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Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals

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ABSTRACT **An important effector of Ca^{2+} signaling in animals and yeast is the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin. However, the biochemical identity of plant calcineurin remained elusive. Here we report the molecular characterization of AtCBL (*Arabidopsis thaliana* calcineurin B-like protein) from *Arabidopsis*. The protein is most similar to mammalian calcineurin B, the regulatory subunit of the phosphatase. AtCBL also shows significant similarity with another Ca^{2+}-binding protein, the neuronal calcium sensor in animals. It contains typical EF-hand motifs with Ca^{2+}-binding capability, as confirmed by *in vitro* Ca^{2+}-binding assays, and it interacts *in vivo* with rat calcineurin A in the yeast two-hybrid system. Interaction of AtCBL1 and rat calcineurin A complemented the salt-sensitive phenotype in a yeast calcineurin B mutant. Cloning of cDNAs revealed that AtCBL proteins are encoded by a family of at least six genes in *Arabidopsis*. Genes for three isoforms were identified in this study. *AtCBL1* mRNA was preferentially expressed in stems and roots and its mRNA levels strongly increased in response to specific stress signals such as drought, cold, and wounding. In contrast, *AtCBL2* and *AtCBL3* are constitutively expressed under all conditions investigated. Our data suggest that AtCBL1 may act as a regulatory subunit of a plant calcineurin-like activity mediating calcium signaling under certain stress conditions.**

Calcium signaling mechanisms are widely employed by all eukaryotic organisms to regulate gene expression and a variety of other cellular processes (1, 2). Ca^{2+} mediates intracellular signaling and regulation of differentiation predominantly through the activity of Ca^{2+}-dependent protein kinases and phosphatases (3). One of the well characterized effector molecules in both animals and fungi is the serine/threonine-specific protein phosphatase calcineurin (4). Calcineurin is a heterodimer composed of a regulatory B subunit (CNB) and a catalytic A subunit (CNA). The enzyme is activated through interaction with Ca^{2+}-stimulated calmodulin (CaM) and acts as an effector of Ca^{2+} signaling (5, 6). Identification of calcineurin as the molecular target for the immunosupressants cyclosporin A and FK506 has established these drugs as useful tools to dissect the function of this phosphatase in various eukaryotic organisms (7, 8). In animals, calcineurin is involved in a variety of signaling pathways, including those for T cell activation (7–9) and neuronal functions (10, 11). In yeast, calcineurin mediates recovery from pheromone arrest and adaptation to high-salt stress (12–15).

By applying immunosuppressive drugs as probes, we have shown that a calcineurin-like activity is critical for the modulation of a plasma membrane ion channel activity in guard cells (16). In addition, a calcineurin enzyme from animal sources regulates ion channels in the plasma membrane and tonoplast of guard cells and aleurone cells (16–18). A recent study (19) also showed that overexpression of yeast calcineurin enhances salt tolerance in transgenic tobacco. Despite increasing evidence for calcineurin function in higher plants, the molecular nature of the plant calcineurin-like activity has remained elusive.

We report here the molecular and biochemical characterization of a CNB-like protein (AtCBL) from *Arabidopsis.* This Ca^{2+}-binding protein is most similar to CNB from animals and is able to interact with the CNA subunit from rat as revealed by protein–protein interaction studies in the yeast two-hybrid system, and this interaction restores the salt tolerance to yeast CNB mutant. *AtCBL1* gene expression is strongly regulated by stress signals such as drought, cold, and wounding, consistent with a possible function of this protein in plant stress responses.

MATERIALS AND METHODS

Cloning, cDNA Sequence Analysis, and DNA Blot. PCRs were performed for 35 cycles (1 min 94°C, 1 min 40°C, 1 min 72°C) using 2 μl of phage stock of an *Arabidopsis* cDNA library (20) as a template. One of the primer pairs (PCD1, 5′-ATCTA(C/T)GA(C/T)ATGGAN-3′; PCD2, 5′-(A/C)(A/C)CIAATIGT-CTT(C/T)TCN-3′ yielded a PCR product of the expected size (shown in Fig. 1*A*). The PCR products were gel purified and cloned into PCRII TA vector as described by the manufacturer (Invitrogen). A 1- to 2-kb size-selected *Arabidopsis* cDNA library (20) was screened to isolate full-length cDNA clones. Clones were sequenced with an AutoRead Sequencing kit (Pharmacia) and analyzed on ALF (Pharmacia). Sequences were analyzed by using DNASTAR software, and comparison to databases was performed by using the BLAST program. DNA isolation and gel blot analyses were carried out as described (21).

Stress Treatments and Northern Blots. Five-week-old *Arabidopsis* seedlings grown under short-day conditions were used for the stress treatments. Wounding was performed by puncturing leaves with a hemostat. Typically, at least 80% of the leaves in the treated pot were wounded. Drought and cold treatments were performed as described (22). Samples were collected at time points indicated in Fig. 6. Total RNA was isolated, blotted, and hybridized with ^{32}P-labeled cDNA probes according to a procedure described previously (21).

