Novel CIPK1-Associated Proteins in Arabidopsis Contain an Evolutionarily Conserved C-Terminal Region That Mediates Nuclear Localization[1]

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Environmental stimuli, including light, pathogens, hormones, and abiotic stresses, elicit changes in the cytosolic Ca^{2+} signatures of plant cells. However, little is known about the molecular mechanisms by which plants sense and transmit the specific cytoplasmic Ca^{2+} signal into the nucleus, where gene regulation occurs to respond appropriately to the stress. In this study, we have identified two novel Arabidopsis (*Arabidopsis thaliana*) proteins specifically associated with Calcineurin B-Like-Interacting Protein Kinase1 (CIPK1), a member of Ser/Thr protein kinases that interact with the calcineurin B-like Ca^{2+}-binding proteins. These two proteins contain a very similar C-terminal region (180 amino acids in length, 81% similarity), which is required and sufficient for both interaction with CIPK1 and translocation to the nucleus. Interestingly, the conserved C-terminal region was also found in many proteins from various eukaryotic organisms, including humans. However, none of them have been characterized so far. Taken together, these findings suggest that the two proteins containing the evolutionarily conserved C-terminal region (ECT1 and ECT2) may play a critical role in relaying the cytosolic Ca^{2+} signals to the nucleus, thereby regulating gene expression.

The calcium ion (Ca^{2+}), as a ubiquitous second messenger, plays a pivotal role in a variety of eukaryotic signal transduction pathways. In mammalian cells, changes in the cytosolic free Ca^{2+} control a wide range of cellular processes, such as gene transcription, fertilization, proliferation, muscle development and contraction, and neurite outgrowth (Berridge et al., 1998, 2000; Crabtree, 2001). In the case of plant cells, the Ca^{2+} signals are involved in the regulation of diverse cellular and developmental processes, including pollen tube growth, root hair elongation, and control of guard cell turgor (Holdaway-Clarke et al., 1997; Wymer et al., 1997; Blatt, 2000). In addition, plant cells also utilize the Ca^{2+} signaling mechanisms to respond to a diverse array of extracellular stimuli, such as light, phytohormones, pathogen attack, and abiotic stresses (Ehrhardt et al., 1996; Baum et al., 1999; Sanders et al., 1999; Grant et al., 2000; Kiegle et al., 2000; MacRobbie, 2000; Evans et al., 2001). However, it is questionable how Ca^{2+} can mediate such a number of disparate signal transduction pathways. One of the possible answers for such specificity can be found in the complexity of the Ca^{2+} signatures, which consist of frequency, magnitude, duration, and subcellular location of the transient increases in cytosolic free Ca^{2+} (Dolmetsch et al., 1997, 1998; Evans et al., 2001; Rudd and Franklin-Tong, 2001). The temporal and spatial parameters of the Ca^{2+} signals can be generated by fluxes across the plasma membrane and the rate at which the cytosolic Ca^{2+} enters and exits intracellular stores, including the endoplasmic reticulum, the Golgi apparatus, the vacuole, and the nucleus (Berridge, 1993; Pesty et al., 1998; Allen et al., 2000; Geisler et al., 2000; Harper, 2001; Ikeda, 2001).

Along with the complex Ca^{2+} signatures, an additional level of specificity in the Ca^{2+} signaling cascades can be achieved by the presence of several families of Ca^{2+}-binding proteins in eukaryotic cells. These Ca^{2+} sensors, which have different characteristics, such as expression pattern, subcellular localization, and Ca^{2+}-binding affinity, detect and transduce changes in the Ca^{2+} signatures to their distinct target proteins. To date, three major families of Ca^{2+} sensors have been identified in plants. Most extensively studied is the family of calmodulins, which were predicted to be composed of 14 genes in the Arabidopsis (*Arabidopsis thaliana*) genome (Luan et al., 2002). Calmodulins have no enzymatic activities themselves and function by interacting with diverse target proteins, such as NAD kinase, Glu decarboxylase, Ca^{2+}-ATPase, and protein kinases (Zielinski, 1998; Snedden and Fromm, 2001; Luan et al., 2002; Reddy et al., 2002). The second major

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family is the Ca^{2+}-dependent protein kinases (CDPKs), which possess both the EF-hand motifs and a protein kinase domain. Binding of Ca^{2+} to the EF-hand motifs alters the protein kinase activity of CDPKs (Cheng et al., 2002; Hrabak et al., 2003). The Arabidopsis genome contains approximately 34 predicted CDPK genes (Hrabak et al., 2003).

The third major family of Ca^{2+} sensors in plants includes a group of calcineurin B-like (CBL) proteins, which are most recently identified and similar to the regulatory B subunit of the protein phosphatase calcineurin in animals (Kudla et al., 1999). Unlike the animal calcineurin B subunit, however, the plant CBL family members mainly associate with a novel family of Ser/Thr protein kinases referred to as CBL-interacting protein kinases (CIPKs; Shi et al., 1999; Halfter et al., 2000; Kim et al., 2000; Albrecht et al., 2001). CIPKs represent a subclass of protein kinases that contains a unique regulatory domain in the C-terminal region, although the kinase domains of CIPKs are most related to those of the yeast Suc nonfermenting 1 (SNF1) protein kinase and the mammalian AMP-dependent protein kinase (Kudla et al., 1999). Upon Ca^{2+} binding, CBLs interact with target CIPKs through the C-terminal regulatory region containing the NAF (or FISL) motif and modulate the kinase activity present in the N-terminal region (Shi et al., 1999; Halfter et al., 2000; Albrecht et al., 2001). Recent studies with Arabidopsis mutants have demonstrated that the CBL-CIPK calcium signaling pathways play an important role in plant responses to a diverse array of external stimuli, including cold, drought, salinity, and abscisic acid (Cheong et al., 2003; Kim et al., 2003; Guo et al., 2004; Pandey et al., 2004).

Among the 10 CBL family members found in Arabidopsis, CBL1 is particularly interesting because expression of the gene is highly inducible in response to multiple stress signals, such as cold, drought, high salt, wounding, and abscisic acid (Kudla et al., 1999; Albrecht et al., 2003; Cheong et al., 2003; Kolukisaoglu et al., 2004). In addition, CBL1 has the myristolyation motif at the N-terminal end, suggesting that CBL1, like CBL4 (SOS3), may recruit the target CIPKs to the plasma membrane to form a complex (Ishitani et al., 2000). CBL1 is currently known to strongly interact with six CIPK family members, including CIPK1 (Kim et al., 2000; Albrecht et al., 2001; Kolukisaoglu et al., 2004). Using both loss-of-function and gain-of-function Arabidopsis mutants, Cheong et al. (2003) have shown that CBL1 actually regulates expression of stress genes such as *RD29A/B*, *Kin1/2*, and *DREB1A/B*, implicating that the membrane-bound CBL1-CIPK1 (or other five CBL1-interacting CIPKs) complex generated by cytosolic Ca^{2+} signatures somehow delivers the information into the nucleus. However, little is known about the molecular mechanisms that underlie such information delivery in the CBL-CIPK signaling network. We speculated that CIPK1 interactor and/or substrate molecules, which can be present in both the cytoplasm and the nucleus, could be one of the candidates that can carry out the signaling role. Therefore, identification of such CIPK1 interactors is essential to understand how the cytosolic Ca^{2+} signatures perceived by CBL1 can be transduced into the nucleus, thereby regulating expression of the stress genes.

In this study, we identified novel Arabidopsis proteins (designated ECT1 and ECT2) specifically associated with CIPK1, which is the primary target of the CBL1 protein (Shi et al., 1999; Kim et al., 2000). In their C-terminal regions, ECT proteins contained a highly conserved 180-amino acid region, which was also found in many proteins from other organisms, including monocot plants and animals. We demonstrated that this evolutionarily conserved region is involved in mediating the nuclear localization of the ECT proteins. Based on these findings, we addressed that the ECT proteins may be one of the candidate signaling molecules that can relay the information from the membrane-bound CBL1-CIPK1 complex to the nucleus.

RESULTS

Isolation of Two Novel Proteins That Interact Specifically with CIPK1

To identify in vivo substrates and/or other interacting proteins of CIPK1, we utilized a yeast screening system that had been used to isolate an in vivo substrate of the SNF1 protein kinase (Yang et al., 1992). The complete coding region of *CIPK1* cDNA was cloned in frame into a GAL4 DNA binding domain vector pGBT9.BS (pGBT.CIPK1), and it was used as a bait to screen the Arabidopsis ACT cDNA expression library. Sequence analysis of the 54 positive clones obtained by this screening revealed that they derived from several different genes, including *CBL1* and *CBL3*. Because CBL1 and CBL3 were previously known to associate with CIPK1 (Shi et al., 1999), isolation of these genes indicated that the yeast two-hybrid screening was successfully carried out.

