Identification of a Calmodulin-binding NAC Protein as a Transcriptional Repressor in Arabidopsis

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Ho Soo Kim1,2,3, Byung Ouk Park1,2, Jae Hyuk Yoo9, Mi Soon Jung12, Sang Min Lee13, Hay Ju Han1, Kyung Eun Kim4, Sun Ho Kim1, Chae Oh Lim14, Dae-Jin Yun15, Sang Yeol Lee15, and Woo Sik Chung14,4

From the 4Division of Applied Life Science (BK21 Program), Plant Molecular Biology and Biotechnology Research Center, and 5Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Korea

Calmodulin (CaM), a ubiquitous calcium-binding protein, regulates diverse cellular functions by modulating the activity of a variety of proteins. However, little is known about how CaM directly regulates transcription. Screening of an Arabidopsis cDNA expression library using horseradish peroxidase-conjugated calmodulin as a probe identified a calmodulin-binding NAC protein (CBNAC). Using gel overlay assays, a Ca2+-dependent CaM-binding domain was identified in the C terminus of this protein. Specific binding of CaM to CaM-binding domain was corroborated by site-directed mutagenesis and a split-ubiquitin assay. Using a PCR-mediated random binding site selection method, we identified a DNA-binding sequence (CBNACBS) for CBNAC, which consisted of a GCTT core sequence flanked on both sides by other frequently repeating sequences (TTGCTTANNNNNAAG). CBNAC was able to bind to CBNACBS, which resulted in the repression of transcription in Arabidopsis protoplasts. Interestingly, the transcriptional repression mediated by CBNAC was enhanced by CaM. These results suggest that CBNAC may be a CaM-regulated transcriptional repressor in Arabidopsis.

In plants one of the earliest cellular responses to external stimuli is a transient increase in cytosolic Ca2+ concentration (1–4) and the subsequent propagation of this signal through the binding of Ca2+ to various Ca2+-sensing proteins, such as calmodulin (CaM),5 calcineurin B-like protein, and calcium-dependent protein kinase (5–8). CaM is known to couple Cal2+ signals to changes in the activity of downstream target proteins via direct interaction. Therefore, understanding of CaM-binding proteins is crucial to determine how Ca2+ signals are transduced to downstream events to trigger cellular responses. The repertoire of CaM targets in plants includes many structurally and functionally diverse proteins involved in various biological processes such as morphogenesis, cell division, cell elongation, ion transport, cytoskeletal organization, and stress tolerance (8–11). CaM also modulates the activity of various transcription factors. In animal cells, some transcription factors are modulated indirectly through phosphorylation by Ca2+/CaM-dependent protein kinase (12). In plants, certain transcription factors of the basic helix-loop-helix family were shown to bind CaM, thus inhibiting their DNA-binding properties by masking the DNA-binding domain (13–15). In addition, it was reported that soybean CaM isoforms differentially bind to AtMYB2 and regulate its DNA-binding activity (16). Therefore, interaction of CaM with transcription factors may be a mechanism that transduces Ca2+ signals to the gene expression machinery to induce specific cellular responses.

There are ~1700 transcription factor genes in the Arabidopsis genome, which have been grouped into >30 gene families based on their DNA-binding domains (17). A plant-specific NAC family, which constitutes one of the largest Arabidopsis transcription factor groups, has been identified in various plant species. More than a hundred NAC proteins are believed to exist in the Arabidopsis genome, but only a few have been characterized (18). NAC proteins share a common structure consisting of a conserved N-terminal NAC domain and a highly variable C-terminal domain. The NAC domain designation is derived from a conserved domain originally associated with the NO APICAL MERISTEM (NAM) gene in Petunia (19) and the ATAFA1, ATAFA2, and CUP-SHAPED COTYLEDON (CUC) genes of Arabidopsis (20). NAC family genes are involved in embryo and shoot meristem development (19), auxin signaling (21), biotic and abiotic stress response (22–25), and secondary cell wall thickenings (26).

Elucidation of the molecular function of NAC proteins began with a report in which two NAC proteins could activate a cauliflower mosaic virus (CaMV) 35S promoter construct in yeast (19). Several NAC proteins (NAC1, AtNAM, and ANAC019) have been shown to bind the CaM-35S promoter at a site overlapping the ocs/as-1 bZIP binding site (21, 27). Furthermore, three ANAC proteins were shown to bind the NAC common recognition sequence of the ERDI promoter (25). The conserved N-terminal NAC domain and the variable C-termin-
nal domain were responsible for DNA binding and transcription activation or repression, respectively. However, the common DNA-binding sequences and the regulation mechanisms of the NAC family are still poorly understood.

