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Novel Protein Kinases Associated with Calcineurin B–like Calcium Sensors in Arabidopsis

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Members of the Arabidopsis calcineurin B–like Ca^{2+} binding protein (AtCBL) family are differentially regulated by stress conditions. One AtCBL plays a role in salt stress; another is implicated in response to other stress signals, including drought, cold, and wounding. In this study, we identified a group of novel protein kinases specifically associated with AtCBL-type Ca^{2+} sensors. In addition to a typical protein kinase domain, they all contain a unique C-terminal region that is both required and sufficient for interaction with the AtCBL-type but not calmodulin-type Ca^{2+} binding proteins from plants. Interactions between the kinases and AtCBLs require micromolar concentrations of Ca^{2+}, suggesting that increases in cellular Ca^{2+} concentrations may trigger the formation of AtCBL–kinase complexes in vivo. Unlike most serine/threonine kinases, the AtCBL-interacting kinase efficiently uses Mn^{2+} to Mg^{2+} as a cofactor and may function as a Mn^{2+} binding protein in the cell. These findings link a new type of Ca^{2+} sensors to a group of novel protein kinases, providing the molecular basis for a unique Ca^{2+} signaling machinery in plant cells.

INTRODUCTION

Among the extracellular signals eliciting changes in Ca^{2+} concentration in the cytoplasm of plant cells are plant hormones, light, stress factors, and pathogenic or symbiotic elicitors (Knight et al., 1991, 1996, 1997; Neuhaus et al., 1993; Trewavas and Knight, 1994; Ehrhardt et al., 1996; McAinsh et al., 1997; Wu et al., 1997). In addition, many intrinsic growth and developmental processes, such as elongation of the root hair and pollen tube, are accompanied by Ca^{2+} transients (Franklin-Tong et al., 1996; Felle and Hepler, 1997; Holdaway-Clarke et al., 1997; Wymer et al., 1997). Because different signals often elicit distinct and specific cellular responses, an interesting question is how do cells distinguish between the Ca^{2+} signals produced by different stimuli?

Studies with both animal and plant cells suggest that a Ca^{2+} signal is represented not only by Ca^{2+} concentration but also by spatial and temporal information, including Ca^{2+} localization and oscillation (Franklin-Tong et al., 1996; Holdaway-Clarke et al., 1997; Dolmetsch et al., 1998; Li et al., 1998). Although such complexity in Ca^{2+} parameters may partially explain the specificity of cellular responses triggered by a particular stimulus, the signaling components that "sense" and "interpret" the Ca^{2+} signals hold the key to linking the changes in these parameters to specific cellular responses.

If Ca^{2+} signaling pathways constitute "molecular relays," the first "runner" after Ca^{2+} should be a component that serves as the Ca^{2+} "sensor" to monitor changes in Ca^{2+} parameters. Such sensors often are proteins that bind Ca^{2+} and, in so doing, change conformation in a Ca^{2+}-dependent manner. Several families of Ca^{2+} sensors have been identified in higher plants. Perhaps the best known is the family of calmodulin (CaM) and CaM-related proteins, which contain four EF-hand domains responsible for Ca^{2+} binding (Zielinski, 1998). These Ca^{2+} sensors are small proteins that have no enzymatic activity themselves and function by interacting with their target proteins (Zielinski, 1998). The second major class is the Ca^{2+}-dependent protein kinases (CDPKs) that contain CaM-like Ca^{2+} binding domains and a kinase domain in a single protein (Roberts and Harmon, 1992). Each individual CDPK protein is expected to detect the changes in the Ca^{2+} parameters and translate these changes into the regulation of a protein kinase activity (Roberts and Harmon, 1992).

Two recent studies have identified genes encoding a new family of Ca^{2+} sensors from Arabidopsis (Liu and Zhu, 1998; Kudla et al., 1999). These proteins are similar to both the

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regulatory B subunit of calcineurin and the neuronal Ca^{2+} sensor (NCS) in animals (Olafsson et al., 1995; Klee et al., 1998). We refer to this unique family of plant Ca^{2+} sensors in Arabidopsis as calcineurin B–like (AtCBL) proteins (Kudla et al., 1999). One member of the *AtCBL* gene family, *AtCBL1*, is highly inducible by stress signals, including drought, cold, and wounding (Kudla et al., 1999). Another member, *SALT OVERLY SENSITIVE 3* (*SOS3*), plays a role in the salt resistance of Arabidopsis (Liu and Zhu, 1998). These results strongly suggest that the AtCBL family of Ca^{2+} sensors is involved in plant signal transduction processes in response to stress conditions. Like CaM, calcineurin B, and NCS, AtCBLs are also small Ca^{2+} binding proteins that do not themselves have enzymatic activity; therefore, identification of their target proteins becomes critical to understanding their functions. In this study, we have identified a group of novel protein kinases from Arabidopsis as common targets for AtCBL proteins.

RESULTS

A Group of Protein Kinases That Interact Specifically with AtCBL1

To identify the target proteins for AtCBL1, a stress-responsive calcium sensor, we conducted yeast two-hybrid screening. An *AtCBL1* cDNA was cloned into a vector expressing the GAL4 DNA binding domain by in-frame fusion and was used to screen the Arabidopsis ACT cDNA expression library for potential AtCBL1 binding proteins (see Methods). Among the many positive clones sequenced, a large proportion represented a group of protein kinases.

We chose one of the protein kinase clones for further analysis in this study by isolating the full-length cDNA. The longest cDNA obtained was 1.7 kb; it contained an open reading frame of 1332 bp and encoded a polypeptide of 444 amino acid residues with an estimated molecular mass of 49 kD and a calculated pI of 6.85. We designated this protein as CIPK1 (for CBL-interacting protein kinase 1). The N-terminal 278–amino acid residues of CIPK1 form a catalytic domain with characteristics of serine/threonine protein kinases. A predicted ATP binding site was found between amino acid residues 26 and 49. Serine/threonine protein kinase motifs were located between residues 139 and 151 and between residues 179 and 192. Based on information derived from the Arabidopsis genomic sequence database (Stanford University, Stanford, CA), the *CIPK1* gene is located on chromosome 3. Comparing the sequences of the cDNA and the genomic region, we found that *CIPK1* contains eight introns and spans 3.6 kb in the genome.

Partial sequences of several other interacting protein kinase clones were identical to genes sequenced by the Arabidopsis genome project (GenBank accession numbers AB010697, AC005623, and Z97336). These genes are referred to as *CIPK2*, *CIPK3*, and *CIPK4*, based on their interaction with AtCBL1. Highly related to each other and to CIPK1, these kinases are most similar to a subfamily of protein kinases represented by SNF1 (for sucrose nonfermenting 1) in yeast and AMP-dependent kinase (AMPK) in mammalian systems (Hardie et al., 1998; Figures 1A and 1B). The sequence identity among CIPKs is ~70% in the kinase domains and ~40% in the C-terminal nonkinase region. Sequence identity between CIPK1 and AMPK or SNF1 is ~50% in the kinase domains (Figure 1B).