We chose two of the novel interactor genes for further analysis and screened Arabidopsis cDNA libraries (CD4-7 and CD4-15) in order to isolate the full-length cDNAs. The two genes were designated *ECT1* and *ECT2*, respectively, because they both have an evolutionarily conserved region in their C-terminal region. The *ECT1* full-length cDNA contained a 1,287-bp open reading frame, which encodes a polypeptide of 428 amino acid residues with an estimated molecular mass of 48 kD. The *ECT2* full-length cDNA consisted of a 1,959-bp open reading frame coding for 652 amino acid residues with an estimated molecular mass of 71 kD. Comparison of deduced amino acid sequences between ECT1 and ECT2 revealed a conserved region (81% similarity) of 180 amino acid residues in the C terminus, although the rest are quite dissimilar (Fig. 1A). Through the GenBank search, we identified nine additional Arabidopsis polypeptides containing a similar C-terminal end (Fig. 1B).

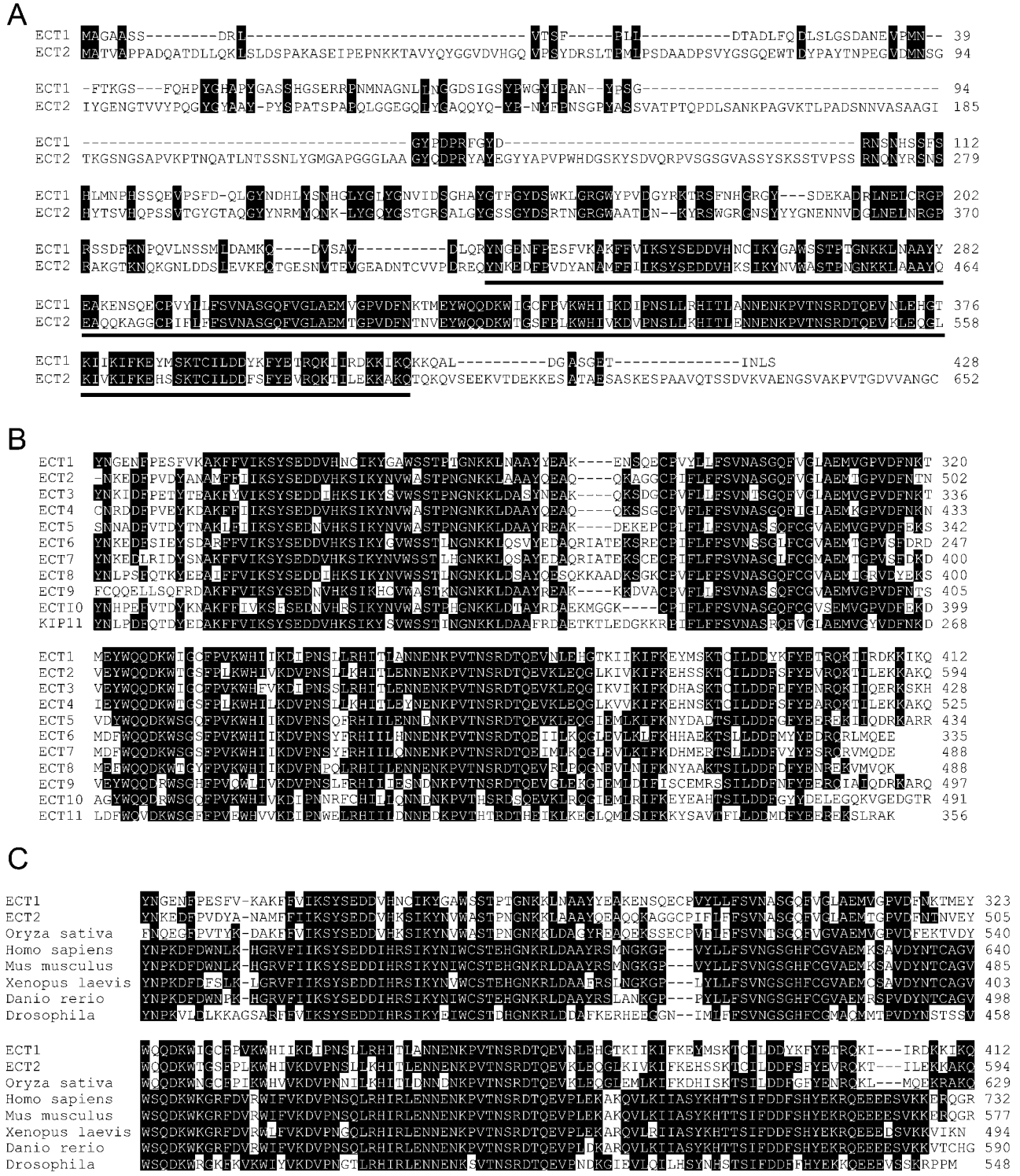

Figure 1. Sequence analysis of ECT1 and ECT2. A, Amino acid sequence alignment of ECT1 (GenBank accession and Arabidopsis Genome Initiative numbers AY894117 and At3g03950) and ECT2 (AY894118; At3g13460). Solid lines below the sequences indicate the 180-amino acid region conserved between ECT1 and ECT2. B, Amino acid sequence comparison of the C-terminal region of the ECT family members found in the Arabidopsis genome. GenBank accession and Arabidopsis Genome Initiative numbers are indicated in the parenthesis next to the gene name: ECT3 (AY056421; At5g61020), ECT4 (AY050425; At1g55500), ECT5 (AY070734; At3g13060), ECT6 (AB022216; At3g17330), ECT7 (AC023673; At1g48110), ECT8 (AF326910; At1g79270), ECT9 (AC069471; At1g27960), ECT10 (AY072018; At5g58190), and ECT11 (AC000132; At1g09810). C, Amino acid sequence alignment of the C-terminal region of the Arabidopsis ECT-like proteins found in the various organisms: rice (AC091811), human (AF432214), mouse (BAC28785), frog (BC068959), zebrafish (BC047846), and fruit fly (AAN71205). Residues with black background indicate identical amino acids, and dashes represent gaps to maximize alignment.

Interestingly, proteins with this feature are also present in other organisms, such as rice (*Oryza sativa*), human (*Homo sapiens*), mouse (*Mus musculus*), frog (*Xenopus laevis*), zebrafish (*Danio rerio*), and fruit fly (*Drosophila melanogaster*; Fig. 1C). Unfortunately, however, none of the genes have been characterized so far in terms of biochemical or biological properties.

Because none of the ECT1 and ECT2 clones isolated by CIPK1 in the yeast two-hybrid screening were full-length cDNAs, we first tested whether the complete form of ECT1 and ECT2 can still maintain the interaction with CIPK1. As shown in Figure 2A, yeast cells that carry both pGBT.CIPK1 and pGAD.ECT1 grew well on the selection medium (SC-HLT), indicating expression of the *HIS3* reporter gene. The yeast cells also expressed the other reporter gene *LacZ*, which encodes β-galactosidase, as determined by the filter-lift assay. In contrast, yeast cells cotransformed with the empty vectors, with pGBT.CIPK1 and pGAD, or with pGBT and pGAD.ECT1, failed to express these two reporter genes. Similar results were obtained in the case of ECT2 (data not shown). These results indicate that the full-length ECT1 and ECT2 proteins respectively interact with CIPK1 in the yeast two-hybrid system. To

