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Molecular Components that Relay the Calcium Singal to Cellular Response in *Arabidopsis*

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INTRODUCTION

Extracellular signals elicit changes in Ca^{2+} concentration in the cytoplasm of plan cells. These signals include plant hormones, light, stress factors, and pathogen or symbiotic elicitors (Knight et al., 1991, 1996, 1997; Trewavas and Knight, 199 Ehrhardt et al., 1996; McAinsh et al., 1997; Neuhaus et al., 1997; Wu et al., 1997 In addition, many intrinsic growth and developmental processes, such a elongation of the root hair and pollen tube, are accompanied by Ca^{2+} transient (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 specif cellular responses, an interesting question is how cells distinguish the Ca^{2+} signa produced by different stimuli.

Recent studies with both animal and plant cells suggest that a Ca^{2+} sign is represented not only by Ca^{2+} concentration but also by spatial and tempor information, including Ca^{2+} localization and oscillation (Franklin-Tong et al., 199 Holdaway-Clarke et al., 1997; Dolmetsch et al., 1998; Li et al., 1998). Althoug such complexity in Ca^{2+} parameters may partially explain the specificity of cellula responses triggered by a particular stimulus, the signaling components tha "sense" and "interpret" the Ca^{2+} signals hold the key to link the changes in Ca parameters to specific cellular responses.

If Ca^{2+} signaling pathways constitute "molecular relays", the first "runner after Ca^{2+} would be a component that serves as the Ca^{2+} "sensor" to monito changes in Ca^{2+} parameters. Such sensors often are proteins that bind Ca^{2+} an change their conformation in a Ca^{2+}-dependent manner. Several families of Ca sensors have been identified in higher plants. Perhaps the best known calmodulin (CaM) and CaM-related proteins, which contain four EF-hand domain responsible for Ca^{2+} binding (Zielinski 1998). These Ca^{2+} sensors are sma proteins that do not have enzymatic activity by themselves and function b interacting with their target proteins (Zielinski, 1998). The second major class

the Ca^{2+}-dependent protein kinases (CDPK) that contain CaM-like Ca^{2+} bindin 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 parameters and translate these changes into the regulation of a protein kinas activity.

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 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 these unique plant Ca^{2+} sensors 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)*, has been shown to play a role in salt resistance of *Arabidopsis* (Liu and Zhu, 1998). These results strongly suggest that the AtCBL family of Ca^{2+} sensors are 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 have enzymatic activity by themselves. 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 Interact Specifically with AtCBL1

To identify the target proteins for AtCBL1, a stress-responsive calcium sensor, we carried out yeast two-hybrid screening. An AtCBL1 cDNA was cloned into a vector expressing the GAL4 DNA binding domain by in-frame fusion and used for screening *Arabidopsis* ACT cDNA expression library (see Methods). Among the many positive clones sequenced, a major proportion represents 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, contained an open reading frame of 1332 bp, and encoded a polypeptide of 444 amino acid residues with a molecular mass of 49 kD and a calculated pI of 6.85. We designated this protein as CIPK1 (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. An ATP binding site was found between amino acid residue 26 and 49. Two serine/threonine protein

kinase motifs located in residues 139-151 and 179-192, respectively. Based on the *Arabidopsis* genomic sequence database (AtDB, Stanford University, Stanford, CA), *CIPK1* gene is located on chromosome three. Comparing the sequences of the cDNA and the genomic region, we found that the *CIPK1* gene contains eight introns and span 3.6 kb in the genome.

Partial sequences of several other interacting protein kinase clones are identical to genes sequenced by the *Arabidopsis* genome projects (GenBank accession numbers: AB010697, AC005623, and Z97336). These genes are referred to as *CIPK2, CIPK3,* and *CIPK4* based on their interaction with AtCBL1. It is worth noting that a region of 20-30 amino acids in the middle of the C-terminal domain has a high degree of homology.

We analyzed the *CIPK1* gene by using DNA and RNA gel blotting procedures. These studies (Figures 2A and 2B) show that *CIPK1* is a single-copy gene, although a number of closely related sequences were detected from the genomic database (Figure 1). Together with the sequence comparison, this suggests that individual CIPKs in Figure 1 may differ significantly in structure and function, although they all interact with and are potentially regulated by AtCBL-type Ca^{2+} sensors. The RNA gel blot analysis shows that the *CIPK1* gene is expressed in all organs including roots, leaves, stems, and developing flowers. Although *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 C-terminal, non-kinase region may be involved in the interaction with AtCBL1. Yeast two-hybrid system was employed 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 DNA binding domain and activation domain, producing plasmid 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 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. These yeast cells also expressed another reporter gene encoding -galactosidase. These results suggest

that the interaction between AtCBL1 and CIPK1 was independent from vectors that express the proteins (J. Shi and S. Luan, data not shown).