Plasmid Construction. Full-length cDNA of *AtCBL1* was amplified by PCR using *Pfu* DNA polymerase and primer PCS1 (5′-CCATGGATCCAGGCTGCTTCCACTCAAAG-3′) and M13 reverse primer. The cDNA was cloned as a *Bam*HI/*Pst*I fragment into yeast vector pGBT9.BS (23), giving rise to pBDAtCBL1. To generate plasmid pBDrCNB, rat CNB cDNA (24) was cloned as a *Bam*HI/*Xho*I fragment into the same vector after PCR amplification with primers PRCNBF (5′-CTAGTGGATC-CCGGAAATGAGGCAAGT-3′) and PRCNBR (5′-TAT-

Abbreviations: CNA, calcineurin A (catalytic) subunit; CNB, calcineurin B (regulatory) subunit; CaM, calmodulin; GST, glutathione *S*-transferase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF076251 to AF076253).

A Commentary on this article begins on page 4216.

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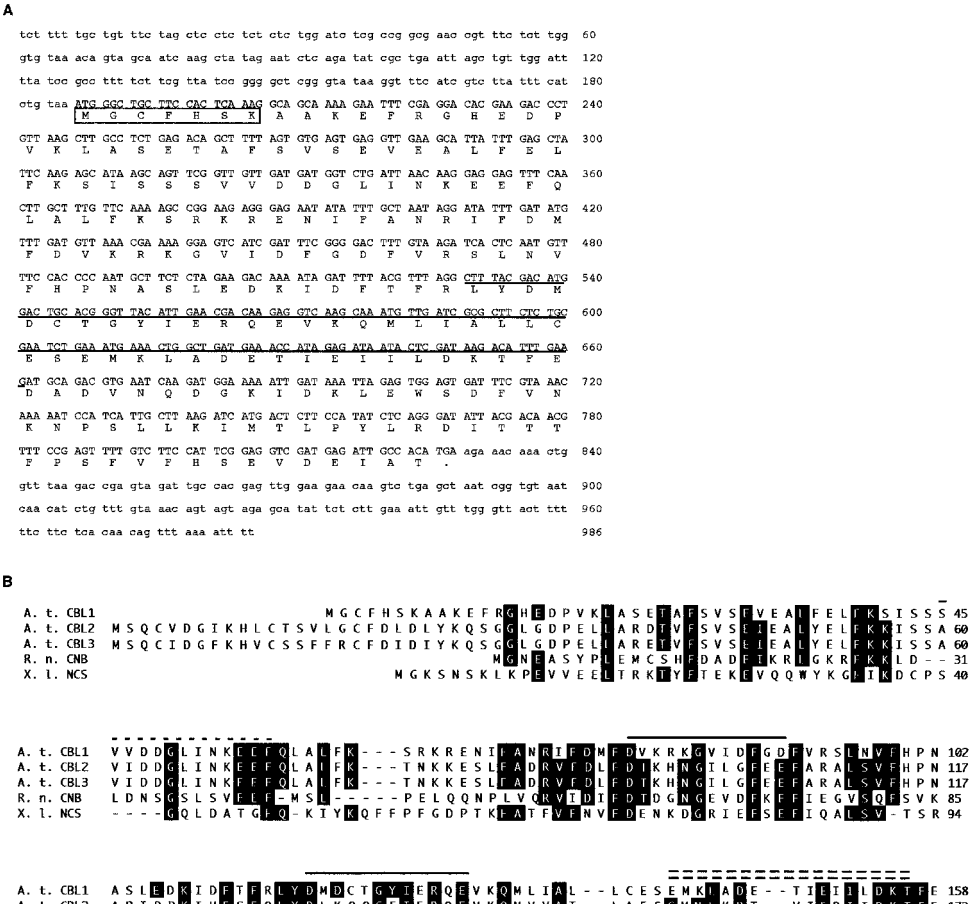

FIG. 1. Sequence analyses of *AtCBL1* cDNA and AtCBL proteins. (*A*) cDNA and deduced amino acid sequences of *AtCBL1*. The coding region of the cDNA is presented in capital letters and the noncoding regions in lowercase letters. The boxed sequence contains a typical myristoylation site (MGXXXSK). The underlined sequence represents the cDNA fragment identified by PCR (see *Materials and Methods*). An in-frame stop codon (taa) is immediately upstream from the ATG starting codon. (*B*) Alignment of the amino acid sequences of three AtCBL proteins from *Arabidopsis* (A. t. CBL1, A. t. CBL2, A. t. CBL3) with rat CNB (R. n. CNB), and *Xenopus* neuronal calcium sensor (X. l. NCS). Alignment was generated by using the CLUSTAL method with DNASTAR software. Amino acids identical to the consensus sequence of the alignment are shown on black background. The numbers on the right indicate the amino acid position. Dashes indicate gaps introduced to improve the alignment. Solid lines above the sequence indicate position of EF-hand regions. The single-dashed line represents a variation of the Ca^{2+}-binding domain. The double-dashed line denotes the CNA interaction domain.