The C-terminal nonkinase region of ~150 amino acids in all CIPK proteins shows high similarity among these proteins but is completely different from that of SNF1 or AMPK. A database search identified two putative protein kinases from sorghum (Annen and Stockhaus, 1998) that are most similar to CIPK1 and are likely to be the sorghum orthologs of CIPK1. A wheat protein kinase, WPK4, also shares substantial sequence identity with CIPK1 in both the kinase and the C-terminal nonkinase domains (Sano and Youssefian, 1994). The C-terminal regions of these protein kinases are compared in Figure 1C, which shows that a region of 20 to 30 amino acids in the middle of the C-terminal domain is highly similar among these proteins.

We analyzed the *CIPK1* gene by using DNA and RNA gel blotting procedures. The results (Figures 2A and 2B) show that *CIPK1* is a single-copy gene, although several closely related sequences were identified in the genomic database (Figure 1A). The RNA gel blot analysis shows that the *CIPK1* gene is expressed in all organs, including roots, leaves, stems, and developing flowers. Although the *AtCBL1* gene is highly inducible by stress conditions, including drought, cold, and wounding (Kudla et al., 1999), *CIPK1* mRNA accumulation was not affected by these stress signals (K.-N. Kim and S. Luan, data not shown).

The Unique C-Terminal Region of CIPK1 Is Required and Sufficient for Interaction with AtCBL1 in the Yeast Two-Hybrid System

Because all CIPKs have a similar C-terminal region that is unique to these proteins, we speculate that this nonkinase region may be involved in the interaction with AtCBL1. A yeast two-hybrid system was used to evaluate in vivo interaction between various domains of CIPK1 and AtCBL1.

The complete coding region of CIPK1 cDNA was cloned into yeast expression vectors containing a DNA binding domain and an activation domain, producing plasmids pGBT.CIPK1 and pGAD.CIPK1, respectively. Similar constructs were also made for AtCBL1, generating pGBT.AtCBL1 and pGAD.AtCBL1. Neither CIPK1 nor AtCBL1 activated reporter gene expression when cotransformed with an empty vector. In contrast, the yeast strain cotransformed with pGBT.CIPK1 and pGAD.AtCBL1 or with pGBT.AtCBL1 and pGAD.CIPK1 grew well on the selection medium. The yeast cells also expressed another reporter gene, which en-

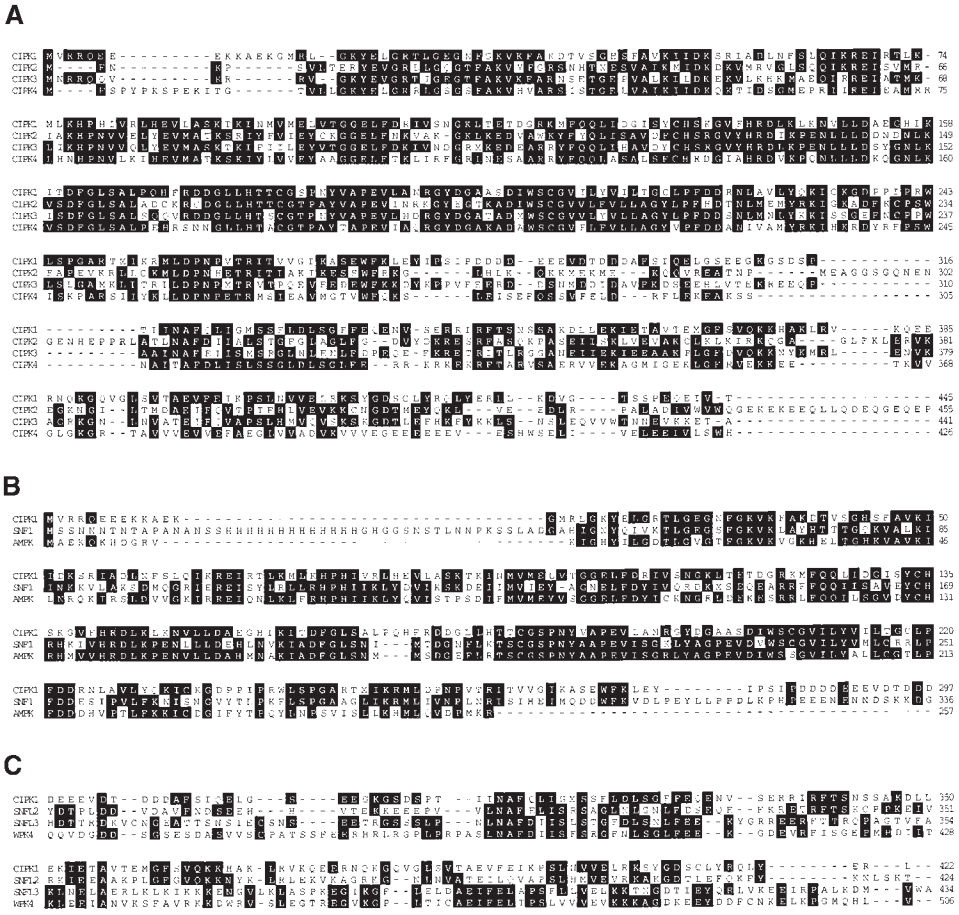

Figure 1. Sequence Analysis of CIPKs.

(A) Amino acid sequence alignment of CIPK1 and other CIPKs. CIPK1, CIPK2, CIPK3, and CIPK4 are identical to gene products with the following GenBank accession numbers: AB022219, AB010697, AC005623, and Z97336.

(B) Amino acid sequence alignment of the kinase domains in CIPK1, SNF1, and AMPK protein kinases. The rat AMPK α subunit and yeast SNF1 have GenBank accession numbers P06782 and P54645, respectively.

(C) Alignment of the C-terminal regions of CIPK1, sorghum protein kinases (SNFL2 and SNFL3), and WPK4 from wheat. GenBank accession numbers for SNFL2, SNFL3, and WPK4 are Y12464, Y12465, and AB011670, respectively.

Residues with a black background indicate identical amino acids. Dashes indicate gaps introduced into the amino acid sequences to optimize the alignments.

coded β-galactosidase. These results suggest that the interaction between AtCBL1 and CIPK1 was independent of the vectors that express the proteins (J. Shi and S. Luan, data not shown).

To map the CIPK1 region responsible for the interaction with AtCBL1, we made a series of deletion constructs by cloning CIPK1 fragments into the pGAD.GH vector. The constructs then were used to transform the yeast strain Y190, which carried plasmid pGBT.AtCBL1. Interactions were assayed by streaking the transformants on the selection medium to determine the expression of the nutritional reporter gene *HIS3.* Expression of the other reporter gene, *LacZ*, was detected by measuring β-galactosidase activity. As shown in Figure 3, full-length CIPK1 interacted with AtCBL1. However, the protein kinase domains in the N-terminal 292–amino acid region did not interact with AtCBL1, which suggests that the C-terminal region of the protein is required for interaction. This is consistent with the fact that all CIPKs contain a similar C-terminal region, one that is unique to this group of protein kinases. Furthermore, the C-terminal CIPK1 construct containing 169 amino acids interacted strongly with AtCBL1, demonstrating that the C-terminal domain of CIPK1 is sufficient for the interaction. In this study, the shortest polypeptide fragment sufficient for interacting with AtCBL1 was identified as a 123–amino acid stretch located between amino acid residues 276 and 398.