To map the CIPK1 region responsible for interaction with AtCBL1, a series of deletion constructs were made by cloning CIPK1 fragments into pGAD.GH vector. The constructs were then used to transform the yeast strain Y190 that carried plasmid pGBT.AtCBL1. Interactions were assayed by streaking the transforments on the selection medium to determine the expression of nutritional reporter gene *HIS3*. Expression of the other reporter gene *LacZ* was detected by measuring -galactosidase activity. As shown in Figure 3, full-length CIPK1 interacted with AtCBL1. The protein kinase domains located in the N-terminal 292 amino acid region did not interact with AtCBL1, suggesting that 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 that is unique to this group of protein kinases. Furthermore, the C-terminal domain 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 residue 276 and 398. It is noteworthy that the AtCBL1 interaction with an independent C-terminal region was stronger than the interaction with full-length CIPK1 (Figure 3). Together, interaction analyses using the yeast two-hybrid system demonstrated that the C-terminal, non-kinase 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 shares significant homology (50-60% identity) with other members of AtCBLs (Kudla et al., 1999) and AtCBL4/SOS3 (Liu and Zhu, 1998), we tested whether other isoforms of AtCBL family would also interact with CIPK1 in a similar fashion. 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 calmodulin 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 calmodulin differs significantly.

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 interaction between the Ca^{2+} sensor protein and CIPK1, we expressed both CIPK1 and AtCBL1 in *E.coli* and purified the recombinant proteins. CIPK1 was first expressed as a glutathione *S*-transferase (GST) fusion protein and subsequently cleaved and purified as a 49-kD protein. In a similar manner, AtCBL1 was also expressed as a GST-fusion protein and can be cleaved into a 22-kD form (data not shown).

For the interaction assays, we used the GST-CIPK1 as the "bait" and a cleaved form of AtCBL1 as the "prey". GST-CIPK1 was retained on the glutathione beads before the cleaved AtCBL1 was incubated with the same beads (Methods). As depicted in Figures 5A and 5B, the interaction between CIPK1 and AtCBL1 proteins occured 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 (C169), but not the N-terminal kinase domain (K292), of CIPK1 interacted strongly with AtCBL1. These protein-protein interaction results are consistent with the observations using yeast two-hybrid assays.

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 levels of Ca^{2+} dramatically increased the interaction (Figure 5). This finding fits the common paradigm in which Ca^{2+} triggers interaction between its sensors and their target proteins (Zielinski, 1998). This also 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+} (Figure 5). This constitutive interaction may explain why AtCBL1 interacted with the C-terminal region of CIPK1 more strongly in yeast cells. More importantly, this observation suggests that Ca^{2+}-regulated interaction between CIPK1 and AtCBL1 requires a 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 CIPK1-AtCBL1 interaction.

It is also worth noting that Ca^{2+} availability changed the mobility

pattern of AtCBL1 protein in the SDS-PAGE analysis (lanes 5 and 6, Figure 5), confirming the effectiveness of 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 GST-CIPK1 fusion and the purified CIPK1 after cleavage, we assayed its kinase activity in an autophosphorylation reaction. As shown in Figure 6A, both the GST fusion and the cleaved form of CIPK1 autophosphorylated in the presence of Mg^{2+} or Mn^{2+}. Interestingly, Mn^{2+} seems to be a much more effective cofactor than Mg^{2+}.

Because typical tyrosine kinases in animals often prefer Mn^{2+} as a cofactor (discussed in Schinkmann and Blenis, 1997), we suspected that CIPK1 may be capable of phosphorylating tyrosine residues. We determined the amino acid specificity of CIPK1 autophosphorylation by phosphoamino acid analysis. Figure 6B shows that CIPK30 was autophosphorylated on serine and threonine residues but not on a tyrosine residue, suggesing that CIPK1 is a serine/threonine kinase with uncommon cofactor preference.

To determine the relative substrate specificity for CIPK1, we assayed the kinase activity against several commonly used protein substrates for protein kinases. These include casein, myelin basic protein, histone H1, and histone IIIS. None of these protein substrates was phosphorylated at a significant level when compared with the autophosphorylation level (data not shown). This may suggest that autophosphorylation is physiologically relavent or that CIPK1 has strict substrate specificity.