Here we isolated a CaM-binding NAC-domain gene, CBNAC, from an Arabidopsis cDNA expression library and identified the specific nucleotide sequences necessary for CBNAC binding of DNA. Furthermore, we showed that CBNAC functioned as a transcriptional repressor and its activity was enhanced by CaM.

**EXPERIMENTAL PROCEDURES**

**Screening of Arabidopsis cDNA Expression Library**—Screening of the Arabidopsis cDNA expression library using HRP-conjugated AtCaM2 (AtCaM2::HRP) was carried out as described in previous reports (28, 29).

**Construction of CBNAC cDNA Deletion Mutants of Site-directed Mutagenesis**—For mapping of the CaM-binding domain, several CBNAC serial fragment constructs were generated in a pGEX-5X series vector. The full-length CBNAC cDNA clone was amplified by PCR with a forward (5′)-primer containing an EcoRI site (5′-GAATTCGATGGGTATCCAAGAAACTGAC-3′) and a reverse (3′) primer containing a XhoI site (5′-CTCGAGTGAACTCACCAGTGTCCTCCA-3′). The PCR product was cloned in pGEM-T Easy Vector (Promega, Madison, WI) and sequenced to verify the correct DNA sequence. The construct was subcloned into pGEX-5X expression vector and digested with EcoRI and XhoI.

This full-length glutathione S-transferase (GST) fusion construct was designated D0 (encompassing CBNAC amino acids 1–512). Serial fragment constructs (D1–D6) were additionally generated by PCR using the following forward (F) and reverse (R) primers that contained at their 5′-ends EcoRI (5′-GAATTCATGGGTGCTGTATCGATGGAG-3′) and XhoI (5′-CTCGAG) restriction sites, respectively: D1 (a DNA amino acids 1–100), F-5′-GAATTCATGGGTATCCAAGAAACTGAC-3′, and R-5′-CTCGAGGCTACGATCTTATACCGGAGGAGTGGGTTGCAACGGTAATGGTGCGTGTGATGGTT-3′; D2 (aDNA amino acids 101–200), F-5′-GAATTCATGCTCAAGAGACTTATTAAGAAGAAGCTTTA-3′, R-5′-CTCGAGAAACACGAGCATCAGGAGGAGGAGG-3′; D3 (aDNA amino acids 201–300), F-5′-GAATTCGAGGAAACATCGATATTATCCA-3′, R-5′-CTCGAGTGGAATCTCTCGAATCTGACGTA-3′; D4 (aDNA amino acids 301–400), F-5′-GAATTCTCAGGTTATCATCTTTAGGGAAAAACGGTTCTAACCACCTT-3′; D5 (aDNA amino acids 401–470), F-5′-GAATTCAGGCTTGCAGTTTCTACGCCAAGGAGGAGG-3′, R-5′-CTCGAGTGCAATCTCTTCTTTACCCATTACAGGAGAG-3′; and D6 (aDNA amino acids 471–512), F-5′-GAATTCGAGGCGAGGTACAGCTTACAGG-3′, R-5′-CTCGAGTGCAATCTCCAGCGTTGTCTTCCATCACAGG-3′. Further PCR reactions were performed to clone other NAC genes using the following primers: for AtCaM1 (AtCaM1::HRP), F-containing EcoRI (5′-GAATTCATGGGTGCTGTATCGATGGAG-3′) and XhoI (5′-CTCGAG), R-containing XhoI (5′-CTCGAGGACTCACCAGTGTCCTCCA-3′), and for AtCaM2 (AtCaM2::HRP), F-containing EcoRI (5′-GAATTCATGGGTGCTGTATCGATGGAG-3′) and XhoI (5′-CTCGAG), R-containing XhoI (5′-CTCGAGGACTCACCAGTGTCCTCCA-3′); and for CUC2 (AtCu53950), F-containing EcoRI (5′-GAATTCATGGGTGCTGTATCGATGGAG-3′) and XhoI (5′-CTCGAG), R-containing XhoI (5′-CTCGAGGACTCACCAGTGTCCTCCA-3′).

**Amplified products were cloned into pGEM-T and subcloned into a pGEX-5X expression vector using the appropriate restriction enzymes sites.**

To identify critical residues in the interactions between CaM and CBNAC, several point mutations were introduced into GST::CBNAC and GST::D6 (CaMBD) clones. Substitution of single amino acids was performed using a QuikChange™ site-directed mutagenesis kit (Stratagene). The following primers were employed: for W487R, F-5′-AGAAAGAGCGAGAGGACGGAGAAGGCTGTGCTGTT3′, R-5′-CACTTACCGTTGCAACACCATCTCTGCCATGCC-3′; for A491R, F-5′-GGAGGGAGCATGAGGAAGTGTCTGATGATGTCGTTAGAAGGCTAATG-3′, R-5′-AACCATTACGACGACAGCTTTCGCTTACACCCTT-3′; and for A496R, F-5′-AACCTTGCACTGACTTACCTACCAACCTCCATCACACCGACACAATTAC-3′.