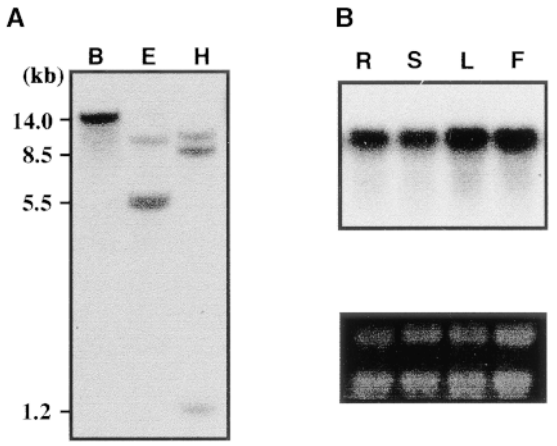

Figure 2. DNA and RNA Gel Blot Analyses of the *CIPK1* Gene.

(A) DNA gel blot of the *CIPK1* gene using the *CIPK1* cDNA (coding region) as a probe. Each lane was loaded with 5 μg of genomic DNA digested with BamHI (B), EcoRI (E), or HindIII (H). The lengths of the DNA fragments in kilobases are indicated at the left.

(B) RNA gel blot of *CIPK1* mRNA hybridized with labeled *CIPK1* cDNA. Lanes show *CIPK1* mRNA abundance in roots (R), stems (S), leaves (L), and developing flowers (F). Shown at bottom are the rRNA bands in the RNA gel stained with ethidium bromide. Each lane contained 10 μg of total RNA.

In the middle of this 123–amino acid region is the domain that is highly conserved among CIPKs, sorghum kinases, and WPK4 (Figure 1C). This domain is predicted to form a long α helix or a coiled coil, a typical protein–protein interaction module.

Interestingly, the AtCBL1 interaction with an independent C-terminal region was stronger than that with full-length CIPK1 (Figure 3). Together, these interaction analyses using the yeast two-hybrid system demonstrated that the C-terminal nonkinase region of CIPK1 is both required and sufficient for interaction with AtCBL1.

CIPK1 Interacts with Other AtCBL Proteins but Not with CaM

We are also interested in the structural basis for the AtCBL1 and CIPK1 interaction. Because AtCBL1 is rather similar (50 to 60% identity) to other AtCBLs (Kudla et al., 1999) and AtCBL4/SOS3 (Liu and Zhu, 1998), we tested whether other isoforms of the AtCBL family also would interact with CIPK1 the way that AtCBL1 does. Figure 4 shows an interaction between CIPK1 and AtCBL1, AtCBL3, or AtCBL4/SOS3. (Because AtCBL2 and AtCBL3 are 93% identical [Kudla et al., 1999], we did not include AtCBL2 in this assay.) Indeed, all of the AtCBLs tested here interacted with the CIPK1 full-length protein or the C-terminal region but not with the N-terminal kinase domains.

To determine whether other EF-hand-containing proteins also interact with CIPK1, we used CaM53 from petunia in the interaction assays and found no interaction between CIPK1 and CaM53 (Figure 4). This finding suggests that AtCBL proteins may retain similar structural domains for interaction with CIPK1 but that CaM differs substantially.

AtCBL1 Interacts with CIPK1 in a Ca^{2+}-Dependent Manner

To further confirm the interaction between CIPK1 and AtCBL1 and to determine whether Ca^{2+} regulates the interaction between the Ca^{2+} sensor protein and CIPK1, we expressed both CIPK1 and AtCBL1 in *Escherichia coli* and purified the recombinant proteins. As shown in Figure 5A, CIPK1 was first expressed as a glutathione *S*-transferase (GST) fusion protein and subsequently cleaved and purified as a 49-kD protein. Similarly, AtCBL1 also was expressed as a GST fusion protein and was cleaved into a 22-kD form (Figure 5B).

For the interaction assays, we used GST–CIPK1 as the "bait" and a cleaved form of AtCBL1 as the "prey." GST–CIPK1 was retained on glutathione-conjugated beads before cleaved AtCBL1 was incubated with the same beads (see Methods). As depicted in Figures 6A and 6B, the CIPK1 and AtCBL1 proteins did interact in vitro. We also expressed the N-terminal 292–amino acid kinase domain and the 169–amino acid C-terminal region of CIPK1 as GST fusion proteins and assayed their interaction with the AtCBL1 protein. The C-terminal region of CIPK1 (C169), but not the N-terminal kinase domain (K292), interacted strongly with AtCBL1. These protein–protein interaction results are consistent with the observations in the yeast two-hybrid assays.

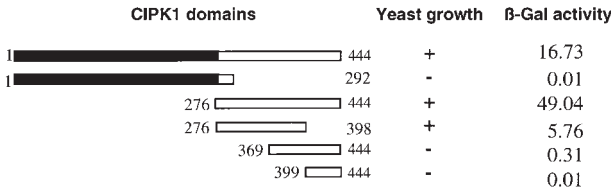

Figure 3. The C-Terminal Region of CIPK1 Is Responsible for the Interaction with AtCBL1.

Different regions of *CIPK1* cDNA were cloned into the pGAD vector and transformed into pGBH.AtCBL1-containing Y190 yeast cells. Yeast growth was monitored on the selection medium and scored as growth (+) and nongrowth (−). β-Galactosidase (β-Gal) activity was measured as described in Methods. The solid bar indicates the kinase domain of CIPK1. The amino acid positions that flank each protein fragment are indicated.

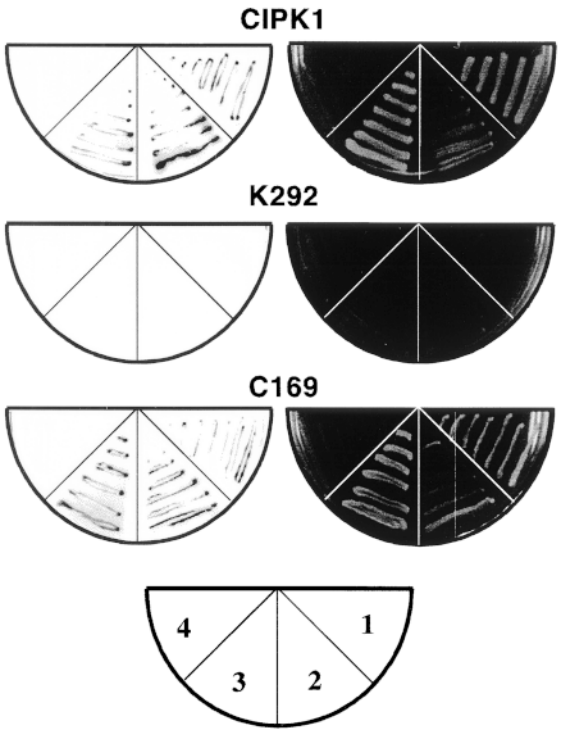

Figure 4. Other AtCBLs Interact with CIPK1 but CaM Does Not.

The half circles at right indicate growth of the yeast strains on the selection medium. The half circles at left show the filter assay results. Three constructs of CIPK1 were used for the interaction assay: the full-length protein (CIPK1), the kinase domain (K292), and the C-terminal region (C169). The half circle at bottom shows the arrangement of the yeast strains containing pGAD.CIPK1 and pGBD.X, where X indicates various cDNAs, including AtCBL1 (1), AtCBL3 (2), AtCBL4/SOS3 (3), and petunia CaM53 (4).