If AtCBL1 interacts with CIPK1 in a Ca^{2+}-regulated manner, it is likely that AtCBL1 serves as a regulatory entity 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 the modulation of kinase activity, alteration of the cellular localization, or changes in the substrate specificity/availability. In this study, we initiated studies to test these possibilities. We have tested whether kinase activity of CIPK1 was modulated by AtCBL1 interaction. Because CIPK1 did not efficiently phosphorylate any generic substrate tested thus far, we monitored its autophosphorylation activity in the presence or absence of AtCBL1. Under the conditions used in this study, we have not observed any significant effect of AtCBL1 or other AtCBL members on CIPK1 autokinase activity. This may indicate that the AtCBL1 interaction regulates CIPK1 function by other mechanisms. It is also possible that AtCBL proteins may regulate the protein

kinase activity of CIPK1 toward a specific substrate. This possibility could be tested after a physiological substrate of CIPK1 is identified.

AtCBL1 Affinity Chromatography Purified CIPK1 from *Arabidopsis* Plants

In both yeast two-hybrid system and in protein-protein interaction assays, CIPK1 is specifically associated with AtCBL1. To further confirm the interaction between these two proteins, we performed an affinity purification procedure using AtCBL1 as an affinity reagent to retrieve CIPK1 from *Arabidopsis* protein extract. As shown in Figure 7A, GST-AtCBL1, but not GST, copurified two proteins that were strongly labeled with gamma-^{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 7B). 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 approximately 1000-fold during the AtCBL1 affinity procedure (considering both band intensity and the amount of total protein loaded onto the gel). Another phospholabeled protein (approximately 51 kD) also copurified with GST-AtCBL1. This protein could be 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 this protein. Results obtained from this affinity purification procedure strongly suggest that the specific interaction between AtCBL1 and CIPK1 takes place in *Arabidopsis* plants.

DISCUSSION

Ca^{2+} serves as a ubiquitous second messenger in all eukaryotic systems. A typical Ca^{2+} signaling process starts 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 with similarity 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* that is also most similar to calcineurin B and NCS (Liu and Zhu, 1998). The AtCBL proteins and SOS3 are highly homologous (50-60% 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 identified from eukaryotic organisms ranging from yeast to mammals (Klee et al., 1988; 1998). It consists of two subunits, calcineurin A and calcineurin B, that function as catalytic and regulatory subunit, respectively. Calcineurin B and CaM both contain four EF hand Ca^{2+} binding motifs, but they belong to distinct group of Ca^{2+} sensors. Both calcineurin B and CaM interact with calcineurin A. The interaction of calcineurin B stablizes the structure of calcineurin A, whereas the CaM interaction significantly increases protein phosphatase activity (Klee et al., 1998). Although a number of 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 that include frequenin (NCS orthologs), recoverin, and the guanylyl cyclase-activating protein (reviewed by Braunewell and Gundelfinger, 1999; Iacovelli et al., 1999). All members of this family of Ca^{2+} sensors, referred as recoverin-type Ca^{2+} sensors, contain three typical EF-hand motifs instead of four found in CaM and calcineurin B. Like CaM and calcineurin B, recoverin-type Ca^{2+} sensors also function by interacting with their target proteins (Calvert et al., 1995; Chen et al., 1995; Schaad et al., 1996).

Here we identify 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, demonstrated that these two proteins are associated in a Ca^{2+}-dependent fashion. This finding suggests that Ca^{2+} binding may induce a conformational change in AtCBL1 and triggered an interaction with its target protein, CIPK1. The AtCBL-interacting domain in CIPK1 is the C-terminal non-kinase 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, all of the homologous sequences appear to contain a protein kinase domain in the N-terminal region of the gene product (data not shown). We speculate that CIPK1 C-terminal regulatory region may be present only in a group of 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 are apparently not sufficient for interacting with CIPK1. CaM (and probably other types of Ca^{2+} sensor) does not recognize CIPK1 as a target protein; therefore, the AtCBL-CIPK interaction provides a new machanism 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 may also 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 yet been found from higher plants. Based on the structural analysis of the calcineurin A and B complex (Kissinger et al., 1995; Griffith et al., 1995), calcineurin B interacts with a long alpha helix formed by a region close to the C terminus of calcineurin A. The secondary structure prediction of the C-terminal domain of CIPK1 also revealed a long helical structure formed by amino acids located in the AtCBL-interacting domain. It is possible that the long alpha helices in calcineurin A and CIPK1 bear structural similarity, which explains why AtCBL1 interacts with both rat calcineurin A and CIPK1.