TCTAGACTCGAGTCACACATCTACCAC-3′). Rat CNA cDNA (25) was amplified with primers PRCNAF (5′-CTAGTGGATCCCTCCGAGCCCAAGGCG-3′) and PRCNAR (5′-ATATCTAGAGAATTCTCACTGAATATTGCT-3′), cloned as *Bam*HI/*Eco*RI fragment into plasmid pGAD.GH (23), and named pADrCNA. Primer PRCNAF and PRCNAR were used to generate plasmid pADdrCNA harboring nucleotide positions 1–1185 of the coding region of rat CNA in vector pGAD.GH. The nucleotide sequences of all constructs were verified by sequencing.

Expression and Purification of Recombinant AtCBL1 Protein. The coding region of *AtCBL1* cDNA was amplified with *Pfu* DNA polymerase (Stratagene) and subcloned into pGEX-4T-3 vector (Pharmacia). The resulting clones were verified by DNA sequencing. Overexpression of glutathione *S*-transferase (GST)-AtCBL1 fusion protein was performed as described previously (21, 26) with the following modifications: upon induction with 0.25 mM isopropyl β-D-thiogalactopyranoside (IPTG), bacterial cultures were pelleted and resuspended in a buffer containing 100 mM NaCl, 50 mM Tris·HCl at pH 8.0, 2 μM PMSF, 1 mM benzamidine, and 2 mM EDTA before lysis by sonication. The cell lysate was pelleted at 15,000 × *g* to collect the supernatant containing the fusion protein, which was subsequently affinity purified by glutathione-Sepharose 4B (Pharmacia).

Gel-Shift and $^{45}Ca^{2+}$-Overlay Assays. GST-AtCBL1 fusion protein was prepared as described above and analyzed by SDS/PAGE in the presence of 5 mM EGTA or 5 mM $CaCl_2$ buffer. Proteins were stained with Coomassie blue to monitor the mobility pattern of the proteins. For the $^{45}Ca^{2+}$-overlay assay, recombinant proteins were analyzed by SDS/PAGE, transferred to nitrocellulose membrane, and blotted by $^{45}Ca^{2+}$ as previously described (27).

Yeast Two-Hybrid Assays. Yeast strain SMY3 (28) was transformed by the polyethylene glycol/lithium acetate method as described (29). Transformants were plated on synthetic complete (SC) medium lacking Trp and Leu. Interaction assays were performed on plates containing 25 mM 3-aminotriazole in SC without Trp, Leu, and His. β-Galactosidase activity was monitored in a filter-lift assay (30) and was also assayed with chlorophenyl-red β-D-galactopyranoside (Boehringer Mannheim) as substrate (31, 32). β-Galactosidase activity (units) was calculated as follows: A_{574} of the supernatant ×1000/[reaction time (min) × culture volume used for the assay (ml) × OD_{600} of the culture].

RESULTS

Cloning and Sequence Analysis of *Arabidopsis* CLB1. Extensive peptide and DNA alignments of known CNA and CNB sequences were performed to develop a homology-based cloning strategy. Highly conserved domains identified in CNA proteins from different species are also conserved in several plant phosphatases that belong to the PP1/PP2A subtypes. In contrast, the CNB subunit is highly conserved and contains unique sequences that are absent from other Ca^{2+}-binding

proteins. Therefore, we focused our efforts on the identification of the regulatory subunit of plant calcineurin by a systematic PCR approach.

One of the primer pairs (PCD1, PCD2) produced a product of the expected size, and its sequence showed significant similarity to known CNB proteins in a BLASTX search (PAM 120 score of 61). When this cDNA was used to screen an *Arabidopsis* cDNA library, 12 clones were identified, one of which was fully sequenced. The 986-bp-long cDNA contains an ORF of 213 aa, corresponding to a predicted polypeptide of 22.5 kDa (Fig. 1*A*). Database analysis with the amino acid sequence revealed a significant similarity to CNB proteins from different organisms (PAM 120 scores of 136 to CNB from rat, mouse, and human a in BLASTP search), and a much lower degree of similarity to CaM and CaM-related sequences (PAM 120 score of 77 to CaM from rat and *Arabidopsis*). The identified clone was therefore termed *AtCBL1* (*Arabidopsis thaliana* calcineurin B-like protein 1). Like CNB, AtCBL1 contains a conserved site for myristoylation at the N terminus (MGXXXSK). As shown in Fig. 1*B*, the amino acid sequence of AtCBL1 shows 32% identity (56% similarity) compared with CNB from rat. In addition, the AtCBL1 protein also shares significant similarity to another Ca^{2+}-binding protein referred to as neuronal calcium sensor from *Xenopus,* although the homology is limited to the Ca^{2+}-binding domains (33). In contrast, the similarity to CNB is not restricted to the Ca^{2+}-binding regions of the proteins. In particular, the CNA-binding domain in CNB is highly conserved in the AtCBL1 protein (24), indicating that AtCBL1 may represent a plant homolog of CNB from other organisms.