We also found that Ca^{2+} was required for the interaction between AtCBL1 and CIPK1. In the presence of EGTA to chelate Ca^{2+}, CIPK1–AtCBL1 interaction was not detected. The addition of micromolar concentrations of Ca^{2+} dramatically increased the interaction (Figure 6). This finding fits the common paradigm in which Ca^{2+} triggers interaction between its sensors and their target proteins (Zielinski, 1998), and it supports the hypothesis that AtCBL1 may serve as a Ca^{2+} sensor that interacts with protein targets (including CIPK1) to initiate regulatory pathways in plant cells. Interestingly, the AtCBL1 interaction with the C-terminal region of CIPK1 is much less dependent on Ca^{2+} than is its interaction with the full-length CIPK1 protein (Figure 6). This constitutive interaction may explain why AtCBL1 in yeast cells interacts with the C-terminal region of CIPK1 more strongly than with full-length CIPK1 protein. More importantly, this observation suggests that Ca^{2+}-regulated interaction between CIPK1 and AtCBL1 requires structural information presented by the full-length CIPK1 protein, although the Ca^{2+}-independent interaction can occur with the C-terminal region alone. In other words, Ca^{2+} regulation further increases the specificity of the CIPK1–AtCBL1 interaction.

Importantly, Ca^{2+} availability also changed the mobility pattern of AtCBL1 in SDS-PAGE analysis (Figure 6, lanes 5 and 6), confirming the effectiveness of the EGTA/Ca^{2+} treatments used in this study.

CIPK1 Is a Novel Serine/Threonine Protein Kinase

To characterize the CIPK1 function and the possible regulation by AtCBL1 interaction, we studied the kinase activity of CIPK1. Using both the GST–CIPK1 fusion protein and the purified CIPK1 after cleavage (Figure 5), we assayed their kinase activities in an autophosphorylation reaction. As shown in Figure 7A, both the GST fusion form and the cleaved form of CIPK1 autophosphorylated themselves in the presence of Mg^{2+} or Mn^{2+}. Interestingly, Mn^{2+} seemed to be a much more effective cofactor than Mg^{2+}.

We then tested whether CIPK1 prefers Mn^{2+} over Mg^{2+} as a cofactor. We assayed autophosphorylation in the presence of various concentrations of the two divalent cations. Mg^{2+} only weakly activated CIPK1 autophosphorylation in the millimolar range, 5 mM being the apparent optimal concentration for this reaction. In contrast, Mn^{2+} strongly activated CIPK1 at much lower concentrations, in the micromolar range (Figure 7B). At 10 mM, Mn^{2+} apparently induced a mobility shift

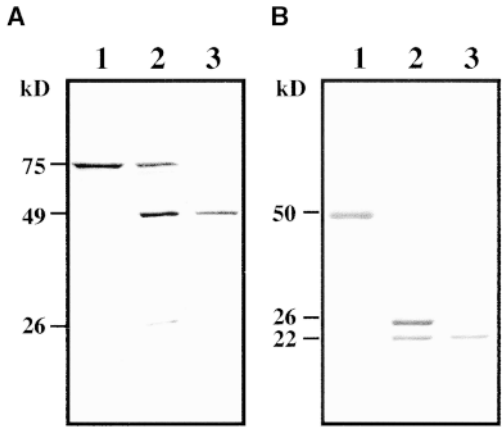

Figure 5. Expression of Recombinant CIPK1 and AtCBL1 Proteins.

(A) GST–CIPK1 expression. Lanes 1 to 3 contain the GST–CIPK1 fusion protein, the cleaved forms of the CIPK1 and GST proteins, and purified CIPK1, respectively.

(B) GST–AtCBL1 expression. Lanes 1 to 3 contain the GST–AtCBL1 fusion protein, the cleaved forms of the AtCBL1 and GST proteins, and purified AtCBL1, respectively.

In **(A)** and **(B)**, the molecular masses of the proteins are indicated at left in kilodaltons. The proteins were analyzed by SDS-PAGE, and the gel was stained with Coomassie blue.

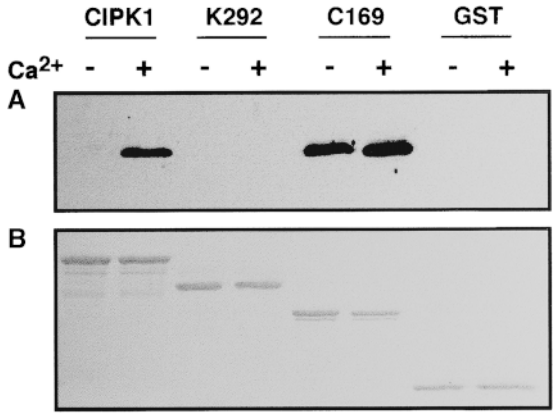

Figure 6. CIPK1 Interacts with AtCBL1 in a Ca^{2+}-Dependent Manner.

Various fragments of CIPK1 were used to make GST fusion proteins to "pull down" cleaved AtCBL1 protein in the presence of 1 mM EGTA (−) or 0.2 mM $CaCl_2$ (+). Full-length CIPK1, the kinase domain (K292), and the C-terminal domain (C169) were used for the interaction assays. The GST protein (GST) was used as a negative control.

(A) A protein immunoblot using anti-AtCBL1 antibody as a probe. Note a gel shift pattern of AtCBL1 in the presence or absence of Ca^{2+}.

(B) A Coomassie blue–stained SDS-PAGE gel indicating the amount of fusion proteins used in each binding assay.

of a portion of the CIPK1 protein during SDS-PAGE analysis. When the concentration reached 20 mM, all of the CIPK1 protein changed into a form with a higher apparent molecular mass. This gel shift pattern was not observed with GST or with the molecular mass marker proteins (data not shown). The protein mobility shift indicates that CIPK1 is a Mn^{2+} binding protein (Figure 7B).

Because typical tyrosine kinases in animals often prefer Mn^{2+} as a cofactor (discussed in Schinkmann and Blenis, 1997), we suspected that CIPK1 might be capable of phosphorylating tyrosine residues. We determined the amino acid specificity of CIPK1 autophosphorylation by phosphoamino acid analysis. Figure 7C shows that CIPK1 was autophosphorylated on serine and threonine residues but not on a tyrosine residue, which suggests that CIPK1 is a serine/threonine kinase with an uncommon cofactor preference.

To determine the relative substrate specificity for CIPK1, we assayed the kinase activity against several proteins commonly used as substrates for protein kinases, including casein, myelin basic protein, histone H1, and histone IIIS. None of these protein substrates was phosphorylated to any important extent when compared with the autophosphorylation results. The low extent of phosphorylation for casein and myelin basic protein shown in Figure 7D suggests either that autophosphorylation is physiologically relevant or that CIPK1 has strict substrate specificity.

If AtCBL1 interacts with CIPK1 in a Ca^{2+}-regulated manner, AtCBL1 is likely to serve as a regulator of CIPK1. How does the AtCBL1 interaction regulate CIPK1 function? According to studies with other Ca^{2+} sensor–target interactions (Klee et al., 1998; Zielinski, 1998), regulation mechanisms may include modulation of the kinase activity, alteration of the cellular localization, or changes in the substrate specificity/availability. In initiating studies to test these possibilities, we explored whether the kinase activity of CIPK1 was modulated by the AtCBL1 interaction. Because CIPK1 did not efficiently phosphorylate any generic substrate that we have tested thus far, we monitored its autophosphorylation activity in the presence or absence of AtCBL1. Under the conditions used , we observed no significant effect of AtCBL1 or other AtCBL members on CIPK1 autophosphorylation activity. This may indicate that the AtCBL1 interaction regulates CIPK1 function by other mechanisms. Alternatively, AtCBL proteins may regulate the protein kinase activity of CIPK1 toward a specific substrate only. This possibility can be tested once a physiological substrate of CIPK1 has been identified.