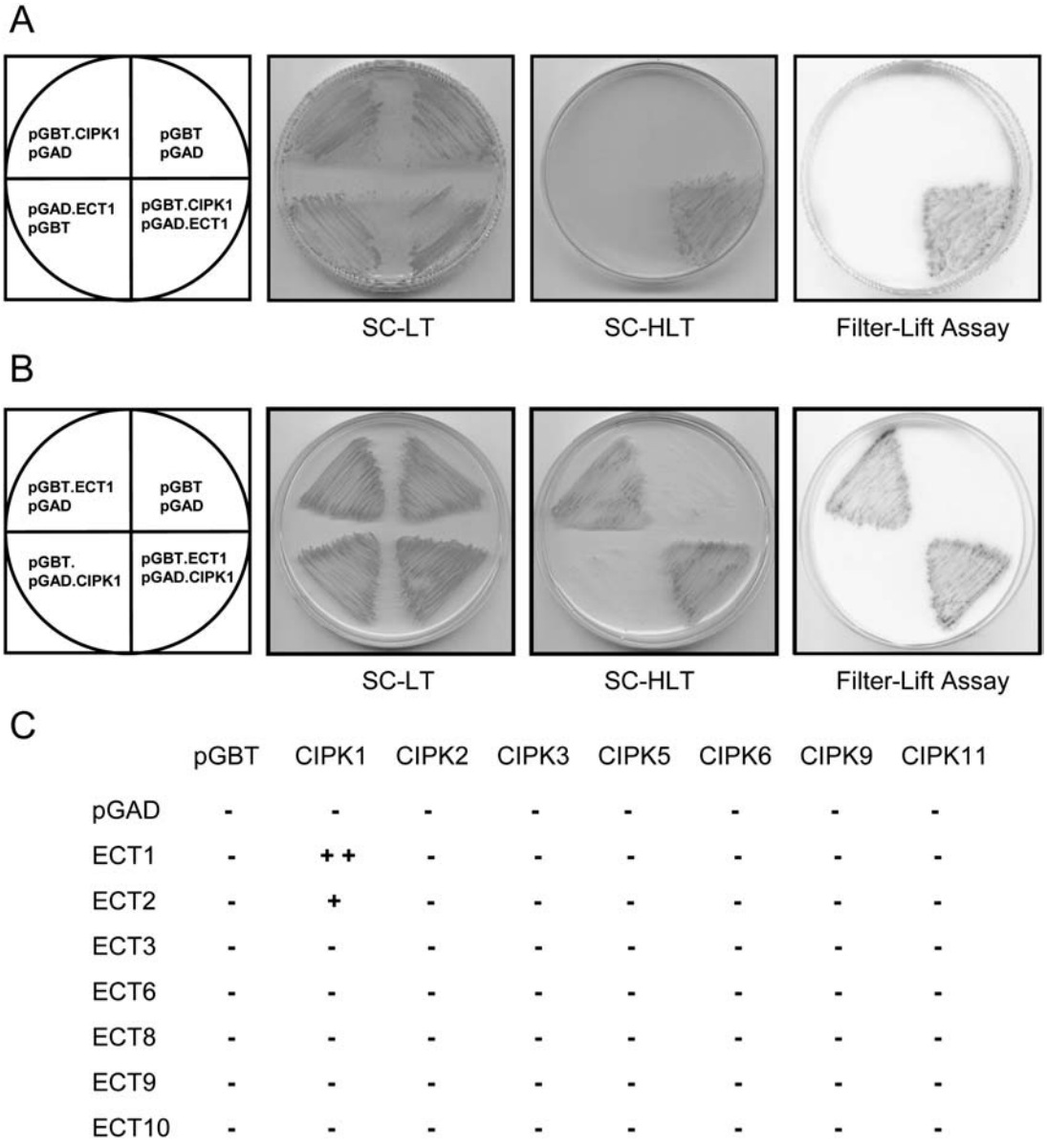

Figure 2. Yeast two-hybrid assays. A, ECT1 interacts with CIPK1. The circle at left shows the arrangement of the Y190 yeast cells carrying the indicated pGBT and pGAD plasmids. The second and third panels display yeast growth on synthetic complete media lacking Leu and Trp (SC-LT) and lacking His, Leu, and Trp (SC-HLT), respectively. The last panel shows β-galactosidase activity (filter-lift assay). B, Vector-swapping test. C, Interaction specificity between CIPK1 and ECT1 (ECT2). Yeast growth on the selection media (SC-HLT) was scored as growth (+) and no growth (−).

determine whether the interaction is vector independent, we also created the pGBT.ECT1 and pGAD.CIPK1 plasmids and performed the vector-swapping analysis. It should be noted that yeast cells harboring pGBT.ECT1 and the empty pGAD vector, not to mention yeast cells carrying pGBT.ECT1 and pGAD.CIPK1, expressed the *HIS3* and *LacZ* genes (Fig. 2B). This indicates that the ECT1 protein, when fused to the DNA binding domain of the GAL4 transcription factor, is able to autonomously activate expression of the reporter genes. Such autonomous activation activity of ECT1 provides a possibility that they may function as a transcription coactivator because they do not appear to have a DNA-binding motif. Deletion analysis demonstrated that the ECT1 N-terminal region lacking the conserved C-terminal end is required and sufficient for the autoactivation activity (data not shown).

Meanwhile, we decided to investigate whether the ECT1 and ECT2 proteins can also interact with other members of the CIPK family, which consists of 25 genes in the Arabidopsis genome (Kolukisaoglu et al., 2004). We also investigated whether CIPK1 interacts with other ECT family members. As shown in Figure 2C, both ECT1 and ECT2 interacted only with CIPK1, but not with other CIPK members tested so far, which include CIPK2, CIPK3, CIPK5, CIPK6, CIPK9, and CIPK11 (Kim et al., 2000; Albrecht et al., 2001). In addition, CIPK1 did not interact with other ECT family members such as ECT3, ECT6, ECT8, ECT9, and ECT10. These results suggest that CIPK1 specifically interacts with the ECT1 and ECT2 proteins.

The Conserved C-Terminal Region of ECT1 and ECT2 Is Required and Sufficient for Interaction with the Kinase Domain of CIPK1 in the Yeast Two-Hybrid System

Because both ECT1 and ECT2 have a similar C-terminal region, we presumed that the conserved amino acids might be involved in the interaction with CIPK1. To verify this, we created several deletion constructs by cloning ECT1 and ECT2 fragments into the pGAD.GH vector (Fig. 3). In addition, we also used the CIPK1 deletion constructs, pGBT.K292 and pGBT.C169 (Shi et al., 1999), to determine the CIPK1 domain necessary for the interaction. Combinations of these pGAD and pGBT constructs were then used to cotransform the yeast strain Y190. Interactions were determined by monitoring growth of the transformants on the selection medium and by measuring β-galactosidase activity. As shown in Figure 3, the full-length ECT1 and ECT2 proteins interacted with CIPK1, whereas the N-terminal region of ECT1 and ECT2 (ECT1N and ECT2N) did not interact. These results suggest that the conserved C-terminal region of the ECT proteins is indispensable for the interaction with CIPK1. Furthermore, the ECT1C and ECT2C mutants lacking the N-terminal region did interact with CIPK1 almost at the same intensity as their full-length forms did, revealing that the conserved C-terminal region of the ECT proteins is sufficient for the interaction.

The kinase domain of CIPK1 (K292) alone interacted with the full-length ECT1 and ECT2 proteins as well as the N-terminal deletion mutants ECT1C and ECT2C. However, it did not interact with the C-terminal deletion mutants ECT1N and ECT2N. Taken together, these results indicated that the CIPK1 kinase domain is required and sufficient for the interaction with the ECT proteins. It is noteworthy that neither the full-length nor the N-terminal deleted forms of the ECT proteins interacted with the nonkinase domain of CIPK1 (C169), which contains the NAF domain involved in interaction with the CBL family (Albrecht et al., 2001).

ECT1 Physically Interacts with CIPK1 in Vitro

Yeast two-hybrid assays demonstrated that CIPK1 interacts with the ECT1 and ECT2 proteins. To corroborate the interactions, we chose ECT1 for further analysis because it interacted with CIPK1 at higher strength than ECT2. Using the glutathione *S*-transferase (GST) gene fusion system, we purified the recombinant proteins of ECT1, ECT1N, and ECT1C produced in *Escherichia coli* and removed the GST protein via thrombin digestion (Fig. 4). The CIPK1, K292, and

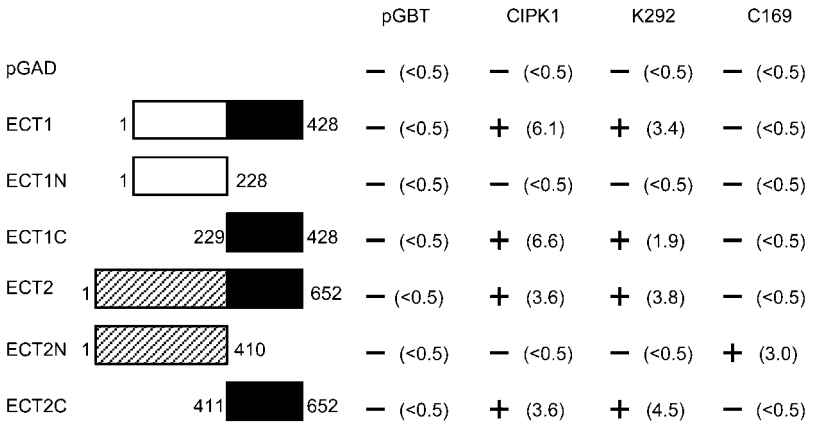

Figure 3. Identification of interaction domains in ECTs and CIPK1. Different regions of ECT1 and ECT2 were respectively cloned into the pGAD.GH vector and transformed into the Y190 yeast cells carrying pGBT, pGBT.CIPK1, pGBT.K292, or pGBT.C169. Yeast growth on the selection media (SC-HLT) was scored as growth (+) and no growth (−). Numbers in the parentheses represent units of β-galactosidase activity. Black boxes indicate the C-terminal region containing the conserved 180-amino acid residues between ECT1 and ECT2. Numbers that flank the boxes indicate the beginning and the ending positions of each protein fragment.