**Expression of Recombinant Proteins in E. coli and CaM Binding Assay**—All clones were individually introduced into E. coli BL21(DE3)pLYS5 and expressed. Expression of GST fusion proteins was induced by application of 1 mM isopropyl β-D-thiogalactopyranoside for 5 h at 25 °C. Cells were harvested, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 μg/ml lysosome) and incubated on ice for 20 min. The mixture was sonicated for 1 min using a 50% pulse and then centrifuged at 6000 × g for 10 min to remove cell debris. Recombinant proteins were purified on a glutathione-Sepharose 4B column (Amersham Biosciences). From each clone, 1 μg of purified protein was separated on 10% SDS-polyacrylamide gel and transferred onto Immobilon-P membrane (polyvinylidene difluoride) after which expressed GST fusion proteins were detected using a polyclonal GST-specific antibody. To examine the CaM-binding ability of recombinant proteins, a duplicate blot was probed with AtCaM2::HRP conjugate in the presence of 1 mM CaCl₂ or 5 mM EGTA. The CaM:HRP overlay assay was carried out as described previously (29). Bound CaM was visualized using an ECL detection system (Amersham Biosciences).

**Yeast Split-ubiquitin Assay**—The yeast split-ubiquitin assay was performed as described previously (30). Saccharomyces cerevisiae strain JD53 was used in all of the experiments. AtCaM2 and CBNAC (WT and mutant construct) cDNA were cloned into pMet-Ste14-Cub::R-Ura3, replacing yeast Ste14. AtCaM2 and CBNAC (WT and mutant construct) cDNA were cloned into modified versions of the pCup-Nub-Sec62 vector, replacing yeast Sec62 (31). Interactions between each pair of proteins were tested on selective medium containing 1 mg/ml 5-FOA and selective medium lacking uracil. Plates were incubated at 30 °C for 3–5 days, unless specified otherwise.

**Heparin Affinity Chromatography**—A Heparin Sepharose 6 Fast Flow (Amersham Biosciences) column was pre-equilibrated with the following buffer: 25 mM HEPES-KOH (pH 7.5), 150 mM NaCl, and 0.5 mM CaCl₂. After the first wash step (0.1 M NaCl), the columns were eluted with 0.5 M NaCl. Finally, protein was detected with either a polyclonal antibody against GST or an anti-GST monoclonal antibody.
40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Soluble fraction proteins obtained from transformed *E. coli* were dialyzed against this buffer at 4 °C using VSWP-25 filters (Millipore Corp.) and loaded onto the column. After washing with 10 column volumes of buffer, proteins were eluted with the same buffer containing 100 mM to 1 M KCl, as indicated.

**Subcellular Localization Assay**—To determine the subcellular localization of CBNAC, the C terminus of CBNAC was fused with the N terminus of smGFP under control of the CaMV 35S promoter. Full-length CBNAC cDNA was obtained by PCR using a 5′ primer containing a BamHI site and a 3′ primer containing an Smal site. The PCR product was inserted into the pUC::smGFP expression vector (32). Plasmids were introduced by polyethylene glycol-mediated transformation (33) into Arabidopsis protoplasts prepared from leaf tissues. Expression of the fusion constructs was monitored at 24 h after transformation by fluorescence microscopy using a Zeiss Axioplan fluorescence microscope (Jena, Germany), and the images were captured with a cooled charge-coupled device camera. Filter sets XF116 (exciter, 474AF20; dichroic, 500DRLP; and emitter, 605DF50) (Omega, Inc., Brattleboro, VT) were used for observing green fluorescent protein and red fluorescent protein, respectively.

**Electrophoretic Mobility Shift Assay**—DNA probes were generated by annealing oligonucleotides spanning the regions of interest and filling in the 5′ overhangs with a Klenow fragment polymerase (Takara, Tokyo, Japan), [32P]dATP, and unlabeled dNTPs (final concentration, 0.25 mM each). The DNA binding reaction was allowed to proceed at 25 °C for 20 min in binding buffer (20 mM Hepes/KOH (pH 7.9), 0.5 mM dithiothreitol, 0.1 mM EDTA), 50 mM KCl, 15% glycerol, 1 μg of poly(dI-dC) and 0.5 μg of bacterially produced fusion protein purified with glutathione-Sepharose. The mixture was then incubated at 25 °C for 30 min after adding 40,000 cpm of [32P]-labeled DNA probes (Table 1). The reaction mixture was subjected to electrophoresis on 8% polyacrylamide gel in 0.5× TBE buffer at 80 V for 3 h. The gel was dried, mounted for autoradiography with intensifying screens, and exposed at −70 °C.