AtCBL1 Affinity Chromatography Purifies CIPK1 from Arabidopsis Plants

In both the yeast two-hybrid system and protein–protein interaction assays, CIPK1 was specifically associated with AtCBL1. To further confirm the interaction between these two proteins, we performed an affinity purification procedure by using AtCBL1 as an affinity reagent to retrieve CIPK1 from an Arabidopsis protein extract. As shown in Figure 8A, GST–AtCBL1, but not GST, copurified two proteins that were strongly labeled with γ-^{32}P-ATP in the presence of Mn^{2+}. One of the two proteins comigrated with a protein recognized by the anti-CIPK1 antibody (Figure 8B); moreover, this protein migrated at 49 kD, the expected size of native CIPK1. Comparing the amount of CIPK1 protein detected in the total protein extract and in the affinity-purified fraction, CIPK1 was enriched ~1000-fold during the AtCBL1 affinity procedure (considering both band intensity and the amount of total protein loaded onto the gel). The other phospholabeled protein (~51 kD) that copurified with GST–AtCBL1 could be either a CIPK1-like protein kinase that interacted with AtCBL1 or an endogenous substrate for CIPK1. Further purification and characterization are required to determine the molecular nature of the second protein. Results of the affinity purification procedure strongly suggest that AtCBL and CIPK1 specifically interact in Arabidopsis plants.

DISCUSSION

A New Family of Ca^{2+} Sensors and Their Target Kinases

Ca^{2+} serves as a ubiquitous second messenger in all eukaryotic systems. A typical Ca^{2+} signaling process starts

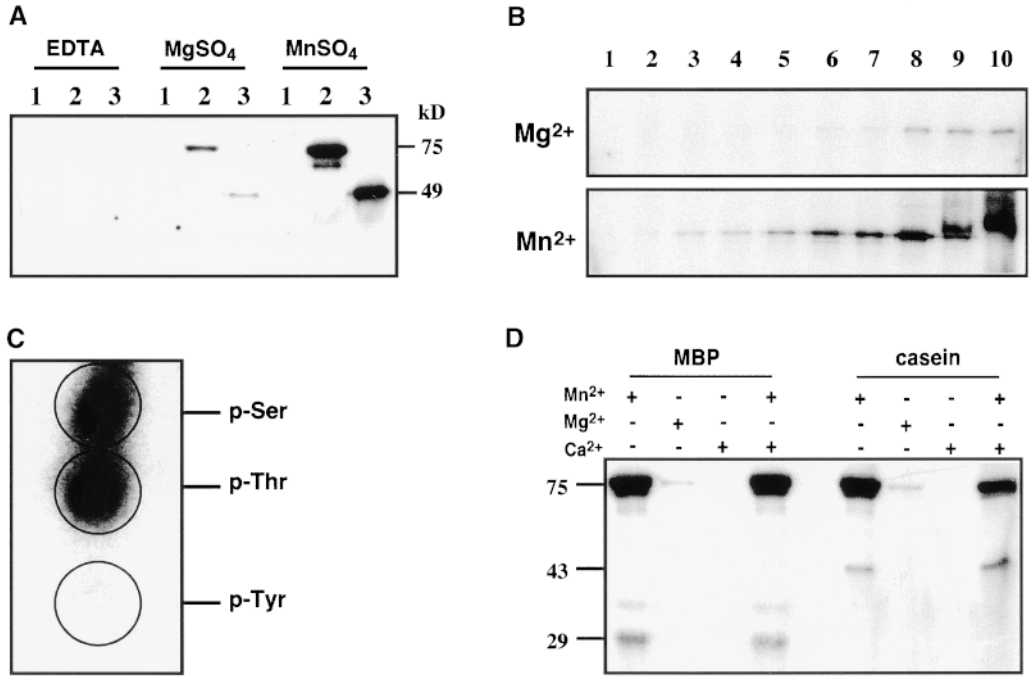

Figure 7. Protein Kinase Activity of CIPK1.

(A) Autophosphorylation of GST–CIPK1 and CIPK1. The autokinase assays were performed in the presence of EDTA, $MgSO_4$, or $MnSO_4$. One of the following three proteins was included in each assay: lanes 1, GST; lanes 2, GST–CIPK1; and lanes 3, CIPK1 (in cleaved and purified form as shown in Figure 5). The molecular mass in kilodaltons is indicated at right.

(B) Cofactor preference of CIPK1. Autokinase activity of the CIPK1 protein in the presence of various concentrations of $MgSO_4$ or $MnSO_4$ is presented as the density of autoradiographic bands. Lane 1, kinase activity with 1 mM EDTA in the assay buffer; lanes 2 to 10, activity in the presence, respectively, of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 5, 10, or 20 mM $MgSO_4$ (Mg^{2+}) or $MnSO_4$ (Mn^{2+}).

(C) Phosphoamino acid analysis of CIPK1 autophosphorylation. Positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated as p-Ser, p-Thr, and p-Tyr, respectively.

(D) Low kinase activity of CIPK1 against generic substrates. Phosphorylation of myelin basic protein (MBP) and casein was determined in the presence (+) or absence (−) of various divalent cations. The molecular masses of GST–CIPK1, casein, and myelin basic protein are 75, 43, and 29 kD, respectively (as indicated at left).

with a protein sensor that binds Ca^{2+} and interacts with other signaling proteins (the "targets") to relay the signal (Vogel, 1994; Crivici and Ikura, 1995; Zielinski, 1998). We recently isolated three *AtCBL* genes encoding novel Ca^{2+} sensors that were similar to both the calcineurin B subunit and NCS (Kudla et al., 1999; Trewavas, 1999). An independent study searching for salt-tolerance genes in Arabidopsis identified *SOS3*, which is also similar to calcineurin B and NCS (Liu and Zhu, 1998). The AtCBL proteins and SOS3 are highly similar in structure (50 to 60% amino acid identity) and collectively represent a new family of calcium sensors in plants.