C169 proteins were expressed and purified as previously (Shi et al., 1999). The cleaved CIPK1 and ECT1 proteins were used to produce polyclonal antibodies from rabbit. For the pull-down assays, we mixed GST-CIPK1 (bait) with the cleaved forms of ECT1, ECT1N, or ECT1C (preys) and checked whether GST beads pulled down the preys with the immunoblot analyses. The anti-ECT1 antibody purified by the ECT1 antigen was used as a probe. As shown in Figure 5A, the CIPK1 and ECT1 proteins did interact in vitro. Moreover, CIPK1 interacted with the C-terminal region of ECT1 (ECT1C) but not with the N-terminal region (ECT1N). The GST protein alone used as a negative control did not pull down any of the preys. These interaction patterns were further assured by the results of the additional pull-down assays using the switched bait and prey proteins (Fig. 5B). As expected, the immunoblot probed by the anti-CIPK1 antibody displayed the CIPK1 protein band only in the lanes of GST-ECT1 and GST-ECT1C baits but not in the lanes of GST control and GST-ECT1N. These in vitro protein-protein interaction patterns between CIPK1 and ECT1 are consistent with the results in the yeast two-hybrid assays.

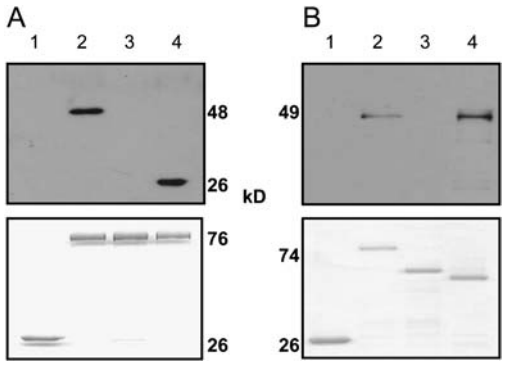

Figure 5. CIPK1 physically interacts with ECT1 in vitro. A, ECT1 and ECT1C proteins pulled down by GST-CIPK1. The GST-CIPK1 fusion protein was used as a bait to pull down the preys, which include ECT1 (lane 2), ECT1N (lane 3), and ECT1C (lane 4). For a negative control (lane 1), the GST and ECT1 proteins were used as bait and prey, respectively. Top panel is an immunoblot probed with rabbit anti-ECT1 antibody. Bottom panel is a Coomassie Brilliant Blue-stained SDS-PAGE gel indicating the amount of bait proteins used in each pull-down assay. B, CIPK1 protein pulled down by GST-ECT1 and GST-ECT1N. Each lane contains a different bait: GST (lane 1), GST-ECT1 (lane 2), GST-ECT1N (lane 3), and GST-ECT1C (lane 4). The CIPK1 protein was used as a prey. Top panel is an immunoblot probed with rabbit anti-CIPK1 antibody. Bottom panel is a Coomassie Brilliant Blue-stained SDS-PAGE gel indicating the amount of bait proteins used in each pull-down assay.

ECT1 Affinity Chromatography Purifies CIPK1 from Arabidopsis Plants

To further confirm the interaction between ECT1 and CIPK1, we attempted to purify CIPK1 from an Arabidopsis total protein extract using ECT1 as an affinity reagent. In our previous report, we have shown that CIPK1 is strongly expressed in the roots (Shi et al., 1999). Therefore, we prepared and used the total protein extracted from the Arabidopsis roots for the affinity purification experiment. As shown in Figure 6, GST-ECT1 purified a 49-kD protein, which was recognized by the anti-CIPK1 antibody. In contrast, GST control failed to retrieve the 49-kD protein, the expected size of CIPK1. These results strongly suggest a possibility of the CIPK1-ECT1 interaction in Arabidopsis plant cells.

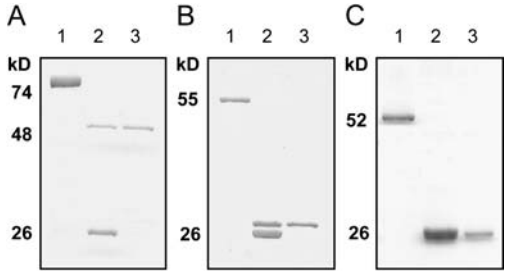

Figure 4. Expression and purification of recombinant ECT1 and its deletion mutant proteins. A, GST-ECT1 purification. Lanes 1 to 3 contain the GST-ECT1 fusion protein, the thrombin-digested forms of the GST and ECT1, and purified ECT1, respectively. B, GST-ECT1N expression. Lanes 1 to 3 contain the GST-ECT1N fusion protein, the thrombin-digested forms of the GST and ECT1N, and purified ECT1N, respectively. C, Lanes 1 to 3 contain the GST-ECT1C fusion protein, the thrombin-digested forms of the GST and ECT1C, and purified ECT1C, respectively. 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 Brilliant Blue.

Expression Patterns of *ECT1*

The Arabidopsis genome contains 11 ECT1-like genes as mentioned before. Although polypeptides encoded by these genes share a very similar C-terminal region, their N-terminal region is quite different from each other in terms of sizes and sequences. This finding suggested that a single copy of the *ECT1* gene is present in the Arabidopsis genome, which was in fact confirmed by genomic southern-blot analysis using the partial cDNA of the ECT1 gene (ECT1N) as a probe (data not shown). To gain insight into possible function of ECT1, we initially attempted to investigate expression patterns of the *ECT1* gene by northern-blot analyses using total RNAs extracted from the various organs of 5-week-old Arabidopsis wild-type plants (ecotype Columbia-0), including roots, stems, leaves, and flowers. However, the *ECT1* mRNA levels were too low to be detected in most organs; the flowers alone displayed a very faint band of the *ECT1* transcript only after long exposure (data not shown). Therefore, we used a more sensitive technique, real-time RT-PCR analysis, and determined the relative levels of the *ECT1* transcripts in such organs. ECT1 primers were designed to span exon-intron boundaries, thereby producing a 233-bp PCR

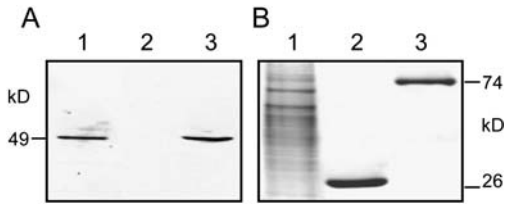

Figure 6. Affinity purification of CIPK1 by GST-ECT1 from the Arabidopsis root total protein extract. A, An immunoblot probed with rabbit anti-CIPK1 antibody. The proteins, which were purified by the GST (lane 2) and GST-ECT1 (lane 3) affinity beads from the plant extract, were characterized by western-blot analysis using anti-CIPK1 antibody as probe. Total protein extract (6 μg) from the Arabidopsis roots was included in lane 1 to show the size of the CIPK1 protein. B, A Coomassie Brilliant Blue-stained SDS-PAGE gel. Lane 1 contains 6 μg of total protein extract from the Arabidopsis roots. Lanes 2 and 3 show the GST and GST-ECT1 proteins, respectively, which were used as affinity beads. The molecular masses of the proteins are indicated at left and right in kilodaltons.

fragment only from the cDNA template, but not from the genomic DNA. The same strategy was applied to the primers of *Actin2*, a housekeeping gene used as an internal standard. As depicted in Figure 7A, flowers expressed the highest level of the *ECT1* transcripts, consistent with the RNA gel-blot result. Other organs, such as the roots, stems, and leaves, also expressed the ECT1 transcripts, although the levels were significantly lower. Furthermore, we have found that *ECT1* mRNA accumulation was not affected by stress conditions, including cold, drought, wounding, and high salt (data not shown).