**PCR-mediated Random Binding Site Selection (RBSS)**—The *in vitro* binding site selection procedure was derived from a method by Pollock and Treisman (34) with modifications described below. Two different oligonucleotides, R1 and R2, were synthesized and used as primers in two independent binding site selection experiments. The R1 oligonucleotide (5′-CGCTACGTGGAGAACAGAAGCTTTGTAANNNNNNNNNNNNNNNATAGGATCCCTACCTAGACAGACAC-3′), was a randomized sequence of 15 nucleotides flanked at its 5′-end by 26 nucleotides containing a HindIII site and at its 3′-end by 25 nucleotides containing a BamHI site. PCR amplification was performed with primers TNF16 F-(5′-CGCTACGTGGAGAACAGAAGCTTTGTAANNNNNNNNNNNNNNNATAGGATCCCTACCTAGACAGACAC-3′) and TNR20 R-(5′-GTCTGTCTAGGTAGGAGGAT-3′). The R2 oligonucleotide (5′-CGCTACGTGGAGAACAGAAGCTTTGTAANNNNNNNNNNNNNNNATAGGATCCCTACCTAGACAGACAC-3′), was composed of 21 and 20 random nucleotides flanking the 5′- and 3′-ends of the GCTT sequence, respectively. PCR amplification was performed with primers TNF16 F-(5′-CGCTACGTGGAGAACAGAAGCTTTGTAANNNNNNNNNNNNNNNATAGGATCCCTACCTAGACAGACAC-3′) and TNR16 R-(5′-GTCTGTCTAGGTAGGAGGAT-3′). To produce double-stranded, random binding DNA sequences 400 pmol of R1 and 800 pmol of TNR20 (or R2 and TNR16) were annealed, and second strand synthesis was catalyzed by Klenow fragment polymerase in the presence of dNTPs (final concentration, 0.25 mM each). The DNA binding assay was performed as described previously (32). Out of the five selection cycles (binding/mobility shift/elution/PCR amplification) conducted, the first three selections were performed using binding conditions of 50 mM KCl, whereas the latter two selections were carried out at 100 mM and 150 mM KCl, respectively. The final pool of oligonucleotides was digested with BamHI and HindIII, ligated into pBluescript II SK(+/(Stratagene), and transformed into XL-1 Blue MRF. Sequences of the inserted DNA were determined by sequencing.

**Transient Expression Assay**—A pUC19-derived plasmid containing the β-glucuronidase (GUS) reporter gene under the control of a 35S minimal promoter (35) was used as a reporter plasmid and a CBNAC binding site (CBNACBS) was inserted in front of the CaMV 35S minimal promoter. For effector plasmids, full-length CBNAC or AtCaM2 cDNA was inserted into a plant expression vector (HBT95) containing the 35S C4PPDK promoter and nos terminator (36). Transient expression of these constructs was performed as described previously (37). In each transfection, 2 × 10⁶ protoplasts were transfected with 30 μg of plasmid DNA carrying reporter construct alone, plasmid DNA carrying an effector construct, or control vector DNA. The transfected protoplasts were incubated for 16 h in the dark. A construct carrying the 35S promoter fused to the luciferase gene was used as an internal control in each CBNACBS-35S-GUS reporter construct transfection. In each sample, the GUS activity of the cell lysate was divided by the luciferase activity, thereby normalizing the data to control for variations in transfection efficiency.

**RESULTS**

**Isolation of a CaM-binding NAC Protein in Arabidopsis**—To identify the molecular components of CaM-mediated signaling pathways, we screened an Arabidopsis cDNA expression library using HRP-conjugated CaM as a probe (28, 29). One of clones isolated encoded a NAC transcription factor, designated CBNAC (calmodulin-binding NAC protein). Sequence alignment of CBNAC with five well known Arabidopsis NAC proteins showed a high degree of sequence conservation in the N-terminal NAC domain. The N-terminal NAC domains of AtNAM, ANAC, NAC1, ATAF1, and CUC2 are 58%, 61%, 59%, 64%, and 63% identical to that of CBNAC. However, the C-terminal region of CBNAC showed no significant similarity with other NAC proteins (Fig. 1A).