It is intriguing to find plant Ca^{2+} sensors that resemble both calcineurin B and NCS to a similar degree. Calcineurin is a Ca^{2+}, CaM-dependent protein phosphatase found in eukaryotic organisms ranging from yeast to mammals (Klee et al., 1988, 1998). Its two subunits, calcineurin A and calcineurin B, function as a catalytic subunit and a regulatory subunit, respectively. Calcineurin B and CaM both contain four EF-hand Ca^{2+} binding motifs, but they belong to two different groups. Both calcineurin B and CaM interact with calcineurin A. The interaction of calcineurin B stabilizes the structure of calcineurin A, whereas the CaM interaction substantially increases its protein phosphatase activity (Klee et al., 1998). Although several studies implicate calcineurin-like activities in plant signaling pathways (Luan et al., 1993; Allen and Sanders, 1995; Bethke and Jones, 1997; Pardo et al., 1998), the molecular nature of a typical plant calcineurin remains unclear. The NCS is a subfamily of another type of Ca^{2+} sensors, which includes frequenin (an NCS ortholog), recoverin, and the guanylyl cyclase–activating protein (reviewed in Braunewell and Gundelfinger, 1999; Iacovelli et al., 1999). All members of this family of Ca^{2+} sensors, referred to as recoverin-type Ca^{2+} sensors, contain three typical EF-hand motifs instead of the four found in CaM and calcineurin B. As with CaM and calcineurin B, recoverin-type Ca^{2+} sensors also function by interacting with their target

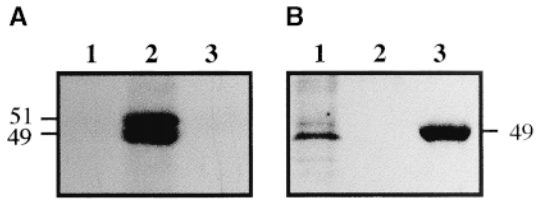

Figure 8. Affinity Purification of CIPK1 by AtCBL1.

(A) Protein phosphorylation pattern. The proteins obtained from the GST (lane 1) or GST–AtCBL1 (lane 2) affinity beads were assayed for autokinase activity in the presence of 2 mM $MnSO_4$. Lane 3 includes the AtCBL1 protein without added plant extract.
(B) Immunoblot analysis. Total protein extract (lane 1), proteins purified by GST affinity beads (lane 2), and proteins purified by GST–AtCBL1 affinity beads (lane 3) were analyzed by immunoblot with use of an anti-CIPK1 antibody as a probe.
The molecular masses of the proteins are indicated at right and left in kilodaltons.

proteins (Calvert et al., 1995; Chen et al., 1995; Schaad et al., 1996).

Here, we have identified a family of novel protein kinases (CIPKs) as target proteins for AtCBLs/SOS3. Our detailed characterization of the interaction between a pair of partner proteins, CIPK1 and AtCBL1, has demonstrated that these two proteins associate in a Ca^{2+}-dependent manner. This finding suggests that Ca^{2+} binding may induce a conformational change in AtCBL1 and hence trigger an interaction with its target protein, CIPK1. The AtCBL-interacting domain in CIPK1 is the C-terminal nonkinase region, indicating that CIPK1 contains two functional domains, the N-terminal kinase domain and a C-terminal regulatory domain. Using the C-terminal regulatory domain as a query in the sequence database search, we found that all of the structurally similar sequences appear to contain a protein kinase domain in the N-terminal region of the gene product (data not shown). We speculate that the CIPK1 C-terminal regulatory region may be present only in CIPK1-related protein kinases and may represent a protein module for specific interaction with AtCBL-type Ca^{2+} sensors. Regarding the specific structural domains in AtCBLs, Ca^{2+} binding EF-hand motifs apparently are not sufficient for interacting with CIPK1. CaM (and probably other types of Ca^{2+} sensors) does not recognize CIPK1 as a target protein; therefore, the AtCBL–CIPK interaction provides a new mechanism for Ca^{2+} signaling in plant cells.

Because Ca^{2+} sensors such as CaM often interact with multiple protein targets, finding CIPKs as partner proteins does not exclude the possibility that AtCBLs also may have other target proteins, such as a calcineurin-like protein phosphatase. Although AtCBL proteins retain structural domains responsible for a weak interaction with an animal calcineurin A subunit in the yeast two-hybrid system (Kudla et al., 1999), a typical PP2B-type protein phosphatase has not been found in higher plants. As structural analysis of the calcineurin A and B complex shows (Griffith et al., 1995; Kissinger et al., 1995), calcineurin B interacts with a long α helix formed by a region close to the C terminus of calcineurin A. The secondary structure prediction of the C-terminal domain of CIPK1 has also revealed a long helical structure formed by amino acids located in the AtCBL-interacting domain. If the long α helices in calcineurin A and CIPK1 are structurally similar, that could explain why AtCBL1 interacts with both rat calcineurin A and CIPK1.

Further studies are required to resolve the mechanism for AtCBL1 regulation of CIPK1 function. In this study, we have shown that the AtCBL1 interaction does not alter the extent of CIPK1 autophosphorylation. However, AtCBL1 might still change the CIPK1 kinase activity against its specific cellular substrate(s). Aside from alterations in catalytic activity, a change in subcellular localization might also regulate the function of a target protein. One such example is recoverin regulation of rhodopsin kinase function. When Ca^{2+} concentrations are increased in the cell, recoverin binds Ca^{2+} and triggers a conformational change that has two consequences: an interaction with rhodopsin kinase, and exposure of a myristoylated site at the N-terminal region of recoverin protein. The fatty acid chain can be inserted into the membrane, thereby translocating the recoverin–rhodopsin kinase complex to the cell membrane (Ames et al., 1997). Translocation of the recoverin–rhodopsin kinase to the membrane potentiates the inhibition of kinase activity by recoverin (Sanada et al., 1996). Compared with recoverin, AtCBL1 also contains a conserved myristoylation site at the N terminus and targets a protein kinase. Whether AtCBL1 can target CIPK1 to the cell membrane and thereby regulate the kinase activity of CIPK1 remains to be determined.

Compared with other kinases, the CIPKs presented in this study share high similarity with the kinase domains of SNF1 and AMPK in yeast and animal cells. Both SNF1 and AMPK are involved in regulation of carbon metabolism in response to metabolic signals (Stone and Walker, 1995; Hardie and Carling, 1997; Hardie et al., 1998). Several SNF1-related protein kinases have been identified in plants and can be divided into two groups. Members of the first group, which includes NPK5 and RKIN1 from tobacco and rye, respectively (Alderson et al., 1991; Muranaka et al., 1994), are considered to be functional homologs of SNF1 for two major reasons. First, the sequence similarity between these plant kinases and SNF1 and AMPK extends the entire length of the proteins. Second, RKIN1 and NPK5 have been shown to functionally complement a yeast mutant that lacks SNF1 function (Alderson et al., 1991; Muranaka et al., 1994). Members of the second group of SNF1-like kinases show high sequence similarity to SNF1 and AMPK—but only in the kinase domain, not in the C-terminal region; consequently, these kinases do not functionally complement the yeast *snf1* mutant. Two putative protein kinases identified from sorghum belong to this group and are referred to as SNF1-like kinases (SNFL2 and SNFL3; see Annen and Stockhaus, 1998).

CIPK1 belongs to the second group of SNF1-related ki-

nases. As described earlier (Figure 1), CIPK1 shares the most similarity to the sorghum protein kinases not only in the kinase domains (>70% amino acid identity) but also in the AtCBL-interacting domain in the C-terminal portion. Another SNF1-like protein kinase, WPK4 from wheat, is regulated by light, cytokinin, and nutrient conditions (Sano and Youssefian, 1994); WPK4 also contains a C-terminal region with substantial similarity to CIPK1 (Figure 1C). In contrast, NPK5 and RKIN1 differ completely from SNF1 in the C-terminal sequence. Because the C-terminal region of SNF1 and AMPK is a regulatory domain for interaction with regulatory subunits (Hardie et al., 1998), diversity in this nonkinase region may provide a mechanism for directing these kinases to different regulatory subunits. Indeed, the C-terminal region of CIPKs (and possibly of SNFL2 and SNFL3 in sorghum and WPK4 in wheat) provides the structural module for interaction with a family of Ca^{2+} sensors. The two groups of SNF1-related kinases in plants may thus be regulated differently by the Ca^{2+}-dependent and the Ca^{2+}-independent pathways; consequently, these two groups may play distinct roles in plant cells. According to studies with yeast and animals, the SNF1 homologs in plants may regulate cellular metabolism in response to metabolic signals. The SNF1-like kinases, such as CIPKs, may play a role in stress response, given their interactions with stress-related Ca^{2+} sensors.