Biochemical analyses of CIPK1 kinase activity revealed two notable features. First, CIPK1 prefers Mn^{2+} for its kinase acitivity. It has been generally observed that receptor tyrosine kinases in animal cells prefer Mn^{2+} to Mg^{2+} as a cofactor, as discusseed by Schinkermann and Blennis (1997). A few recent studies with animal and yeast systems have identified serine/threonine kinases that utilize Mn^{2+} as a preferred cofactor (Su et al., 1996; Schinkermann and Blennis, 1997, Stocchetto et al., 1997), but the functional significance is not clear. Several protein kinases from higher plants are also more active when Mn^{2+} was used as a cofactor. These include the SNF1 homolog NPK5 from tobacco (Muranaka et al., 1994) and RLK5, a receptor-like protein kinase from *Arabidopsis* (Horn and Walker, 1994). In this study, we found that micromolar levels (20-100 μM) of Mn^{2+} are sufficient for activation of CIPK1 (Figure 7). Because plant cells normally contain micromolar levels 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. It is possible that CIPK1 autophosphorylation is a physiologically relavent event as it is for the NPH1 kinase in phototropism (Huala et al., 1997; Christie et al., 1998). It could also suggest that CIPK1 has strict substrate specificity and is active only toward a small number of cellular target proteins.

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 different target proteins will be regulated through protein-protein interactions. A 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. The coding region of the cDNA for *Arabidopsis* calcineurin B-like protein (AtCBL1) was amplified with polymerase chain reaction (PCR) with flanking primers containing BamHI and SalI restriction sites. Resulted PCR products were digested and cloned into pGBT9.BS. Using the *Arabidopsis* ACT cDNA expression library CD4-22 constructed by Kim et al. (1997), the two-hybrid library screening was carried out essentially according to Durfee et al. (1993). Selection for interactions between AtCBL1 and its partner proteins was carried out on the plates containing selection medium that lacks leucine, tryptophan, and histidine (SC-Leu-Trp-His) and was supplemented with 25 mM 3-amino-1,2,4-aminotriazole. The -galactosidase expression of the His^{+} colonies was analyzed using a filter lifting assay as described by Breeden and Nasmyth (1985). Assays for -galactosidase activity were performed in triplicate using chlorophenol red--D-galactopyranoside as the substrate according to Durfee et al. (1993).

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, CA). These contructs were transformed into Y190 before various constructs containing different regions of CIPK1 were introduced into the same strain. The interaction was determined based on the growth of the yeast strain on the selection medium and the filter assay described earlier.

Plant Materials and DNA and RNA Gel Blot Analyses

Plants (*Arabidopsis* ecotype Columbia) were grown in a greenhouse under long-day conditions (16-hr-light and 8-hr-dark cycle) to flowering stage. 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 a 0.8 % agarose gel, transferred to GeneScreen Plus nylon membrane (Du Pont/New England Nuclear, Boston, MA), and probed with the ^{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 a 1.2 % agarose gel, transferred to GeneScreen Plus nylon membrane, and hybridized as described for the DNA gel blot analysis above. The membranes were autoradiographed with Kodak XAR film.

Protein Expression and Purification

Production of 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 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, *E.coli* BL21 cells transformed with the GST fusion constructs were grown at 37 °C for overnight and was subcultured until OD_{600} reached 0.6. Isopropyl--D-thiogalactopyranoside (IPTG) was added to induce the expression of GST-CIPK1 fusion protein. Cells were harvested by centrifugation, and the pellets were resuspended in ice-cold bacterial lysis buffer (50 mM Tris, 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 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 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 attatched to the glutathione-Sepharose 4B 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. To detect AtCBL1, 10-μL of samples were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (NitroBind, Osmonics, Minnetonka, MN). Membranes were blocked in TBST (25 mM Trizma base, 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 containining 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 acitvities were assayed according to Zhang et al. (1998) with modifications. Briefly, phosphorylation was measured as the incorporation of radioactivity from gamma-^{32}P-ATP into the CIPK1 protein (autophosphorylation) or substrate proteins. Assays were performed at room temperature for 30 min in a final volume of 40 μL containing 1 μg GST-CIPK1 fusion protein or 0.5 μg CIPK1, 10 μCi of gamma-^{32}P-ATP, 50 mM Tris, 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+}, and/or Ca^{2+}) and substrate proteins. Reactions were terminated by the addition of 40 μL of 2X SDS-PAGE sample loading buffer followed immediately by boiling for 2 min. The reaction mixtures were analyzed by SDS-PAGE. The gel was fixed in 10% acetic acid and 40% methanol solution 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 gamma-^{32}P-ATP, as described above, and precipated with 25% (w/v) trichloroacetic acid. The pellets were washed with 5% trichloroacetic acid for three times and once with 95% (w/v) ethanol. The labeled protein was hydrolyzed by the addition of 6 N 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 improved one-dimensional electrophoresis as described by Jelinek and Weber (1993). The position of the standards was visualized by ninhydrin (0.2%[w/v] in acetone), and the labeled phosphoamino acids were detected by using 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 into 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 rpm 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 onto the glutathione-Sepharose 4B beads in the presence of extraction buffer containing 0.2 mM $CaCl_2$. After 6 hr of gentle rotation at 4°C, the beads were pelleted and washed five times with the binding buffer as described earlier. The beads were then resuspended 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 before.