To ascertain whether other members of the NAC family bind CaM, AtNAM (At1g52880), ANAC (At1g52890), NAC1 (At1g56010), ATAF1 (At1g01720), and CUC2 (At5g3950) were cloned into a GST fusion vector. Recombinant NAC proteins were produced in *E. coli* and subjected to Western blotting and the CaM::HRP overlay assay. None of the NAC protein
members except CBNAC bound to CaM (Fig. 1B). This finding indicates that CaM binds specifically to CBNAC protein.

**Mapping of a CaMBD in CBNAC—**Comparison of the CaMBDs of many CaM-binding proteins has shown that there are multiple sequence motifs for CaM complex formation (38). Based on the structural characteristics of known CaMBDs, a putative CaMBD motif was predicted in the C terminus of CBNAC, between Lys485 and Gly500 (CaMBD) (Fig. 2A). Within this 16-amino acid stretch, hydrophobic amino acids are present at positions 1 (Trp487), 5 (Ala491), and 10 (Ala496), and several basic residues (two lysines and one arginine) are interspersed between these hydrophobic residues (Fig. 3A). To confirm the presence of the putative CaMBD, we generated GST-fused constructs containing the full-length CBNAC cDNA (designated D0) and six serial fragment constructs (D1, D2, D3, D4, D5, and D6) (Fig. 2A). Recombinant fusion proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes for Western blotting and CaM overlay assays. Expression of the GST fusion proteins was verified by probing the blot with an anti-GST antibody. Two recombinant proteins that contained the putative CaMBD (designated D0 and D6) interacted with HRP-conjugated CaM (CaM::HRP), whereas GST only (GST) and GST fusion proteins lacking the predicted CaM binding region (D1, D2, D3, D4, and...
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Identification of a CaMBD in the C terminus of CBNAC.

A, schematic representation of GST-fused CBNAC, full-length, and serial fragments. The designations D0 and D1–D6 represent GST-fused protein constructs containing full-length clone or indicated clone fragments, respectively. A putative CaMBD in the C terminus and a NAC domain in the N terminus are represented with a black and gray box, respectively. Amino acid positions of each serial fragment are indicated by numbers. D0–D6 represent GST fusion constructs containing full-length CBNAC or the specified fragment. CaM-binding ability is specified as + (CaM binding) or − (no CaM binding). B, CaM binding overlay analysis. GST and GST fusion proteins of full-length (D0) and serial fragment mutants (D1–D6) of CBNAC were expressed in E. coli. Expressed recombinant proteins were analyzed by Coomassie Brilliant Blue staining (Staining) and Western blotting with an anti-GST antibody (Western). CaM overlay assay was performed using AcAM2::HRP in the presence of CaCl₂ and absence (EGTA) of Ca²⁺.

Identification of Critical Residues for the Interaction between CaM and CBNAC.

To identify the critical residues of the CaMBD in CBNAC, we performed site-directed mutagenesis to introduce single amino acid substitutions into the CBNAC full-length (D0) and serial fragment (D6) GST-fused CaMBD. Hydrophobic residues important for CaM binding (Trp⁴⁸⁷, Ala⁴⁹¹, and Ala⁴⁹⁶) residues that formed the 1-5-10 motif in CBNAC) were separately replaced with Arg and denoted W⁴⁸⁷R, A⁴⁹¹R, and A⁴⁹⁶R, respectively (Fig. 3A). Mutant proteins were produced in E. coli and analyzed for CaM binding with a CaM overlay assay. As shown in Fig. 3B, CaM bound to A⁴⁹¹R and A⁴⁹⁶R mutant proteins, but not to W⁴⁸⁷R of CBNAC. These results indicate that the hydrophobic residue Trp⁴⁸⁷ is the critical residue for the interaction between CaM and CBNAC.

In Vivo Interactions between CBNAC and CaM in Yeast—To examine the direct in vivo interactions between CBNAC and CaM, we used the yeast split-ubiquitin assay system, based on the reassembly of N- and C-terminal halves (Nub and Cub) of ubiquitin (30, 31). CBNAC and CaM were fused to the C terminus of Nub and the N terminus of Cub, respectively. The Cub of ubiquitin was linked to an N-terminally modified Ura3p reporter containing Arg at position 1 (RUra3p). When CaM interacts with the CBNAC protein, Nub and Cub reassemble into native-like ubiquitin. This is followed by cleavage of RUra3p by ubiquitin-specific proteases and the rapid degradation of the released RUra3p through the N-end rule pathway of protein degradation (43). Consequently, cells co-expressing CaM-Cub·RUra3p and Nub·CBNAC or CBNAC-Cub·RUra3p and Nub·CaM are unable to grow on plates lacking uracil, but grow on plates containing 5-fluorouracil (5-FOA), which is converted into toxic 5-fluorouracil by RUra3p. In the opposite cases, yeast cells are uracil prototrophs and 5-FOA-sensitive.