AtCBL Is Encoded by a Small Gene Family in *Arabidopsis*. Comparative Southern blot analyses at high and low stringency using *AtCBL1* cDNA as a probe (Fig. 2) indicated that *AtCBL1* is a member of a small gene family. Partial sequencing of the 11 remaining cDNA clones from the primary library screening revealed a second closely related cDNA, which was designated *AtCBL2*. Database searches using *AtCBL1* and *AtCBL2* sequences identified several expressed sequence tags (ESTs) from *Arabidopsis* (GenBank accession nos. N65303, H77209, T20940, T21746). Further analysis revealed that N65303 is identical to *AtCBL1* and H77209/T20940 are identical to *AtCBL2*, respectively. EST T21746, however, represents a previously unknown member of the *AtCBL* gene family and was, therefore, designated *AtCBL3*. The *AtCBL2* and *AtCBL3* cDNAs are both 1,043 bp long and contain ORFs of 216 aa each, corresponding to potential polypeptides of 25.8 kDa. As shown in Fig. 1*B*, the amino acid sequences of AtCBL proteins are highly conserved (63% identity and 79% similarity comparing AtCBL1 with AtCBL2 or AtCBL3; 91% identity and 96% similarity between AtCBL2 and AtCBL3). Comparative Southern blot analyses using all three *AtCBL* cDNAs as probes (Fig. 2) indicate that this gene family consists of five or six members with sufficient similarities to be detected under the stringency conditions used in our experiments (see *Materials and Methods*).

AtCBL1 Encodes a Functional Ca^{2+}-Binding Protein. The presence of conserved EF-hand motifs in the predicted protein sequence of AtCBL suggests that it may function as a Ca^{2+}-binding protein. To examine this possibility, we expressed the recombinant AtCBL1 protein as a GST fusion in *Escherichia coli*. Two approaches were used to confirm that the recombinant protein is a functional Ca^{2+}-binding protein. One was a specific gel-shift assay in which all EF-hand-containing proteins migrate faster during gel electrophoresis when the loading buffer contains high levels of Ca^{2+} instead of EGTA (27). As shown in Fig. 3, the GST-AtCBL1 fusion protein migrates more slowly in the presence of EGTA compared with its migration in Ca^{2+}-containing buffer. As a negative control, the mobility of GST was not affected by the presence of EGTA or Ca^{2+} in the buffer. As a second functional test, we performed a Ca^{2+}-binding assay using the $^{45}Ca^{2+}$-overlay technique (27). The GST-AtCBL1 fusion protein clearly binds Ca^{2+}, whereas the GST protein and the molecular weight markers did not (Fig. 3).

AtCBL1 Interacts *in Vivo* with Rat CNA and Complements a Yeast CNB Mutant. Calcineurin forms a tightly associated heterodimer composed of regulatory B and catalytic A subunits (5, 6). Interaction between the two subunits is necessary for the function of the holoenzyme and the stabilization of its subunits. Since the identity of the AtCBL-interacting protein(s) from plants is unknown, we tested whether AtCBL can interact with the heterologous CNA subunit from rat *in vivo* in the yeast two-hybrid system. To avoid potential interference in our assay by the endogenous yeast CNB, we used a yeast strain (SMY3) harboring a disrupted CNB gene. AtCBL1 was fused to the GAL4 DNA-binding domain (pBDAtCBL1). Rat CNA was fused to the GAL4 transcriptional activation domain

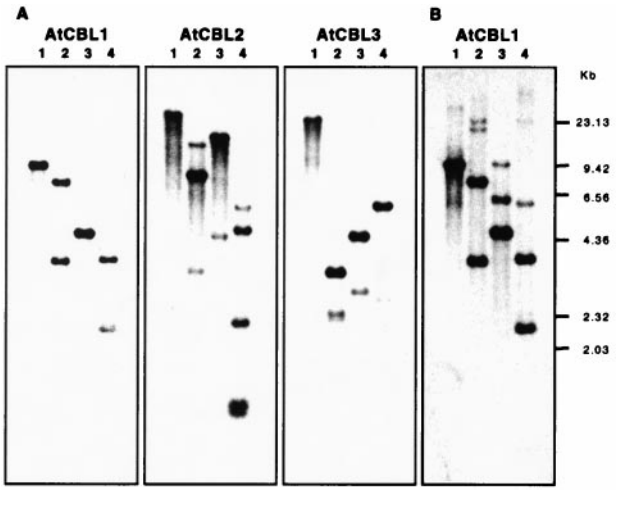

FIG. 2. Genomic organization of *AtCBL* genes. Aliquots (5 μg per digestion) of genomic DNA from *Arabidopsis* plants (Columbia ecotype) were digested with *Bam*HI (lanes 1), *Bgl*II (lanes 2), *Eco*RI (lanes 3), and *Hin*dIII (lanes 4). (*A*) After agarose gel electrophoresis, DNA was blotted onto nylon membranes and hybridized under high-stringency conditions with ^{32}P-labeled probes for *AtCBL1*, *AtCBL2,* and *AtCBL3*, respectively. (*B*) Low-stringency hybridization was performed using *AtCBL1* cDNA as a probe. *Hin*dIII-digested phage λ DNA was used to provide molecular size markers.