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FIGURE LEGENDS

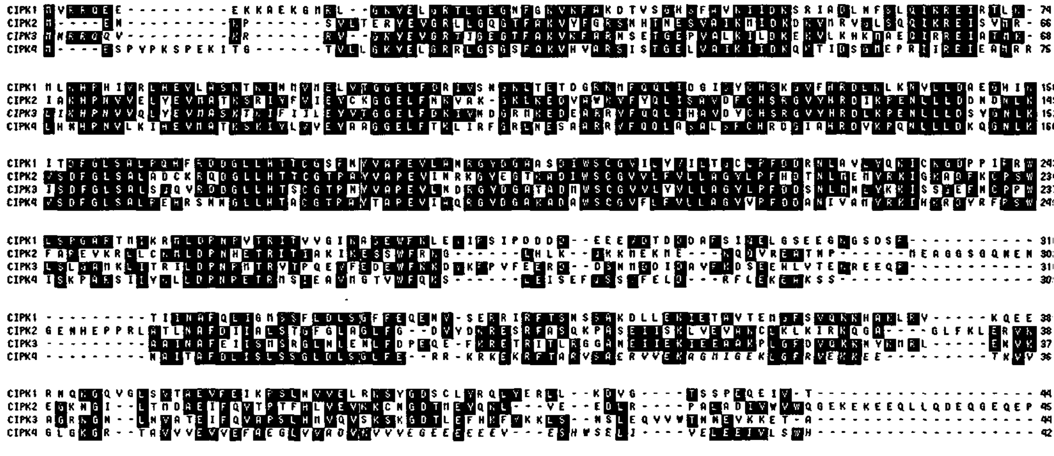

Figure 1. Sequence Analysis of AtCBL1-Interacting Protein Kinases (CIPKs).

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.