Biochemical analyses of CIPK1 kinase activity revealed two notable features. First, CIPK1 prefers Mn^{2+} as a cofactor for its kinase activity. Receptor tyrosine kinases in animal cells generally prefer Mn^{2+} to Mg^{2+} as a cofactor, as discussed by Schinkmann and Blenis (1997). A few recent studies with animal and yeast systems have identified serine/threonine kinases that use Mn^{2+} as a preferred cofactor (Su et al., 1996; Schinkmann and Blenis, 1997; Stocchetto et al., 1997), but the functional significance is not clear. Several protein kinases from higher plants—including the SNF1 homolog NPK5 from tobacco (Muranaka et al., 1994) and RLK5, a receptor-like protein kinase from Arabidopsis (Horn and Walker, 1994)—are also more active when Mn^{2+} is used as a cofactor. In this study, we found that micromolar amounts (20 to 100 μM) of Mn^{2+} are sufficient for activation of CIPK1 (Figure 7B). Because plant cells usually contain micromolar amounts of Mn^{2+}, our observation suggests a physiological role for Mn^{2+} in CIPK1 regulation.

The second notable feature of CIPK1 is its strong autophosphorylation and low activity against generic substrates. Perhaps CIPK1 autophosphorylation is a physiologically relevant event, the way it is for the NPH1 kinase in phototropism (Huala et al., 1997; Christie et al., 1998). Moreover, perhaps CIPK1 has strict substrate specificity and is active towards only a few cellular target proteins.

Combination Codes for Specific Ca^{2+} Signaling

When we were submitting this article, an updated database search revealed at least six other AtCBL isoforms from Arabidopsis besides the ones we characterized. A similar search using the AtCBL-interacting domain sequence of CIPK1 as a query revealed several other putative protein kinases that contain a similar C-terminal region. Apparently, more AtCBL proteins and CIPK-like protein kinases will be identified as the complete genomic sequence of Arabidopsis becomes available. Both AtCBLs and their target protein kinases represent large families of proteins. Our study shows that all of the AtCBL isoforms tested thus far are capable of interacting with the C-terminal domain of CIPK1, and all CIPKs contain a similar C-terminal region for interaction with AtCBLs. Thus, an important issue for future studies of AtCBL–CIPK complexes is to resolve the isoform specificity regarding both interaction and function in plant cells.

We speculate, on the basis of our studies (Kudla et al., 1999; this report), that the specificity for AtCBL–CIPK function may be based on several factors. (1) The expression pattern of these proteins may vary importantly. For example, the *AtCBL1* gene, but not other family members (including *AtCBL2*, *AtCBL3*, and *AtCBL4*/*SOS3*), strongly respond to stress factors, including wounding, cold, and drought (Kudla et al., 1999). A similar regulation can be expected for the expression pattern of the partner proteins, CIPK1 and family. As a result, different cells at any given time may have a distinct pattern of AtCBL–CIPK complexes. (2) Subcellular localization of AtCBLs and CIPKs may differ. Among the four AtCBL proteins characterized, AtCBL1 and AtCBL4/SOS3 have a well-defined sequence motif for myristoylation at the N terminus, but AtCBL2 and AtCBL3 do not have this sequence motif. As a result, AtCBL1 and AtCBL4/SOS3 could be recruited to the cell membranes through attachment of the myristyl acid group, whereas AtCBL2 and AtCBL3 cannot. Clearly, CIPK members interacting with AtCBL1 or AtCBL4/SOS3 may relocate to the cell membrane, and CIPKs interacting with AtCBL2 and AtCBL3 may remain in the cytosol. (3) Interaction affinity between individual partner proteins may vary significantly. When all of the isoforms of CIPKs and AtCBLs are identified, interactions between any pair of members from each family can be assayed, and the affinity of the interaction can be determined. Proteins interacting at higher affinities will out compete those with lower affinities. (4) Individual AtCBLs and CIPKs may function differently in the cell. As is obvious from sequence analysis, CIPKs are distinct protein kinases, and individual CIPKs will probably have different substrate(s) in the cell and regulate distinct cellular pathways. Finally (5), each member of the AtCBL family may have specific targets in addition to the CIPKs identified in this study.

Changes in Ca^{2+} parameters in the cell trigger conformational changes in Ca^{2+} sensors. Depending on which Ca^{2+} sensors are available (CaM, CDPK, or AtCBLs), the function of various different target proteins is regulated through protein–protein interactions. The combination of a certain number of sensors and targets may serve as "combination code" for a specific signaling event. Various combinations of

different sensor–target proteins in a cell form a large and complex network that connects extracellular signals to the cellular response. As more Ca^{2+} sensors and their target proteins are identified, this network becomes more complicated. A subtle difference in a combination event in distinct cell types or at different developmental stages may result in specific cellular response.

METHODS

Two-Hybrid Screening and Assays

A Gal4p-based two-hybrid system (Chien et al., 1991) was used in this study. Vectors pGBT9.BS and pGAD.GH carry the DNA binding domain and the activation domain, respectively. The coding region of the cDNA for *Arabidopsis thaliana* calcineurin B–like protein (AtCBL1) was amplified by the polymerase chain reaction (PCR) with flanking primers containing BamHI and SalI restriction sites. The resulting PCR products were digested and cloned into pGBT9.BS. The two-hybrid library screening was conducted essentially according to Durfee et al. (1993). The Arabidopsis ACT cDNA expression library CD4-22 constructed by Kim et al. (1997) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH). The plasmid library was converted from the phage library by in vivo excision and was used to transform the Y190 strain that expresses the DNA binding domain/AtCBL1 fusion protein. Selection for interactions between AtCBL1 and its partner proteins was performed on plates containing selection medium that lacked leucine, tryptophan, and histidine (SC-Leu-Trp-His) and was supplemented with 25 mM 3-amino-1,2,4-aminotriazole. The β-galactosidase expression of the His^+ colonies was analyzed by using a filter-lifting assay, as described by Breeden and Nasmyth (1985).

For additional interaction assays, the coding region of the cDNA for AtCBL1-interacting protein kinase (CIPK1) was amplified by PCR using flanking primers containing BamHI and SalI restriction sites. The PCR product was digested and cloned into the two-hybrid plasmids. The deletion constructs for CIPK1 were produced by cloning PCR-amplified fragments into the pGAD.GH vector. The sequence of each fusion construct was confirmed by DNA sequencing. Yeast strain Y190, carrying two reporter genes, served as the reporter strain. Two-hybrid plasmids were introduced into Y190 by the lithium acetate method (Ito et al., 1983; Schiestl and Gietz, 1989). The expression of the *HIS3* nutritional reporter gene was assayed by streaking the transformants on the selection medium described above. Assays of β-galactosidase activity were performed in triplicate by using chlorophenol red–β-D-galactopyranoside as the substrate according to Durfee et al. (1993). β-Galactosidase activity was calculated from the following equation:

$$\text{units of } \beta\text{-galactosidase activity} = OD_{420}/(V \times T \times OD_{600})$$

where V is the volume of the culture (mL), T is the reaction time (min), and OD_{600} is the optical density of the yeast cells at 600 nm.