To further analyze the expression patterns of the *ECT1* gene, we created transgenic Arabidopsis plants carrying a chimeric construct, which consists of the putative promoter region of *ECT1* fused to the β-glucuronidase (GUS) reporter gene in pBI101. As shown in Figure 7B, histochemical GUS assays of the transgenic plants indicated that the *ECT1* promoter weakly drove the GUS expression in the shoot and root apices. Interestingly, GUS activity was also detected around the primary root regions where lateral roots were formed, suggesting that ECT1 may be involved in the initiation and/or development of the lateral root. In addition, stamens and carpels in the flowers as well as trichomes in the leaves displayed relatively strong GUS activities, whereas no GUS expression was detected in the mature siliques, germinating seeds, and young seedlings (2–4 d after germination).

Nuclear Localization of ECT1 Is Mediated by the Conserved C-Terminal Region

To investigate the subcellular localization of ECT1, we created the ECT1-GFP fusion construct under the control of the 35S promoter of cauliflower mosaic virus (pMD.ECT1) and introduced into the onion epidermal cells by the particle bombardment procedure. As displayed in Figure 8A, the transient expression analysis showed that the ECT1-green fluorescent protein (GFP) protein was predominantly localized in the nucleus. The GFP control, however, exhibited similar intensities of fluorescence in both the cytoplasm and the nucleus. These results clearly indicate that the ECT1 protein possesses information to direct the nuclear targeting.

Using Arabidopsis transgenic plants expressing the ECT1-GFP fusion protein, we further confirmed nuclear targeting of the ECT1 protein. The Arabidopsis

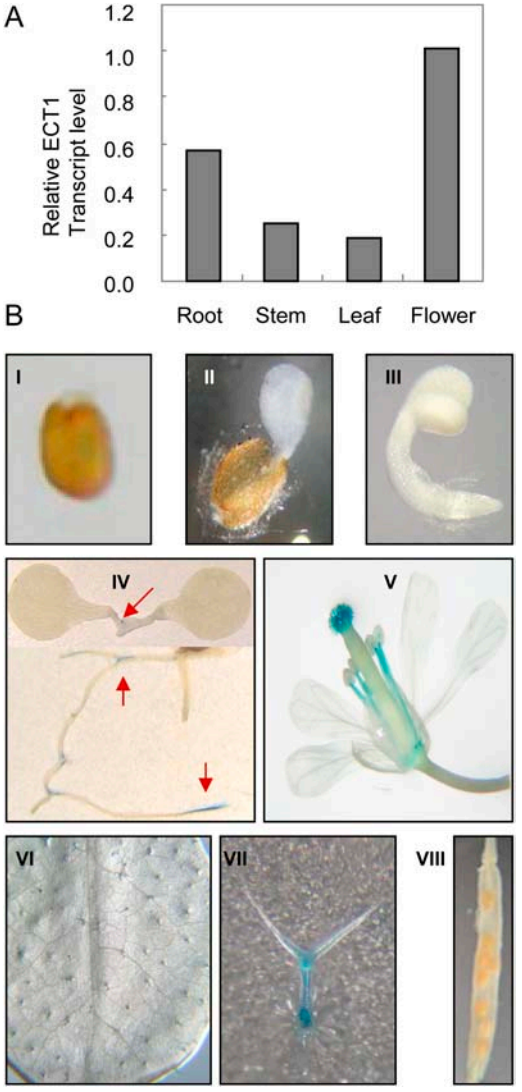

Figure 7. Expression patterns of the *ECT1* gene. A, Real-time RT-PCR analysis of ECT1 transcript levels in different organs of Arabidopsis plants. Total RNA was isolated from various tissues (root, stem, leaf, and flower) of 5-week-old wild-type plants grown under long-day conditions. Real-time RT-PCR was performed with either *ECT1*-specific primers or *Actin2*-specific primers. Bars indicate the relative *ECT1* transcript levels normalized to the housekeeping gene *Actin2* transcript levels. Data present means of triplicate samples. B, Histochemical GUS analysis of *ECT1* promoter-GUS transgenic plants. I and II, a germinating seed; III, a 2-d-old seedling; IV, a 2-week-old seedling. The red arrows indicate the positions where GUS activity was detected. V, Flower; VI, leaf; VII, trichome; VIII, silique.

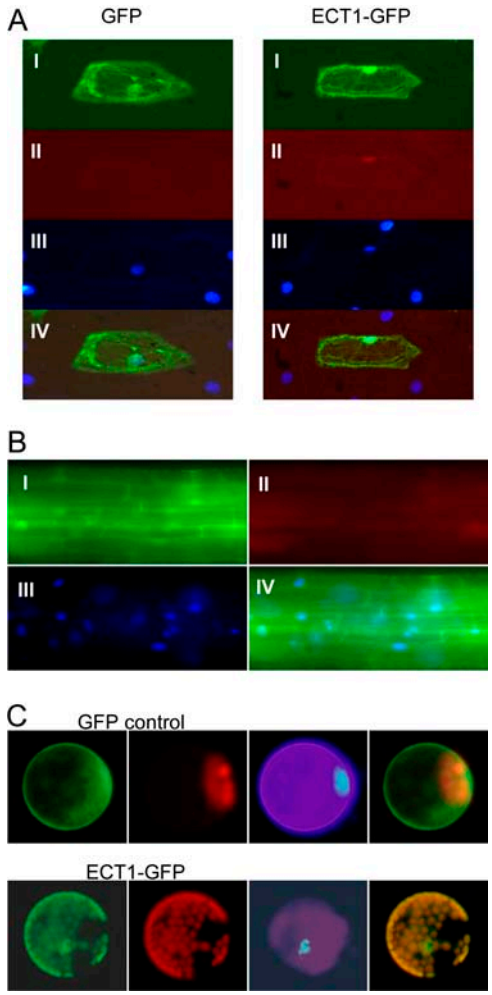

Figure 8. Subcellular localization of the ECT1 protein. A, Transient expression of the GFP control (left) and ECT1-GFP (right) in the epidermal onion cells. The plasmids were respectively introduced into the onion epidermal cells via the biolistic particle delivery system. The cells were analyzed at 18 h after particle bombardment: I and II indicate images of GFP and autofluorescence, respectively. III shows nuclei visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. IV shows the merged image. B, The transgenic Arabidopsis root (elongation zone) expressing ECT1-GFP. I, II, and III indicate images of GFP, autofluorescence, and DAPI, respectively. IV shows the merged image. C, A protoplast from the stably transformed Arabidopsis leaf expressing GFP (top) and ECT1-GFP (bottom), respectively. From the left, GFP, autofluorescence, DAPI, and merged images are displayed.

transgenic plant roots (elongation zone) showed high GFP activities in the regions where nuclei were localized (Fig. 8B). In addition, protoplasts prepared from the stably transformed Arabidopsis leaves showed that ECT1-GFP was localized significantly in the nucleus. In contrast, fluorescence was uniformly detected throughout the protoplast prepared from the Arabidopsis plants expressing the GFP protein alone (Fig. 8C).

To dissect the ECT1 region involved in the nuclear localization, we generated two ECT1 deletion constructs pMD.ECT1N (the N-terminal 228 amino acids) and pMD.ECT1C (the conserved C-terminal region), which express ECT1N-GFP and ECT1C-GFP, respectively, in plant cells. As depicted in Figure 9, transient expression assays showed that ECT1N-GFP was localized in the cytoplasm, whereas ECT1C-GFP was almost exclusively confined in the nucleus. These observations indicate that the ECT1 C-terminal region containing the evolutionarily conserved domain is required and sufficient for the nuclear localization. Interestingly, ECT1C-GFP lacking the N-terminal 228 amino acids was more predominantly localized in the nucleus than was the full-length ECT1-GFP. Therefore, it suggests that the N-terminal region may play an inhibitory role in nuclear targeting of ECT1. Although ECT1 does not appear to carry the known nuclear localization signal, a short stretch of amino acids that mediates the transport of nuclear proteins into the nucleus in ECT1, it is noteworthy that the C-terminal 180 amino acids of ECT1 have a higher proportion of positively charged residues. It is well known that positively charged amino acids are abundant in nuclear localization signals. The ECT1 C-terminal region has 21 Lys, 4 Arg, and 4 His residues.

To determine whether calcium regulates targeting of the ECT1 protein in vivo, we transiently coexpressed ECT1-GFP along with the CBL1 calcium sensor and the interacting partner CIPK1 in the onion epidermal cells, which were subsequently incubated in the elevated or depleted calcium conditions. As shown in Figure 10, addition of Ca^{2+} in the presence of calcium ionophore A23187 facilitated nuclear localization of ECT1-GFP. However, such predominant nuclear localization of ECT1-GFP was not observed in the onion cells incubated with calcium-chelating reagent EGTA. These results suggest a possibility that calcium plays a role in regulating the nuclear localization of ECT1 through the CBL1 calcium sensor.