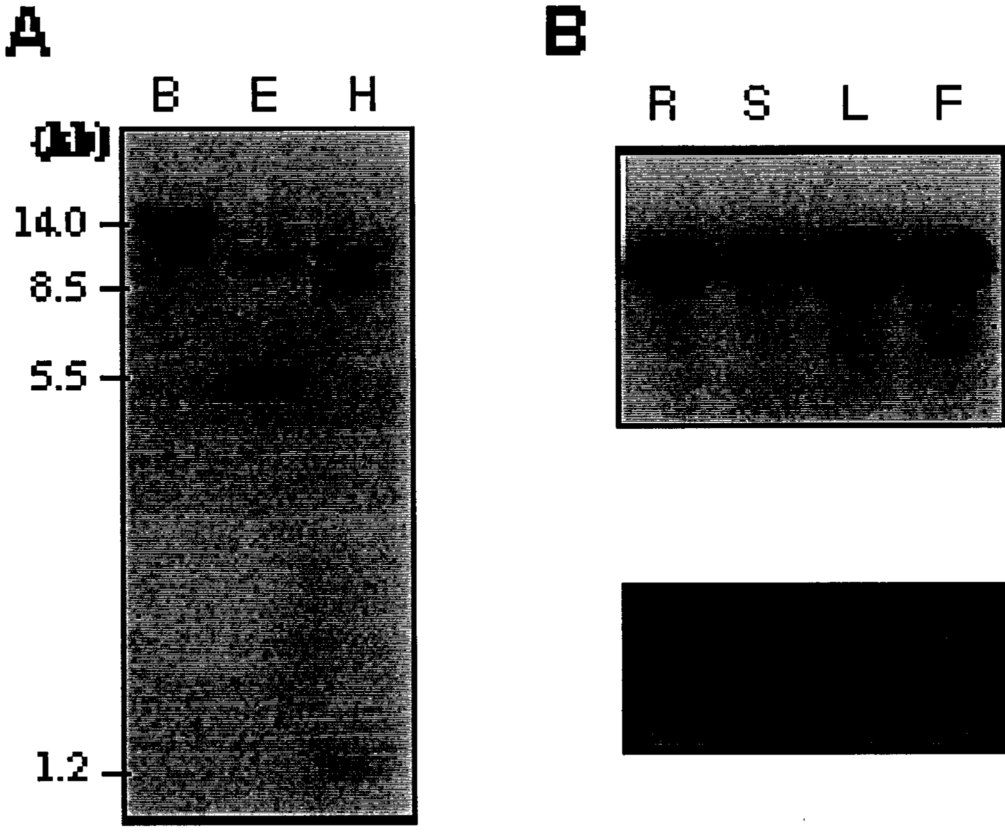

Figure 2. DNA and RNA Blot Analyses of *CIPK1* Gene.

(A) DNA blot of the *CIPK1* gene using *CIPK1* cDNA (coding region) as a probe. Each lane was loaded with 5 μg genomic DNA digested with BamHI (B), EcoRI (E), and HindIII (H), respectively. The length of the DNA fragments in kilobases (kb) were marked at left.

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

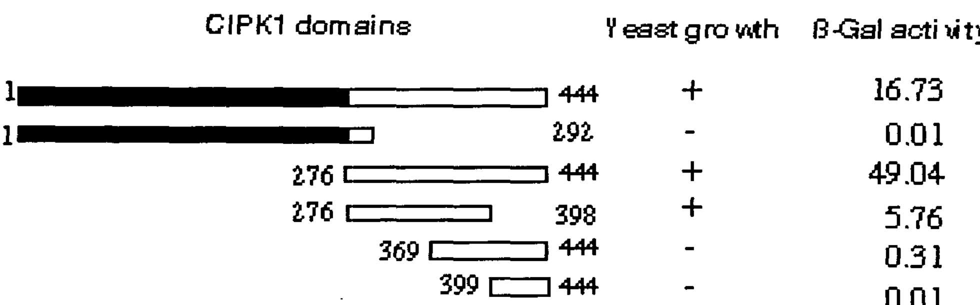

Figure 3. The C-Terminal Region of CIPK1 Is Responsible for the Interaction with AtCBL1.

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 (-) (non-growth). ß-galactosidase (ß-Gal) activity was measured as described in the Methods. The solid bar indicates the kinase domain of CIPK1. The amino acid positions that flank each protein fragment are indicated.