As shown in Fig. 4, cells co-expressing CaM-Cub·RUra3p and Nub·CBNAC or CBNAC-Cub·RUra3p and Nub·CaM were unable
to grow on plates lacking uracil but grew on plates containing 5-FOA, indicating that CBNAC effectively forms stable complexes with CaM in vivo. In negative controls, no interactions were observed between CaM-Cub-RUra3p and Nub or Cub-RUra3p and Nub-CaM. To test the specificity and CaMBD sequence dependence of the interactions, experiments were repeated with the CBNAC CaM-binding negative mutant (W487R) in place of the wild-type CBNAC. CaM/CBNAC mutant (W487R)-transformed cells displayed markedly increased 5-FOA sensitivity and grew well on plates lacking uracil, indicating a lack of CaM binding to this mutant. These results are consistent with the data from the previous CaM binding overlay assay.

CBNAC DNA Binding Capacity—Recent studies suggest that NAC family members, such as NAC1, AtNAM, and ANAC019, may function as transcriptional activators. NAC domains from the Arabidopsis proteins NAC1 and AtNAM were shown to specifically bind a fragment of the CaMV 35S promoter (21, 27), and three ANAC proteins were shown to bind a fragment of the ERD1 promoter (25). To test the ability of CBNAC to bind DNA, we verified that CBNAC binds heparin, a glycosaminoglycan known to interact with DNA-binding proteins (44). A recombinant form of GST-fused CBNAC was expressed in E. coli, and soluble proteins were extracted and loaded onto a heparin-Sepharose column. The column was washed with an excess of loading buffer, and proteins were eluted by stepwise increases in buffer KCl concentration. Western blot analysis of the collected fractions, using a polyclonal antibody, showed that most of the recombinant protein fraction was retained on the column at concentrations up to 300 mM KCl and was completely eluted at 1000 mM KCl (supplemental Fig. S1). No proteins were detected by Western blot analysis of the extracts prepared from E. coli transformed with vector alone (data not shown). These results indicate that recombinant CBNAC protein expressed in E. coli binds tightly to heparin-Sepharose, indicating that CBNAC has potential DNA-binding activity.

Identification of CBNACBS—PCR-mediated RBSS was used to identify a putative CBNACBS. This was accomplished in two phases, each using a different oligonucleotide: the first phase yielded a core binding sequence of four conserved nucleotides, and the second phase elucidated key nucleotide positions flanking the core. R1 oligonucleotides contained various combinations of 15 random nucleotides flanked by PCR primer-binding sites and restriction enzyme digestion sites for cloning (34, 45) (Fig. 5A). DNA binding assays were performed using double-stranded radiolabeled, R1 oligonucleotides incubated with purified recombinant CBNAC protein in the presence of excess nonspecific competitors, poly[dI-dC]. After binding and performing the mobility shift assay, CBNAC-bound R1 oligonucleotides were recovered and PCR-amplified. After five rounds of DNA binding and recovery, the enriched R1 oligonucleotide species were cloned. 50 different clones were sequenced, and the resulting data were compiled (Fig. 5B).
44 of 50 clones contained a selected sequence of 4 nucleotides, GCTT, at various positions on either strand. However, the remaining 6 clones did not contain the GCTT sequence (data not shown). To determine whether the flanking sequences the GCTT motif were important for CBNAC binding, 44 clones were aligned with respect to the GCTT sequence (Fig. 5). Two nucleotides occurred at high frequency at the 5’ end of the GCTT sequence, a T base at position 1 and a T base at position 2. This result suggested that sequences immediately flanking GCTT might play a role in CBNAC binding.

To elucidate the role of the GCTT-flanking sequences, PCR-mediated random binding site selection was repeated using R2 oligonucleotides (Fig. 6A). R2 nucleotides contained the core GCTT sequence flanked by 21 5’ random nucleotides and 20 3’ random nucleotides. After performing five rounds of DNA binding, the mobility shift assay, and oligonucleotide recovery, selected R2 oligonucleotides were PCR-amplified and cloned. 30 clones were randomly chosen and sequenced, aligned, and examined for selected nucleotide positions (Fig. 6, B and C). The results indicated the following: at the 5’-end of GCTT, T nucleotide was preferentially selected in positions 1 and 2; at the 3’-end, nucleotides A, A, A, and G were preferentially selected in positions 1, 8, 9, and 10, respectively. These results indicated that the preferred sequence for CBNAC DNA binding (CBNACBS) is TTGCTTANNNNNNAAG.