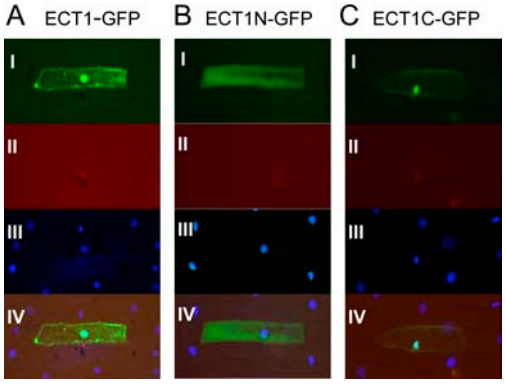

Figure 9. Determination of the ECT1 domains capable of mediating nuclear localization by transient expression assays. A, ECT1-GFP; B, ECT1N-GFP; C, ECT1C-GFP. Each plasmid was introduced into the onion epidermal cells and analyzed as described in Figure 8 legend. I, II, and III indicate images of GFP, autofluorescence, and DAPI, respectively. IV shows the merged image.

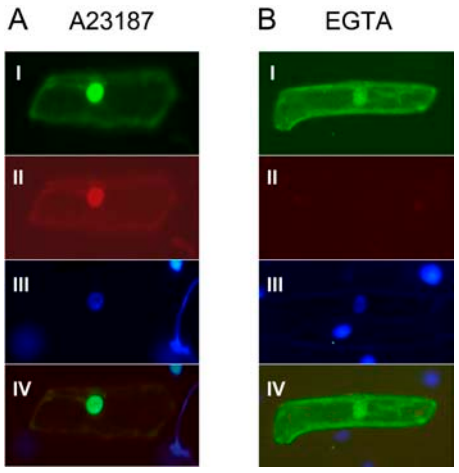

Figure 10. Effect of calcium on the subcellular localization of the ECT1 protein. Plasmids (MAS-CBL1 [Cheong et al., 2003], MAS-CIPK1, and pMD.ECT1) were coprecipitated onto 1.6-μm gold particles and introduced into the onion epidermal cells via the biolistic particle delivery system. Following the particle bombardments, cells were incubated in the indicated conditions and analyzed by a fluorescence microscope. A, Treatment of A23187 along with 10 mM $CaCl_2$. After 18 h incubation in the Murashige and Skoog media, cells were treated for 30 min with calcium ionophore A23187 (3.8 μM) together with 10 mM $CaCl_2$. B, Incubation with 1 mM EGTA for 18 h. I, II, and III indicate images of GFP, autofluorescence, and DAPI, respectively. IV shows the merged image.

DISCUSSION

We have identified novel CIPK1-associated proteins (ECT1 and ECT2), which share conserved 180 amino acids in the C terminus. We have also shown that the conserved region is not only involved in the interaction with CIPK1, but also responsible for the nuclear localization of the ECT protein. These findings implicated the ECT proteins as molecular components in the CBL-CIPK calcium signaling pathways. Because the conserved region is also found in the unknown proteins from other eukaryotes, including animals, in which Ca^{2+} serves as a ubiquitous second messenger, our findings may provide a new insight into understanding of the calcium signal transduction pathways in the eukaryotic system.

Specific Interaction between CIPK and ECT Family Members Confers an Additional Level of Complexity on Deciphering Calcium Signals

Calcium plays a role in a variety of signal transduction pathways in plants and animals (Crabtree, 2001; Evans et al., 2001; Carafoli, 2002; Scrase-Field and Knight, 2003). Although it is not clear how calcium can convey such diverse information, it is currently believed that calcium can accomplish a stimulus-specific cellular response partly due to the complexity of Ca^{2+} signature consisting of the temporal and spatial parameters, not to mention concentration (Dolmetsch et al., 1998; Rudd and Franklin-Tong, 2001). In addition, presence of a number of calcium sensors and their target proteins in the eukaryotic cells increase the level of complexity in deciphering the Ca^{2+} signature.

Among several families of calcium sensors in plants, CBLs are known to form a complex molecular network with their target protein CIPKs (Kim et al., 2000; Albrecht et al., 2001; Kolukisaoglu et al., 2004). The CIPK family proteins contain a unique regulatory domain in the C-terminal region, which is required and sufficient for interacting with CBL Ca^{2+} sensors. (Zhu, 2002; Cheong et al., 2003; Kim et al., 2003; Pandey et al., 2004). Because the Arabidopsis genome contains 10 CBLs and 25 CIPKs and each member of the CBL family specifically interacts with only a subset of CIPKs, they can in theory generate a number of different CBL-CIPK complexes that can mediate a diverse array of signaling processes (Kim et al., 2000; Albrecht et al., 2001; Luan et al., 2002; Kolukisaoglu et al., 2004). In fact, several recent studies have demonstrated that an individual component of the CBL-CIPK network functions specifically in a disparate signal transduction pathway. Perhaps the best known is the CBL4-CIPK24 (also known as SOS3-SOS2) complex, which is specifically involved in mediating ionic stress tolerance (Xiong et al., 2002; Guo et al., 2004). Loss-of-function mutants of either *SOS3* (*CBL4*) or *SOS2* (*CIPK24*) exhibited hypersensitivity to Na^+ (Zhu, 2002).

Using yeast two-hybrid analyses and pull-down assays, we have demonstrated in this study that both ECT1 and ECT2 specifically associate with CIPK1 with different interaction affinities (Figs. 3 and 5). Interestingly, however, other CIPK family members, including CIPK2, CIPK3, CIPK5, CIPK6, and CIPK11, did not interact with the two ECT proteins (Fig. 2). We also found that CIPK1 did not interact with other ECT family members tested so far, which include ECT3, ECT6, ECT10, and ECT11. In addition, ECT11 interacted only with CIPK3, but not with other CIPK family members (data not shown). These findings suggest that each member of the CIPK family may have different ECT partners, as in the case of CBL-CIPK complex formation (Kim et al., 2000; Albrecht et al., 2001; Kolukisaoglu et al., 2004). It is noteworthy that the N-terminal region of the ECT family members varies significantly from each other, indicating that individual ECT proteins probably possess a distinct function that cannot be replaced by other ECT members. Therefore, the specific interaction between 25 CIPK and 11 ECT family members should provide the CBL-CIPK signaling pathways with an additional level of diversity in transducing Ca^{2+} signals, thereby allowing them to mediate more diverse signaling cascades that lead to a variety of distinct cellular or physiological responses. We speculate that formation of the specific CIPK-ECT complex in vivo may be determined by expression pattern, subcellular localization, and interaction affinity of the two proteins.

ECT1 and CBL1 Can Respectively Form a Complex with CIPK1

CBLs activate the kinase activity of CIPKs in a Ca^{2+}-dependent manner by physically binding to their C-terminal regulatory domain (Halfter et al., 2000). It appears that intramolecular interaction between the N-terminal kinase domain and the C-terminal regulatory domain inhibits kinase activity. Removal of the C-terminal regulatory region, including the CBL binding motif, resulted in constitutive activation of the protein kinase that is active in the absence of CBL protein and Ca^{2+} (Guo et al., 2001). More recently, Guo et al. (2004) reported that expression of the constitutively active SOS2 (CIPK24) form, which lacks the C-terminal regulatory domain, was not sufficient to rescue the salt-sensitive phenotype of either *sos2* or *sos3* (*cbl4*) mutants. By contrast, another form of constitutively active SOS2 kinase, which was created by a Thr-168-to-Asp change and therefore retains the structural integrity of the C-terminal regulatory domain, did rescue the phenotype of both mutants. These results revealed that the C-terminal regulatory domain of SOS2 plays an essential role in salt tolerance, implying that the CBL-CIPK interaction itself has additional unknown function in the signaling pathway other than activating target kinase activity. Now, our discovery of the novel ECT family proteins, associated with the kinase domain of CIPKs, presents a clue to unraveling the dual function of the CBL-CIPK interaction. For example, CIPK1 interacts via the C-terminal region with CBL1 in the presence of calcium (Shi et al., 1999). It also interacts with ECT1, and the N-terminal kinase domain is responsible for the interaction (Figs. 3 and 5). Therefore, it is plausible that formation of the CBL1-CIPK1 complex not only activates the N-terminal kinase activity but also influences the interaction affinity between CIPK1 and ECT1 that has a certain function in the signaling pathway. At present, however, we do not know whether CBL1 and ECT1 interact with CIPK1 exclusively or cooperatively. This explanation accounts for why the C-terminal truncated active SOS2 form, which cannot associate with SOS3 (CBL4), failed to rescue either *sos2* or *sos3* (*cbl4*) mutants. We speculate that at least one of the ECT family members probably interacts with the kinase domain of SOS2.