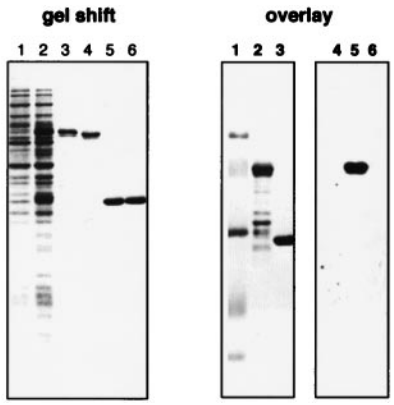

FIG. 3. AtCBL1 is a functional Ca^{2+}-binding protein. In the gel-shift panel, lane 1 and lane 2 were loaded with protein extract prepared before and after isopropyl β-D-thiogalactopyranoside (IPTG) induction, respectively. GST-AtCBL1 fusion protein was purified and incubated in EGTA- (lane 3) or calcium-containing (lane 4) buffer before being analyzed by SDS/PAGE. As a negative control, GST protein was analyzed in the same way (lanes 5 and 6). In the overlay assay, lanes 1–3 show Coomassie blue-stained molecular weight marker, GST-AtCBL1 (0.4 μg), and GST (0.2 μg), respectively. Lanes 4–6 indicate the same samples transferred to the nitrocellulose membrane and assayed by $^{45}Ca^{2+}$ binding. Only the fusion protein in lane 2 shows Ca^{2+}-binding capability (lane 5).

(pADrCNA). As a positive control, rat CNB was also cloned into the binding domain vector (pBDrCNB).

AtCLB1 interacted with the full-length rat CNA *in vivo*, as indicated by growth on selective medium and activation of β-galactosidase activity (Fig. 4 *C* and *E*). As expected, the interaction between the rat CNA and CNB subunits appeared to be much stronger (Fig. 4 *C* and *E*). Control experiments with pBDAtCBL1 and pBDrCNB constructs showed no activation of the yeast reporter gene system (Fig. 4 and Table 1).

CNA interacts not only with the CNB subunit but also with CaM. To exclude potential binding of AtCBL1 to the CaM-binding domain of rat CNA, we generated an additional construct (pADdrCNA). This plasmid expresses a truncated version of the rat CNA catalytic subunit lacking the C-terminal CaM-binding domain. Interestingly, the truncated version of rat CNA (pADdrCNA) lacking the CaM-binding domain interacted more strongly with both rat CNB and AtCLB1. This might be because of a lack of interference from the yeast CaM or better steric accessibility of the CNB-binding domain in the truncated rat CNA protein.

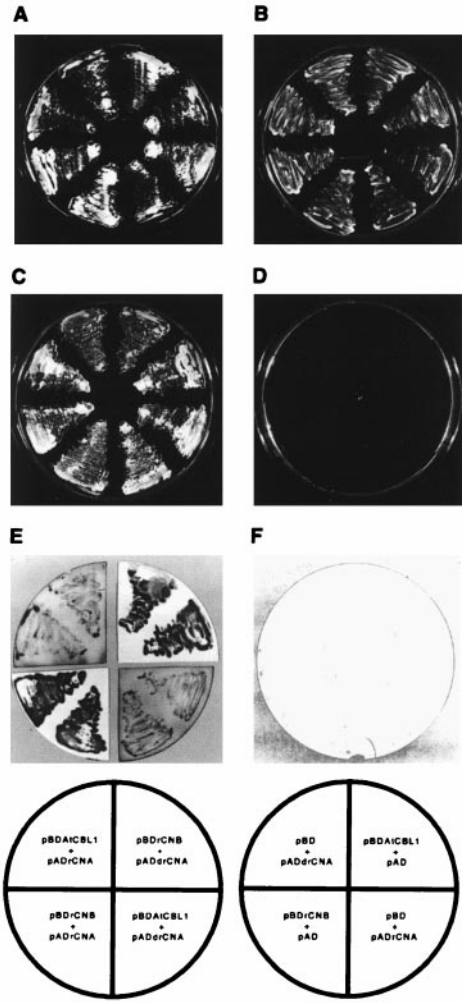

FIG. 4. Interaction between AtCBL1 and rat CNA protein in the yeast two-hybrid system. Growth of yeast transformants on SC −Trp −Leu medium (*A* and *B*), SC −Trp −Leu −His containing 25 mM 3-aminotriazole (*C* and *D*), and the colony color of the transformants determined by the filter lift assay (*E* and *F*) are shown. Plates containing histidine, but lacking tryptophan and leucine, select for the presence of the two plasmids containing the Gal4 DNA-binding and the Gal4 activation domain. Plates lacking tryptophan, leucine, and histidine and supplemented with 25 mM 3-aminotriazole select for a positive interaction between the fusion proteins. The array of the yeasts containing the different constructs is indicated in the scheme at the bottom. The symbols for the constructs were described in the text.

