron-less (22 OsCIPKs) and intron-harbor (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
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 CBLs. NAF domain acts as an autoinhibitory 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 autoinhibition 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 H1, and histone I1S. 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, *cb1* 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 *cb1* 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 amylase 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 aleuron 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 revealed the significance of Ca\(^{2+}\) signal to reinitiate 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 myristoylated 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 Athklt mutation suppresses the sos3 mutation [71] this suggests that the SOS3-SOS2 kinase complex may prevent Na+ influx by down regulating HKT1 gene expression or inactivating the HKT1 protein during salt stress [66]. SOS3 and SOS2 may serve as negative regulators of AthKTI 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]. 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+/Ca2+ 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 transmembrane 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 AtCBL1 and 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

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**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.
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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 both ABA dependent as well as independent pathways. Pathway followed by AtCIPK3 is shown with blue arrows.

**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 unpublished 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.

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.

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