Role of the Evolutionarily Conserved Region in the ECT Family

Plants have developed mechanisms that sense and transduce the cytosolic Ca^{2+} signals induced by environmental stimuli to regulation of gene expression, thereby responding appropriately to the stress. It is known that the CBL1 calcium sensor regulates expression of *RD29A/B*, *Kin1/2*, and *DREB1A/B* genes and plays an important role in plant stress responses, including salt, cold, and drought (Albrecht et al., 2003; Cheong et al., 2003). Recently, we have determined that CBL1, which has the *N*-myristolyation motif, is predominantly bound to the plasma membrane and is not found in the nucleus using the GFP fusion system (data not shown). These results strongly suggested that the cytoplasmic CIPK1 (data not shown), a target protein kinase of CBL1, might be recruited to the membrane under the stressful conditions as the SOS3-SOS2 (CBL4-CIPK24) complex (Ishitani et al., 2000; Kim et al., 2000). In this case, the membrane-bound CBL1-CIPK1 complex should communicate with the nucleus using as yet unidentified signal transducers to regulate expression of the stress genes.

Based on several lines of evidence, we propose that the ECT family protein may function as the novel signal molecules, which can relay the cytoplasmic information into the nucleus. First, ECT1 and ECT2 specifically interact with CIPK1 as determined by the yeast two-hybrid system and pull-down assays. Importantly, the kinase domain of CIPK1 alone was sufficient to form a complex with the conserved region between the two ECT proteins (Figs. 1, 3, and 5). Because the ECT proteins were not phosphorylated in vitro by CIPK1 regardless of Ca^{2+} and CBL1 in the kinase reactions (data not shown), we suspect that the ECT-CIPK1 association itself would be an important factor as in the MEK1-MyoD case (Perry et al., 2001). The MEK1 protein kinase is known to repress MyoD activity not via phosphorylation but via physically binding to the N-terminal end of the MyoD protein. Currently, however, we cannot completely rule out the possibility that the ECT proteins may be in vivo substrate molecules of the CIPK1 protein kinase. Second, the ECT proteins can be localized in both the cytoplasm and the nucleus (Fig. 8). We showed that the conserved amino acids in the C-terminal region of the ECT proteins, which are involved in the physical interaction with CIPK1, are also responsible for the nuclear localization (Fig. 9). Therefore, it is likely that interaction status between CIPK1 and ECT proteins, which can be affected by the CBL1-CIPK1 association, could exert influence on the subcellular localization of the ECT proteins. In fact, our result in Figure 10 supports this idea. Third, yeast cells carrying the DNA-binding domain vector containing the full-length ECT1 (or ECT2) cDNA (pGBT.ECT1 or pGBT.ECT2) and the empty pGAD.GH vector grew on the selection media (SC-HLT) and developed blue color in the filter-lift assay (Fig. 2). This autoactivation activity in the yeast two-hybrid assays suggests that the ECT proteins, which apparently do not have the DNA-binding motif, may act as transcription coactivators that can activate expression of a set of stress genes. Finally, the C-terminal amino acids, conserved among the Arabidopsis ECT family proteins, are also found in many proteins from the eukaryotes, including plants and animals. Interestingly, however, amino acid sequences similar to the ECT C-terminal region were not detected in the prokaryotic proteins. Because calcium acts as a ubiquitous signaling element in the

eukaryotic cells, such evolutionary conservation suggests that the ECT C-terminal region may play a critical role in the calcium signal transduction pathways.

Taken together, it appears that ECT1 and ECT2 interact specifically with CIPK1; therefore, these two ECT proteins may be involved in the CIPK1 signaling pathway. Further molecular genetic and biochemical analyses will unravel the biological function of the ECT family proteins, and it will certainly contribute to our understanding of how the CBL-CIPK network controls gene expression.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening and Assays

The Arabidopsis (*Arabidopsis thaliana*) λ-ACT cDNA expression library (CD4-22) constructed by Kim et al. (1997) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH). The two-hybrid library screening was carried out essentially according to Durfee et al. (1993). Briefly, the plasmid library was obtained from the phage library by in vivo excision and used to transform Y190 strain that expresses a bait protein (CIPK1). Transformants were plated onto the synthetic medium that lacks Leu, Trp, and His (SC-Leu-Trp-His). To inhibit background growth of yeast cells, 25 mM 3-amino-1,2,4-aminotriazole was also supplemented to the medium. Colonies that appeared within 5 d of incubation were selected for further analyses. For yeast two-hybrid interaction assays, genes of interest were first cloned into either the activation domain (pGAD.GH) or the DNA-binding domain (pGBT9.BS) vectors. Then, the two plasmids were introduced into yeast strain Y190 carrying two reporter genes by the lithium acetate method (Ito et al., 1983; Schiestl and Gietz, 1989). Yeast cells carrying both plasmids were selected on the synthetic medium lacking Leu and Trp (SC-Leu-Trp). The yeast cells were streaked on the SC-Leu-Trp-His plate to determine the expression of *HIS3* nutritional reporter. The β-galactosidase expression of the His^+ colonies was analyzed by filter-lift assays as described below.

Assays of β-Galactosidase

Transformants were streaked onto the selective medium (SC-Leu-Trp) and grown for 1 d at 30°C. Filter-lift assays for blue color development were performed overnight at 30°C as described by Breeden and Nasmyth (1985). For quantitative assays, transformants were grown at 30°C to mid-log phase (OD_{600} = 0.5–1.0) in the SC-Leu-Trp liquid medium. Activity of β-galactosidase was measured at OD_{574} using chlorophenol red-β-D-galactopyranoside as the substrate according to Durfee et al. (1993) and expressed in units. The equation below was used to calculate β-galactosidase activity:

$$\text{Units of } \beta\text{-galactosidase activity} = 1000 \times OD_{574}/(V \times T \times OD_{600}),$$

where V is volume of culture in mL, T is reaction time in min, and OD_{600} is yeast cell density.

Cloning of the Full-Length ECT1 and ECT2 cDNAs

In order to isolate the full-length cDNAs encoding ECT1 and ECT2, respectively, the ^{32}P-labeled probes were prepared from CIPK1-interacting ECT clones, which encode only the C-terminal regions of the two ECTs. Arabidopsis cDNA libraries (CD4-7 and CD4-15) obtained from the Arabidopsis Biological Resource Center were screened essentially as described by Kim et al. (2000). Plasmids containing the inserts were obtained from the positive plaques, and their DNA sequences were analyzed with software from DNASTAR.

Purification of GST Fusion Proteins

GST fusion proteins were purified essentially according to the protocols described in the GST gene fusion system (Amersham Biosciences). Briefly, *Escherichia coli* BL21 cells carrying a GST fusion construct were grown at 37°C overnight and were subcultured until the OD_{600} reached 0.5 to approximately 0.6. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.3 mM in order to induce the GST fusion protein. Following 3 h induction at 20°C, cells were harvested by centrifugation, resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM DTT, 5 mM EDTA, and 1 mM EGTA), and lysed by sonication. Triton X-100 was added to a final concentration of 1%. After 1 h incubation on ice, 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 beads were washed six times with ice-cold washing buffer (50 mM Tris-HCl, pH 7.4, and 100 mM NaCl). The GST fusion protein was eluted with 10 mM reduced glutathione in washing buffer from the beads.

Preparation of Polyclonal Antibodies against CIPK1 and ECT1

The protein of interest was expressed in *E. coli* and purified with glutathione-Sepharose 4B beads. After thrombin cleavage to remove the GST protein, the purified protein was separated by SDS-PAGE and visualized with Coomassie staining. The protein band of interest was carved out, washed extensively with deionized water, and used to immunize a rabbit (New Zealand white/male). For immunization, the rabbit was injected four times with 0.5 mg of the protein at an interval of 2 weeks. Following incubation at 37°C to inactivate complements, the rabbit whole blood collected from the celiac artery and heart was centrifuged to produce the blood serum. The polyclonal antibodies were purified from the blood antiserum by affinity chromatography using the antigen coupled to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences).

Pull-Down Assay and Immunoblot Analysis

Pull-down assay and western-blot analysis were performed as described previously (Shi et al., 1999). Briefly, GST fusion proteins attached to the glutathione-Sepharose 4B beads were incubated at 4°C with prey proteins lacking the GST protein in the binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.05% Tween 20, 1 mM EDTA, and 1 mM PMSF). Pull-down samples were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon-P; Millipore) to detect via immunoblot analysis the prey proteins, which were precipitated by the GST fusion bait proteins.