Table 1. Quantitative analyses of AtCBL1–rat CNA interactions

Gal4 DNA-binding domain (BD)	Gal4 activating domain (AD)	Colony color	β-Galactosidase activity, units*
BD	AD	White	0.012
BDrCNB	AD	White	0.013
BD	AD	White	0.018
BD	ADrCNA	White	0.255
BDAtCBL1	AD	White	0.133
BDrCNB	ADdrCNA	Blue	133.31
BDrCNB	ADdrCNA	Pale blue	7.43
BDAtCBL1	ADdrCNA	Pale blue	6.58
BDAtCBL1	ADrCNA	Pale blue	1.00

*The β-galactosidase activity was mesured as described in *Materials and Methods*. The numbers shown are the means of three independent measurements.

To exclude the possibility that the GAL4 DNA-binding or activation domain contributed to the observed interactions, domain swapping experiments were performed by replacing the DNA-binding domain of each construct with the transcriptional activating domain and *vice versa*. Identical results were obtained when these constructs were used (data not shown).

Quantitative analysis of the *in vivo* interactions revealed a reproducible increase in β-galactosidase expression when the truncated version of rat CNA was used in the interaction assay (Table 1). For the interaction with AtCBL1, β-galactosidase activity increased from 8-fold (pBDAtCBL1 + pADrCNA) to 50-fold (pBDAtCBL1 + pADdrCNA) compared with the background level of expression (pBDAtCBL1 + pAD). The interactions between the rat calcineurin subunits were even more robust. The estimated activities of β-galactosidase were 7-fold (pBDrCNB + pADrCNA) or 20-fold (pBDrCNB + pADdrCNA) higher than the corresponding activities observed with AtCBL1. The observed activity resulting from the interaction between AtCBL1 and the truncated rat CNA further suggests that the AtCBL1 protein most likely binds to the CNB-binding domain of rat CNA. Because none of the other Ca^{2+}-binding proteins has been shown to interact with the CNB-binding region of CNA, the results shown here indicate that AtCBL1 may function as a regulatory subunit of a plant calcineurin-like protein.

To further support this idea, we performed yeast complementation experiments. Yeast calcineurin mutant strains are highly sensitive to high-salt conditions (13–15). To test whether interaction of AtCBL1 with rat CNA is functionally significant, the yeast strains with various plasmid combinations were grown on either normal or high-salt medium. As shown in Fig. 5, CNB mutant (SMY3) transformed with different plasmids grew well in the absence of LiCl (Fig. 5 *A* and *B*). In the presence of 200 mM LiCl, mutant or mutant transformed with control plasmids either did not grew or grew very poorly (Fig. 5 *D* and *E*). In contrast, yeast strains that contained rat CNA/CNB or rat CNA/AtCBL1 plasmids grew at a rate similar to that of wild-type strain on high-salt medium (Fig. 5 *A* and *C*). This result suggests that AtCBL1 together with rat CNA can complement the defect of yeast CNB mutant in salt tolerance. Because AtCBL1 alone did not complement yeast CNB mutant, we speculate that AtCBL1 does not efficiently interact with yeast CNA.

Differential Expression Pattern of *AtCBL* Genes in Response to Stress Conditions. Tissue specificity or signal responsiveness of gene expression often reflect the function of the corresponding gene products in plant development and signaling. Members of the AtCBL gene family may be expressed differently and therefore have distinct functions. We investigated the expression pattern of all three *AtCBL* genes by Northern blot analyses using total RNA from various tissues of *Arabidopsis* plants. As shown in Fig. 6*A*, the *AtCBL1* gene is highly expressed in roots and stems. The expression level is lower in leaves and undetectable in flowers. In contrast, *AtCBL3* is expressed at similar levels in all tissues analyzed,