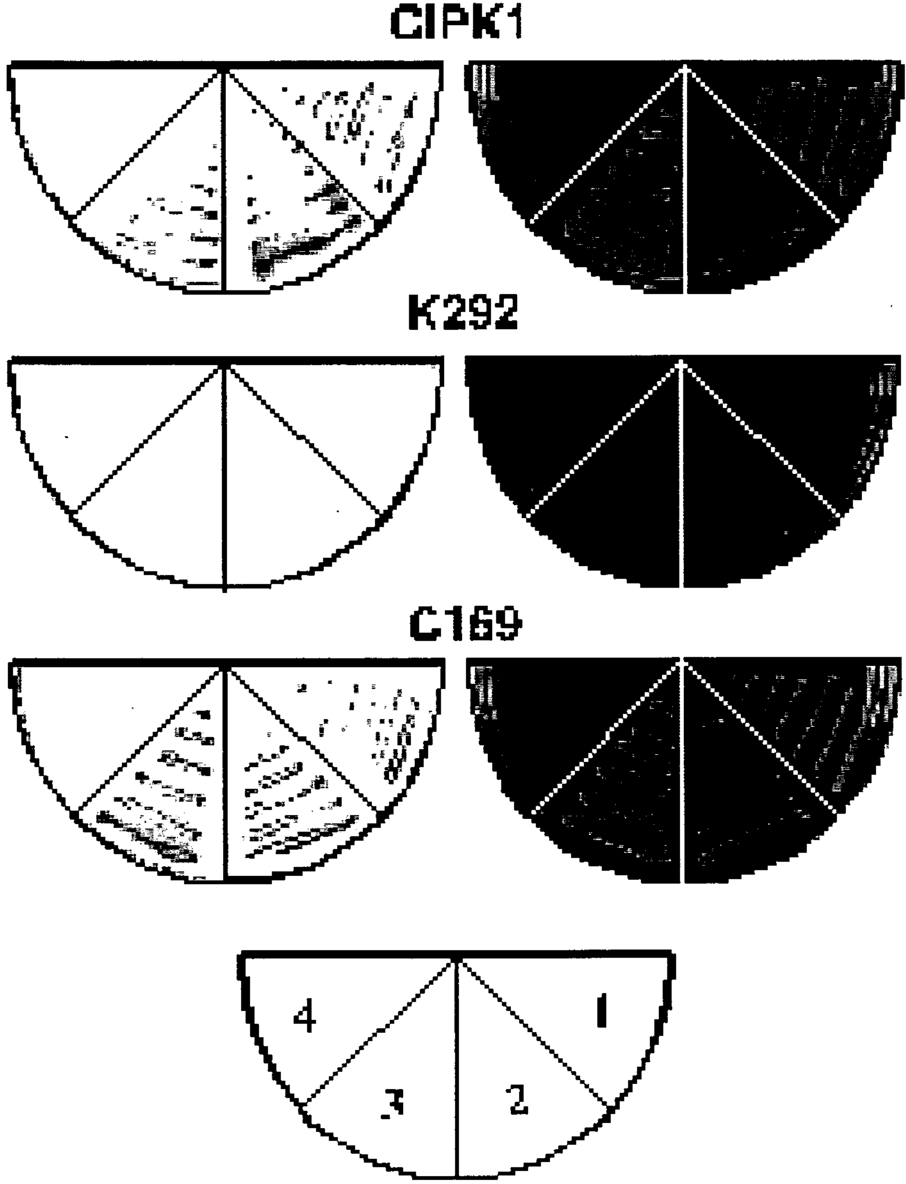

Figure 4. Other AtCBLs, But Not CaM, Interact with CIPK1.
The half circles on the right indicate growth of the yeast strains on selection medium. The half circles at left show the filter assay results. Three constructs of CIPK1 were used for the interaction assay. These include the full-length protein (CIPK1), the kinase domain (K292), and C-terminal region (C169). The bottom half circle shows the arrangement of the yeast strains containing pGAD.CIPK1 and pGBD.X. X indicates various cDNAs including AtCBL1 (1), AtCBL3 (2), AtCBL4/SOS3 (3), and petunia CaM53 (4).

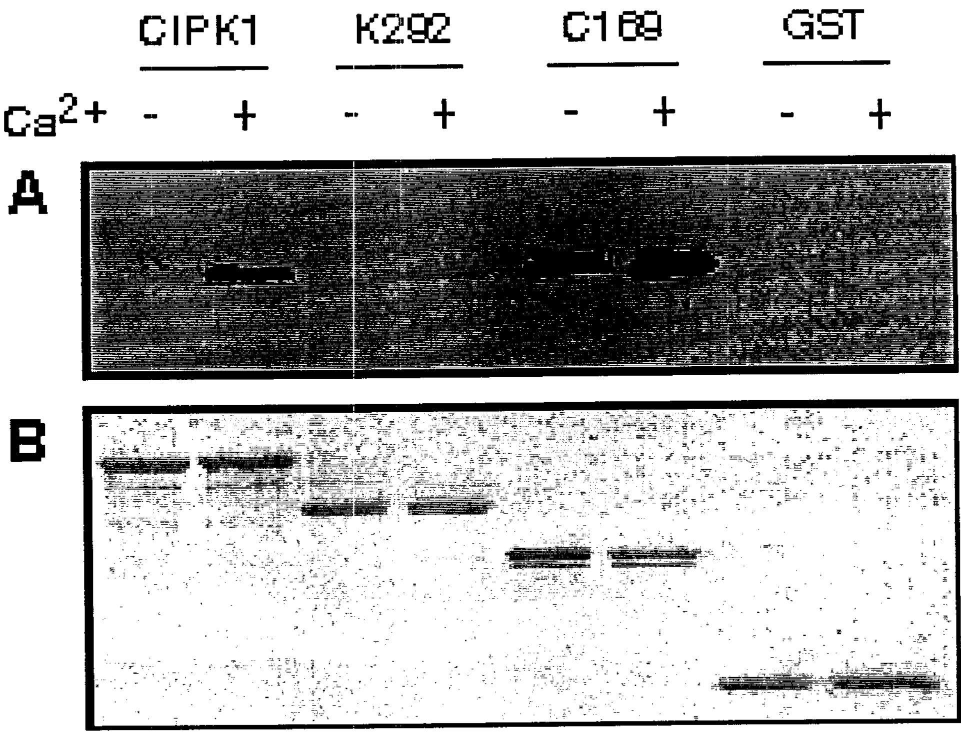

Figure 5. CIPK1 Interacts with AtCBL1 in a Ca^{2+}-Dependent Manner.
Various fragments of CIPK1 were made as GST fusion proteins and used to "pull-down" cleaved AtCBL1 protein in the presence of 1 mM EGTA (-) or 0.2 mM of $CaCl_2$ (+). Full-length CIPK1, the kinase domain (K292), and the C-terminal domain (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.