For interaction assays of CIPK1 with other AtCBL proteins and calmodulin (CaM), individual cDNA sequences for these proteins were cloned into the bait vector as described for AtCBL1. The petunia CaM53 in the binding-domain vector was a kind gift from M. Grudetz and W. Gruissem (University of California, Berkeley). These constructs were transformed into Y190 before various constructs containing different regions of CIPK1 were introduced into the same strain. The interaction was determined on the basis of the growth of the yeast strain on the selection medium and the filter assay described above.

Plant Materials and DNA and RNA Gel Blot Analyses

Plants (Arabidopsis ecotype Columbia) were grown to flowering stage in a greenhouse under long-day conditions (16-hr-light/8-hr-dark cycle). Roots, leaves, stems, and flowers were harvested, frozen in liquid nitrogen, and stored at −80°C, if not used immediately.

For DNA gel blot analysis, genomic DNA (5 μg) isolated from Arabidopsis leaves was digested with 30 units of various restriction enzymes at 37°C for 4 hr. The restriction fragments were resolved in 0.8% agarose gels, transferred to GeneScreen Plus nylon membrane (Du Pont–New England Nuclear, Boston, MA), and probed with ^{32}P-labeled *CIPK1* cDNA as described previously (Gupta et al., 1998).

For RNA gel blot analysis, total RNA (10 μg) from roots, stems, leaves, and flowers was resolved by electrophoresis in 1.2% agarose gel, transferred to GeneScreen Plus nylon membrane, and hybridized as described for the DNA gel blot analysis. The membranes were autoradiographed with Kodak XAR film.

Protein Expression and Purification

Production of a glutathione *S*-transferase (GST)–AtCBL1 fusion protein was described previously (Kudla et al., 1999). The cDNA sequences corresponding to various CIPK1 fragments were cloned into a pGEX-4T-3 expression vector, and the fusion proteins were produced by a procedure described previously, with modifications (Xu et al., 1998a, 1998b; Kudla et al., 1999). Briefly, *Escherichia coli* BL21 cells transformed with the GST fusion constructs were grown at 37°C overnight and were subcultured until the OD_{600} reached 0.6. Isopropyl-β-D-thiogalactopyranoside was added to induce the expression of the GST–CIPK1 fusion protein. Cells were harvested by centrifugation, and the pellets were resuspended in ice-cold bacterial lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 100 μM phenylmethylsulfonyl fluoride [PMSF], 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM benzamidine, 5 mM EDTA, and 1 mM EGTA). Bacterial cells were lysed by sonication. Triton X-100 was added to a final concentration of 1%. After incubation on ice for 1 hr, the cell lysate was centrifuged at 10,000*g* for 10 min at 4°C. Glutathione–Sepharose 4B beads were added to the supernatant and incubated with gentle shaking for 45 min at 4°C. The Sepharose beads were washed four times with the cell lysis buffer and twice with PBS. The GST–CIPK fusion protein was eluted with 10 mM glutathione in 50 mM Tris-HCl and 100 mM NaCl from Sepharose beads. The eluted protein was either used directly or cleaved by thrombin and purified.

Protein–Protein Interaction Assays

Purified AtCBL1 protein (5 μg) was mixed in a final volume of 200 μL with the same amount of GST fusion proteins attached to the glutathione–Sepharose beads in the presence of binding buffer (50 mM Tris-HCl, pH 6.7, 100 mM NaCl, 0.05% Tween 20, and 1 mM PMSF) supplemented with either 0.2 mM $CaCl_2$ or 1 mM EGTA as described previously (Millward et al., 1998). After gentle rotation for 2 hr, the

beads were centrifuged and washed three times with the binding buffer and then eluted with 40 μL of 10 mM glutathione. Ten-microliter samples were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (NitroBind; Osmonics, Minnetonka, MN) to detect AtCBL1. Membranes were blocked with TBST (25 mM Tris-HCl, 192 mM glycine, 20% methanol, 0.05% SDS, and 0.5% Tween 20) containing 5% nonfat dry milk and incubated with the anti-AtCBL1 antibody for 1 hr in TBST containing 1% nonfat dry milk. After four rinses in TBST, bound antibodies were detected with peroxidase-conjugated secondary antibodies and a chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Protein Kinase Assay

Kinase activities were assayed according to Zhang et al. (1998) with modifications. Briefly, phosphorylation was measured as the incorporation of radioactivity from γ-^{32}P-ATP into the CIPK1 protein (autophosphorylation) or into the substrate proteins. Assays were performed at room temperature for 30 min in a final volume of 40 μL containing 1 μg of GST–CIPK1 fusion protein or 0.5 μg of CIPK1, 10 μCi of γ-^{32}P-ATP, 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 0.1 mM EGTA in the presence or absence of various divalent cations (Mg^{2+}, Mn^{2+}, or Ca^{2+}) and substrate proteins. Reactions were terminated by adding 40 μL of 2 × SDS-PAGE sample loading buffer, followed immediately by boiling for 2 min. The reaction mixtures were analyzed by SDS-PAGE. Gels were fixed in a solution of 10% acetic acid and 40% methanol and stained with Coomassie Brilliant Blue R 250. The dried gel was autoradiographed with Kodak XAR film.

Phosphoamino Acid Analysis

Purified GST–CIPK was autophosphorylated in the presence of γ-^{32}P-ATP, as described above, and precipitated with 25% (w/v) trichloroacetic acid. The pellets were washed three times with 5% trichloroacetic acid and once with 95% (w/v) ethanol. The labeled protein was hydrolyzed by adding 6 M HCl and incubated for 1 hr at 110°C. The sample was dried and mixed with nonradioactive L-phosphoserine, L-phosphothreonine, and L-phosphotyrosine, which served as carriers and internal standards. The phosphoamino acids were resolved by one-dimensional electrophoresis as described by Jelinek and Weber (1993). The positions of the standards were visualized by using ninhydrin (0.2% [w/v] in acetone), and the labeled phosphoamino acids were detected by autoradiography.

Affinity Purification of CIPK1 from Total Arabidopsis Proteins

Three-week-old Arabidopsis (ecotype Columbia) roots were ground in liquid nitrogen with a pestle and mortar to a fine powder and transferred to a microcentrifuge tube containing extraction buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.05% Tween 20, 1 mM EDTA, 1 mM PMSF, 5 mg/mL leupeptin, and 5 mg/mL aprotinin). For each gram of the powder, 2 mL of the buffer was added and vortexed vigorously for 15 sec. After 15 min of incubation on ice, cell debris was removed by centrifugation at 14,000*g* for 10 min at 4°C. For affinity purification analysis, 200 μg of the total protein extract was mixed with 10 μg of GST–AtCBL1 immobilized on the glutathione–Sepharose 4B beads in the presence of an extraction buffer containing 0.2 mM $CaCl_2$. After 6 hr of gentle rotation at 4°C, the beads were centrifuged, and the pellets were washed five times with the binding buffer described earlier. After resuspension in a buffer containing 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl, the beads were used for both protein immunoblot and autokinase assays as described above.

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