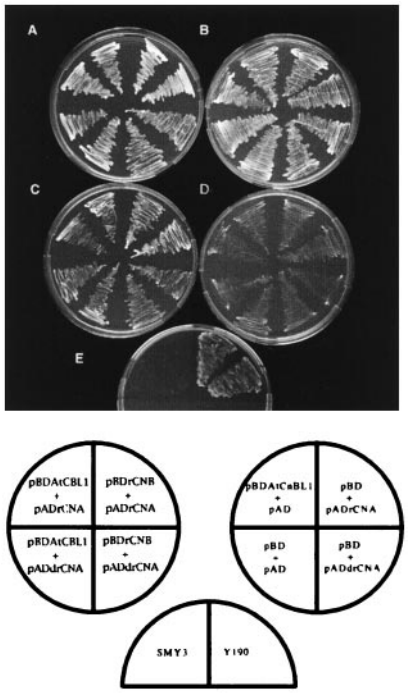

FIG. 5. Complementation of yeast CNB mutant by interaction of rat CNA and AtCBL1. Yeast CNB mutant strain SMY3 (28) was transformed with various combinations of plasmids indicated in the circles. Annotations of the plasmids are shown in *Materials and Methods* and are the same as used in Fig. 4 and Table 1. The same combinations were used in *A* and *C* (left circle in the diagram at the bottom), and in *B* and *D* (right circle). Mutant (SMY3) and a wild-type (Y190) controls in *E* are shown in the half-circle. Yeast strains were grown on the synthetic medium containing 0 mM (*A* and *B*) and 200 mM (*C*, *D*, and *E*) LiCl.

whereas *AtCBL2* is preferentially expressed in roots. Together, these results suggest that *AtCBL* genes are differentially expressed in different tissues under normal growth conditions.

To test whether plant calcineurin-like proteins could play a role in plant stress responses, we examined the expression patterns of these genes under various stress conditions, including drought, wounding, cold, heat shock, and mechanical touch. As shown in Fig. 6*B*, wounding, drought, and cold treatment strongly increased mRNA levels of *AtCBL1*, whereas heat shock or mechanical touch had no effect (data not shown). In contrast, expression of *AtCBL2* and *AtCBL3* did not respond to these stimuli. These experiments may indicate that different isoforms of the AtCBL protein may have distinct distribution patterns in *Arabidopsis* plants and may function in different pathways. Note that induction of AtCBL1 in response to wounding, drought, and cold stress corresponds to the well established Ca^{2+} involvement in these signaling pathways.

DISCUSSION

Increasing evidence indicates that many fundamental steps and components of the cellular signaling machinery are conserved during the evolution of eukaryotic organisms. The detailed mechanisms, however, may become adapted to function in divergent processes in animals, yeast, and plants. Recent studies have suggested that this could also be true for the Ca^{2+}-dependent phosphatase calcineurin, an important regulator of various cellular processes in animals and yeast.

In this study, we characterized AtCBL, a potential regulatory subunit of a calcineurin-like activity from *Arabidopsis.* Different isoforms of this protein are encoded by a small gene family consisting of at least six genes. We identified and characterized three members of this family. The cDNAs encode proteins with significant similarity to CNB from diverse organisms. Although the amino acid sequences of the Ca^{2+}-binding EF-hand motifs are

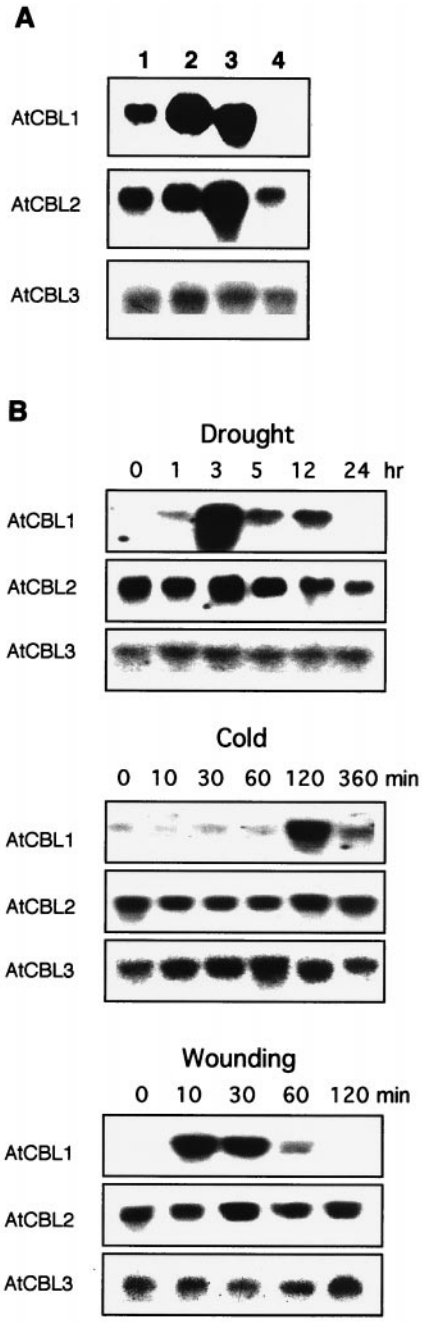

FIG. 6. Expression pattern of three members of *AtCBL* gene family. (*A*) mRNA levels in leaves (lane 1), stems (lane 2), roots (lane 3), and flowers (lane 4). (*B*) mRNA accumulation under the stress conditions drought, cold, and wounding. A 10-μg sample of total RNA was loaded into each lane for RNA gel analysis using *AtCBL* cDNAs as hybridization probes.

highly conserved, the sequence conservation also extends to the entire protein. Most strikingly, the region required for interaction with the CNA subunit is most highly conserved. This domain is located upstream of the fourth EF-hand domain and forms a helical contact surface for CNA interaction in animal homologues (24, 34, 35). The observed similarity to CaM is significantly lower than the observed degree of conservation with CNB. Another Ca^{2+}-binding protein, neuronal calcium sensor, shares significant sequence homology with AtCBL, but the homology is limited to the EF-hand regions (Fig. 1*B*). These sequence features suggest that AtCBL is structurally most similar to CNB.