Analysis of Selected Binding Sites—To analyze in more detail the CBNACBS with respect to the different flanking sequences of GCTT, we divided the RBSS sequences into four groups (Table 1): in Group 1, most sequences contained consensus flanking sequences (CBNACBS). Sequence comparisons are made relative to the Group 1 consensus sequence (CBNACBS). N letters in Groups 2–4 indicate positions flanking the GCTT core motif that do not match CBNACBS.

**Table 1**

| Group | Representative Sequence | Binding affinity |
|-------|-------------------------|-----------------|
| Group 1 | Consensus: TT GCTT ANNNNNNAAG | ++ + |
| Group 2 | A1 | TT GCTT AINNNNNNAAG | ++ + |
| Group 3 | A21 | TT GCTT AINNNNNGCT + |
| Group 4 | A22 | TT GCTT AINNNNNGCA + |
| Group 5 | A23 | TT GCTT AINNNNNNACG + |
| Group 6 | A24 | TT GCTT AINNNNNNAGC + |

* Sequences of selected clones are divided into four groups according to the consensus sequence for DNA binding derived from analysis of RBSS oligonucleotides. Sequence comparisons are made relative to the Group 1 consensus sequence (CBNACBS). N letters in Groups 2–4 indicate positions flanking the GCTT core motif that do not match CBNACBS.

**Analysis of Selected Binding Sites**—To analyze in more detail the CBNACBS with respect to the different flanking sequences of GCTT, we divided the RBSS sequences into four groups (Table 1):

In Group 1, most sequences contained consensus flanking sequences...
sequences on both sides of the GCTT motif; in Groups 2 and 3, sequences contained consensus-flanking sequences on only the 5' and 3' side, respectively; in Group 4, sequences contained no consensus-flanking sequences at either end. Individual oligonucleotides from each group were labeled and used in the electrophoretic mobility shift assay with recombinant CBNAC protein (Fig. 7A). The DNA binding affinity of CBNAC was calculated from the autoradiogram using scanning densitometry as shown in Fig. 7B. All three oligonucleotides in Groups 1 and 3 bound to CBNAC with high affinity. In contrast, all of the oligonucleotides in Groups 2 and 4 bound to CBNAC with moderate affinity, except for two, A23 and A43, which bound very weakly.

Subcellular Localization of CBNAC—To provide further evidence that CBNAC is a transcription factor we examined its presumed localization in the nucleus. A plasmid expressing GFP-tagged-CBNAC protein under the control of 35S promoter (Fig. 8A, CBNAC::smGFP) was constructed, and the subcellular localization of the CBNAC-GFP fusion protein was investigated in Arabidopsis protoplasts. Red fluorescent protein fused to the nuclear localization signal (NLS) peptide from the SV40 large T antigen was used as a positive control (NLS::RFP, Fig. 8A). As shown in Fig. 8B, CBNAC::smGFP protein was predominantly localized in the nucleus. In contrast, smGFP alone was equally distributed in the nucleus and cytosol. This result shows that CBNAC is a nuclear protein, which is consistent with its putative role as a transcription factor.

CBNAC Functions as a Transcriptional Repressor—To test whether CBNACBS derived from in vitro PCR-mediated RBSS functions in vivo, we generated a β-glucuronidase (GUS) reporter plasmid by fusing CBNACBS (Table 1, A11) to a region upstream from the 35S minimal promoter in pDel 151-8 (35), and by generating effector plasmids consisting of the 35S promoter fused to CBNAC (wild type and mutant) or CaM (Fig. 9A). The effects of CBNAC and/or CaM on the expression of the reporter gene were tested in Arabidopsis protoplasts. As shown in Fig. 9B, the expression of CBNAC alone in the protoplast was able to significantly suppress the expression of the GUS reporter gene when compared with the vector control. Furthermore, >2-fold additional repression of GUS reporter expression was observed in cells when CaM and CBNAC were co-expressed. However, GUS expression was not significantly changed by the co-expression of CaM and the CaM-binding-negative CBNAC mutant (W487R) that is incapable of binding.
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**A**

**Effectors**

- CaMV35S
- CBNAC or CaM
- Nos

**Reporter**

- CBNACBS
- TATA box
- GUS
- Nos

**DISCUSSION**

CBNAC Is a CaM-binding Transcription Repressor Protein

CaM plays an important role in modulating metabolism, cytoskeleton, ionic balance, and protein modification through many kinds of CaM-binding proteins in different signaling pathways (8–11). CaM also regulates the transcriptional activities of transcription factors. CaM actually binds transcription factors and activates or inhibits their DNA-binding properties (13–16, 32). In this report, we isolated a CaM-binding NAC transcription factor (CBNAC) that can function as a transcriptional repressor. We also identified the DNA-binding sequence of CBNAC.