Affinity Purification of CIPK1 from Arabidopsis Total Proteins

Total protein preparation and affinity purification were performed as described previously (Shi et al., 1999). Briefly, total proteins were prepared from 4-week-old Arabidopsis (ecotype Columbia) roots with 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). The GST-ECT1 protein immobilized on the glutathione-Sepharose 4B beads was mixed with the total protein extract. After 4 h of incubation, the beads were washed six times with the binding buffer described above. The affinity-purified products were subjected to immunoblot analysis to detect the Arabidopsis CIPK1 protein.

Real-Time RT-PCR Analysis

Real-time quantitative RT-PCR was performed on the Rotor-Gene Real-Time Centrifugal DNA Amplification System (Corbett Research) using the QuantiTect SYBR Green RT-PCR kit (Qiagen) as described by the manufacturer. Briefly, total RNAs from plant tissues were isolated using the TRIzol reagent (Invitrogen), and 1 μg of total RNA served as a template in a 10-μL reaction containing 0.25 μM each primer and 2× QuantiTect SYBR Green RT-PCR Master Mix and QuantiTect RT Mix. After reverse transcription at 50°C for 20 min, samples were denatured at 95°C for 15 min and then subject to 35 PCR cycles consisting of 95°C denaturation for 15 s, 55°C annealing for 20 s, and 72°C extension for 20 s. Detection of amplification products was performed at the end of the extension period at 72°C and analyzed using Rotor-Gene software. Specificity of the amplified transcripts was verified by monitoring melting curves generated after each run. ECT1 primers were designed to produce a 233-bp PCR fragment from the cDNA template: K8

forward, 5′-TAGATCTCCAGCGCTACAATGGGGA-3′; K8 reverse, 5′-CAAATTGCCCACTTGCATTCACCGA-3′. Part of the housekeeping gene *Actin2* mRNA, 134 bp in length, was always coamplified by a pair of primers (forward primer, 5′-GAGATCACCGCTCTTGCACCTAGCA-3′; reverse primer, 5′-CTCACTCTTTGAAATCCACATCTGT-3′) and used as an internal standard.

Analysis of *ECT1* Promoter-GUS Expression

The *ECT1* promoter-GUS construct (pBI.ECT1) was transformed into *Agrobacterium tumefaciens* strain GV3101 and introduced into Arabidopsis plants by the floral dip method (Clough and Bent, 1998). Transformants were selected as described previously (Kim et al., 2003). Histochemical GUS assays of the transgenic plants were performed according to the protocol described by Jefferson et al. (1987).

Subcellular Localization of GFP Fusion Proteins

Transgenic Arabidopsis plants expressing the ECT1-GFP chimeric protein were generated as described above. Mesophyll protoplasts were prepared from the T_2 Arabidopsis leaves described by Kovtun et al. (2000) and were imaged under the Olympus BX51 fluorescence microscope with XF116-2, XF06, and XF111-2 filter sets (Omega). To carry out transient expression assays, the pMD.ECT1, pMD.ECT1N, and pMD.ECT1C plasmids were respectively delivered into the onion epidermal cells according to the procedures described by Kim and Guiltinan (1999) with minor modifications. Particle bombardments were performed using the Biolistic PDS-1000/He system (Bio-Rad) with 1,100 pounds $inch^{-2}$ rupture discs under a vacuum of 26 inches of Hg. After bombardment, tissues were maintained in parafilm-sealed half-strength Murashige and Skoog plates at 23°C for 24 h and analyzed by fluorescence microscopy. Nuclei were stained with 1 μg/mL of DAPI (Sigma-Aldrich) in phosphate-buffered saline buffer for 5 min at room temperature and examined by a fluorescence microscope at an excitation wavelength of 350 nm.

Construction of Plasmids

The pGBT.CIPK1, pGBT.K292, and pGAD.KC169 plasmids were constructed as described previously (Shi et al., 1999). To create pGAD.ECT1, the coding region of the ECT1 cDNA was PCR amplified with a pair of primers K8-5 and K8-4. The PCR product was digested with *Eco*RI/*Sal*I and ligated into pGAD.GH. The plasmid pGAD.ECT1N, which lacks 200 amino acid residues in the C-terminal region, was constructed by cloning the PCR product amplified with the K8-5 and K8-9 primers into the *Eco*RI/*Sal*I sites of the pGAD.GH plasmid. Primers K8-10 and K8-4 were used to PCR amplify the N-terminal region of ECT1. The amplified PCR product was digested with *Eco*RI and *Sal*I and cloned into pGAD.GH to produce pGAD.ECT1C. To generate the pGAD.ECT2 plasmid, primers K6-1 and K6-2 were used to amplify the coding region of the ECT2 cDNA. The resulting PCR product was then cloned into the *Bam*HI/*Sal*I sites of the pGAD.GH vector. To make the pGAD.ECT2N construct, PCR product generated by a pair of primers, K6-1 and K6-3, was digested with *Bam*HI and *Sal*I restriction enzymes and then ligated with pGAD.GH. The pGAD.ECT2C plasmid was constructed by cloning the PCR fragment, which was amplified with K6-4 and K6-3 primers, into the *Eco*RI/*Sal*I sites of pGAD.GH. To create constructs pGEX.ECT1, pGEX.ECT1N, and pGEX.ECT1C, the plasmids pGAD.ECT1, pGAD.ECT1N, and pGAD.ECT1C, respectively, were digested with *Eco*RI and *Sal*I restriction enzymes, and then each resulting insert was introduced into the pGEX-4T-3 vector.

A transcriptional fusion of the *ECT1* promoter to a GUS reporter gene was made as follows: We performed PCR on the Arabidopsis (Columbia-0) genomic DNA with K8-PF2 and K8-PR primers in order to amplify the 5′ flanking DNA sequences between −1816 and +1 relative to the translation start codon (ATG) of the *ECT1* gene. After digestion with *Sal*I and *Bam*HI, the 1.8-kb PCR fragment was cloned into the pBI101.1 binary vector (CLONTECH), thereby creating the pBI.ECT1 plasmid. For creation of the ECT1-GFP chimeric construct (pMD.ECT1), primers K8-12 and K8-41 were used to PCR amplify the ECT1 coding region without a stop codon. Following digestion with *Xba*I/*Bam*HI, the PCR product was cloned into the pMD1 binary vector that contains a GFP reporter gene (Sheen et al., 1995). To make the plasmids pMD.ECT1N and pMD.ECT1C, PCR fragments were produced from the ECT1 cDNA with two pairs of forward and reverse primers (K8-12 and K8-31; K8-25 and K8-41, respectively). Following *Xba*I/*Bam*HI digestion, each DNA fragment was cloned into pMD1. All the PCRs were carried out using *Pfu* DNA polymerase (Stratagene) to enhance fidelity. To create CIPK1 overexpression construct (MAS-CIPK1), the full-length *CIPK1* cDNA (Shi et al., 1999) was digested with *Not*I and *Sac*I and cloned into pATC940 vector (Ni et al., 1995). All the constructs above were verified by DNA sequencing.

Oligonucleotide Primers Used in the Plasmid Construction

Primers used in this study are listed below, with restriction enzyme sites underlined. Three additional bases, which were chosen randomly by considering their effect on melting temperature and on dimer and stem-loop formation, were included at the 5′ end of the primers for efficient digestion by restriction enzymes: K8-4, 5′-ATCGTCGACTCAACTCAAGTTAATTGTTT-3′; K8-5, 5′-TATGAATTCTATGGCTGGAGCCGCTTCTT-3′; K8-9, 5′-AATGTCGACTACTGCTGAAACGTCTTGCT-3′; K8-10, 5′-AATGAATTCAGATCTCCAGCGCTACAATG-3′; K8-12, 5′-ATATCTAGAATGGCTGGAGCCGCTTCTTC-3′; K8-25, 5′-ATATCTAGACATGGATCTCCAGCGCTACAA-3′; K8-31, 5′-TATGGATCCTACTGCTGAAACGTCTTGCT-3′; K8-41, 5′-TTAGGATCCACTCAAGTTAATTGTTTCCC-3′; K8-PF2, 5′-TAAGTCGACCGTATGTCACAAAATCCATT-3′; K8-PR, 5′-TATGGATCCTATCGAGAGAAGGCAAAAAA-3′; K6-1, 5′-TCTGGATCCCATGGCTACCGTTGCTCCTC-3′; K6-2, 5′-ATAGTCGACACACCATCCTCTTAGTTAGC-3′; K6-3, 5′-TTAGTCGACGGAACAACACATGTGTTATC-3′; K6-4, 5′-AATGAATTCGAGGAATCAAAACTACCGCT-3′.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY894117 and AY894118.

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