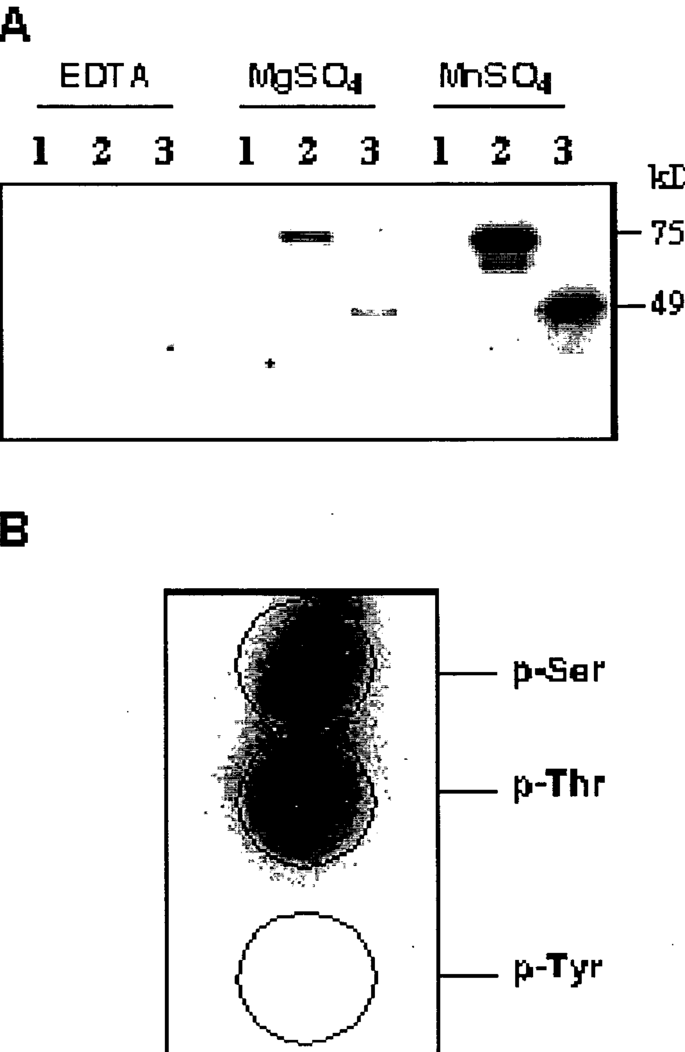

Figure 6. Protein Kinase Activity of CIPK1.

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

(B) Phosphoamino acid analysis of CIPK1 autophosphorylation. The positions of phosphoserine, phosphothreonine, and phosphotyrosine were indicated as p-Ser, p-Thr, and p-Tyr, respectively.

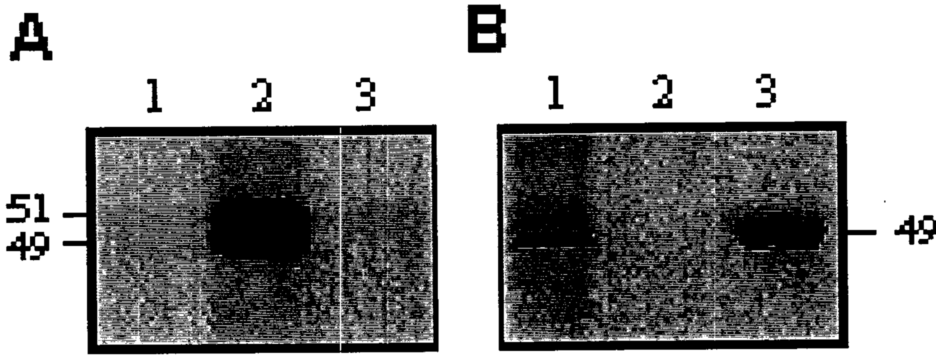

Figure 7. Affinity Purification of CIPK1 by AtCBL1.
(A) Protein phosphorylation pattern. The proteins obtained from the GST (lane 1) or GST-AtCBL1 (lane 2) affinity beads were assayed for autokinase activity in the presence of 2 mM MnSO4. Lane 3 includes the AtCBL1 protein without adding plant extract.
(B) Immuno-blot 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 using anti-CIPK1 antibody as a probe.
The molecular masses of the proteins are indicated at right and left in kilodaltons (kD).