NAC family genes are plant-specific and play roles in a diverse set of developmental and morphogenetic processes, as well as in stress signaling pathways (18). NAC proteins are characterized by the presence of highly conserved NAC domains in their N-terminal regions. However, their C-terminal regions are highly variable, which is thought to impart their distinct and varied individual functions. In this report, we showed that CaM bound to the C-terminal region of CBNAC (Fig. 2), suggesting the regulation of CBNAC activity by CaM binding. Most CaMBDs isolated to date comprise stretches of 16–35 residues that display segregation of basic and polar residues on one side and hydrophobic amino acids on the other, in a helical wheel representation (39, 40, 42). Ca2+-dependent CaM binding motifs have been classified into two major groups based on whether they have the 1-8-14 motif or the 1-5-10 motif (38), where the numbers represent the positions of conserved hydrophobic residues. Based on the conserved structural features of CaMBD, CaM-binding motifs of CBNAC are predicted to be located in the C-terminal region between Lys386 and Gly500 in a 16-amino acid stretch showing the 1-5-10 motif. The position of CaMBD was identified by domain mapping analysis of CBNAC (Fig. 2). We further confirmed the presence of Ca2+-dependent CaMBD of CBNAC by site-directed mutagenesis (Fig. 3). In vivo interactions between CaM and CBNAC were verified by performing the split-ubiquitin assay in yeast, which is widely used for assaying transcriptional regulators (30). When we performed CaM overlay assay using several CaMBD from CaM-binding proteins such as OsMlo (46), AtMyb2 (16), and AtWRKY7 (47), the CaMBD of CBNAC has very similar affinity for CaM with AtWRKY7 CaMBD (a Kd value of 25.8 nM for PDE activation) but weaker affinity than those of MLO and AtMyb2 (data not shown).

In plants, several transcriptional factors such as homeobox1 from rice (Oshox1), a repressor of transcription (HRT) from barley, G-box binding factor 2 from soybean (SGBF-2), and AtMYB4 from Arabidopsis have been shown to be active repressors (48–51). Although several NAC proteins have been shown to function as transcription activators (21, 24, 25, 27), there have been no reports about NAC proteins acting as transcriptional repressors. In this report we showed that, in Arabidopsis protoplasts, CBNAC could repress the basal transcription level of a reporter gene by binding to its cognate CBNACBS (Fig. 9). Furthermore, CaM enhanced the transcriptional repression of CBNAC in protoplasts. This result suggests that CBNAC acts as a transcriptional repressor distinct from other NAC family members. Therefore, we speculate that CBNAC represses the expression of its unknown target genes under normal conditions and that CBNAC repressor activity is enhanced by CaM activation in response to external stimuli. However, the repressor activity of CBNAC in the context of a minimal artificial promoter may not necessarily reflect the in vivo role of CBNAC. Indeed, many transcription factors can act as both activator and repressor (52). Dual activities of transcription fac-
A Calmodulin-binding Transcriptional Repressor in Arabidopsis

microarray analysis to identify CBNAC target genes. By the use of microarray assays using CBNAC ectopic-over-regulation, we suggest defining candidate the physiological effects of CBNAC transcriptional results with other NAC proteins (25). We suggest that identification sequence of three NAC proteins in the promoter of ERD1 gene to be ANNNNNTCNNNNNNACACGCA-TGT, which contains a CACG core DNA-binding motif (underlined). Arabidopsis NAC1 protein was shown to bind a 21-bp segment (CTGACGTAAGGGATGACGCAC) within the −90 region of 35S promoter (21). Independently, it was demonstrated that the AtNAM fusion protein, another NAC protein, bound to a region in the 35S promoter between −70 and −76 (AGGGATC), which is located within the 21-bp 35S promoter segment (27). Interestingly, the GCTT core motif for CBNAC binding was not found within the 21-bp 35S promoter segment. Therefore, we speculate that CBNAC may recognize DNA-binding elements that are different from those of other NAC proteins, although we cannot completely exclude the possibility of CBNAC binding to the DNA-binding sequences of other NAC proteins. In this study, we used the RBSS method to characterize the DNA-binding specificity of CBNAC. This method is convenient and rapid for investigating DNA sequences bound by an individual protein (32, 55, 56). Different approaches used to identify core binding sequences can produce different results with other NAC proteins (25). We suggest that CBNAC binds to GCTT motifs in promoters of unknown genes and regulates their transcription. In the future, to elucidate the physiological effects of CBNAC transcriptional regulation, we suggest defining in vivo CBNAC target genes by the use of microarray assays using CBNAC ectopic-over-expressing transgenic plants. A far informative approach would be the use of chromatin immunoprecipitation microarray analysis to identify CBNAC target genes.

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