AtCBL contains putative EF-hand motifs for Ca^{2+} binding, an intrinsic feature to CNB function as a regulatory subunit of the

protein phosphatase. Both gel-shift experiments and $^{45}Ca^{2+}$-overlay assays revealed that recombinant AtCBL1 is capable of binding calcium. A stringent criterion to establish whether a protein can serve as a regulatory subunit of calcineurin is the demonstration of its ability to interact specifically with the catalytic CNA subunit and form a functional complex. We have investigated the interaction between AtCBL and rat CNA in the yeast two-hybrid system. Our data indicate that AtCBL is capable of interacting with rat CNA *in vivo*, although the affinity is reduced compared with the binding between the two calcineurin subunits from rat. This is not unexpected and may reflect a divergence resulting from the coevolution of both subunits within one organism. As we demonstrated by using a C-terminal truncation of rat CNA, the observed interaction is mediated most likely by the CNA domain required for CNB interaction (24, 35). This result excludes the possibility of an interaction between AtCBL and rat CNA by means of the CaM-binding domain of the catalytic subunit, and it is consistent with the higher structural similarity between AtCBL and CNB as compared with CaM. Indeed, there has not been any known example that shows interaction between a Ca^{2+}-binding protein (other than CNB) and the CNB-binding domain of CNA. The yeast strain we used in the two-hybrid experiments lacks an endogenous active calcineurin because of a disruption of the yeast CNB gene. This mutation renders the strain hypersensitive to LiCl, a well established phenotype of calcineurin mutants in yeast (13–15). We found that the coexpression of *AtCBL1* with full-length or truncated rat CNA restored the Li resistance of this yeast strain (Fig. 5). Taken together, the results support the idea that AtCBL1 may serve as a regulatory subunit of a calcineurin-like activity in plants.

The existence of multiple isoforms of AtCBL in *Arabidopsis* adds to the complexity of AtCBL function in plants. All identified isoforms of AtCBL interact with rat CNA in the yeast two-hybrid system in a similar manner (J.K., unpublished result), which could indicate functional redundancy of AtCBL isoforms. Alternatively, differences in protein structure and gene expression pattern of these isoforms may represent mechanisms for differential regulation of Ca^{2+} signaling in plant cells. For example, AtCBL1, but not AtCBL2 and AtCBL3, contains a conserved N-terminal site for myristoylation that may regulate the localization of the protein. In addition, only the *AtCBL1* gene is induced in response to specific stress factors, such as drought, wounding, and cold, implicating this isoform in stress response. The fact that only a subset of stress factors induce *AtCBL1* expression suggests that it has a specific function in the cellular response to these stimuli. This interpretation is further supported by the rapid and transient induction pattern of *AtCBL1* mRNA levels during stress responses. Also consistent with a function of AtCBL1 in stress response, studies have revealed the occurrence of cellular calcium transients in response to drought and cold (36, 37).

After this work was submitted for publication, Liu and Zhu (38) reported that a salt-tolerance gene in *Arabidopsis* (*SOS3*) encodes a putative Ca^{2+}-binding protein with a high amino acid similarity to the AtCBL proteins. This finding is consistent with calcineurin function in salt tolerance in yeast (13–15) and most likely in plants as well (19). Together, our study on AtCBL proteins, especially stress-responsive expression of AtCBL1, and study on the *SOS3* gene product (38) emphasize a role for calcineurin-like proteins in stress signal transduction. Interestingly, the AtCBL proteins clearly play different roles as compared with SOS3 because they do not complement the defect caused by mutations in SOS3. In addition, unlike *AtCBL1*, *SOS3* gene expression is not regulated by stress conditions (S.L., unpublished results). These studies suggest that AtCBLs and SOS3 are highly related but functionally distinct members of a family of Ca^{2+}-binding proteins in higher plants. Although studies in this report provide strong evidence that this family of proteins potentially serve as a regulatory subunit of calcineurin-like protein phosphatase, confirmation of the functional identity of AtCBLs and SOS3 requires identification of their endogenous protein targets in higher plants. Irrespective of what their functional targets may be, characterization of AtCBL proteins provides an important stepping stone for further understanding the regulation of Ca^{2+} signal transduction in higher plants.

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