

# A NAC transcription factor and SNI1 cooperatively suppress basal pathogen resistance in *Arabidopsis thaliana*

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## ABSTRACT

Transcriptional repression of pathogen defense-related genes is essential for plant growth and development. Several proteins are known to be involved in the transcriptional regulation of plant defense responses. However, mechanisms by which expression of defense-related genes are regulated by repressor proteins are poorly characterized. Here, we describe the *in planta* function of CBNAC, a calmodulin-regulated NAC transcriptional repressor in *Arabidopsis*. A T-DNA insertional mutant (*cbnac1*) displayed enhanced resistance to a virulent strain of the bacterial pathogen *Pseudomonas syringae* DC3000 (*Pst*DC3000), whereas resistance was reduced in transgenic CBNAC overexpression lines. The observed changes in disease resistance were correlated with alterations in *pathogenesis-related protein 1* (*PR1*) gene expression. CBNAC bound directly to the *PR1* promoter. SNI1 (*suppressor of nonexpressor of PR genes1, inducible 1*) was identified as a CBNAC-binding protein. Basal resistance to *Pst*DC3000 and derepression of *PR1* expression was greater in the *cbnac1 sni1* double mutant than in either *cbnac1* or *sni1* mutants. SNI1 enhanced binding of CBNAC to its cognate *PR1* promoter element. CBNAC and SNI1 are hypothesized to work as repressor proteins in the cooperative suppression of plant basal defense.

## INTRODUCTION

Plants, unlike animals, do not possess specialized cells for protection against invading pathogens. When a pathogen challenge is detected, plant defense responses occur through the activation of cellular signal transduction pathways leading to global transcriptional reprogramming. These changes favor immune responses over normal cellular functions (1,2). Equally important is the suppression of immune responses in the absence of a pathogen threat that is necessary for proper plant growth and development. Thus, the induction of defense response to a specific pathogen occurs by a complex signaling network interconnected by crosstalk with networks that regulate response to other stressors, growth and development (3). Research on *Arabidopsis thaliana* has demonstrated that local and systemic resistance responses to biotrophic pathogens such as *Pseudomonas syringae* are mediated by the plant hormone salicylic acid (SA). Accumulation of SA leads to reduction of the oligomeric cytoplasmic form of the transcriptional co-activator NPR1/NIM1 (*nonexpressor of PR genes1*) to a monomeric form that translocates to the nucleus (4–8). Once there, NPR1 interacts with three redundant transcription factors, TGA2, TGA5 and TGA6 to activate expression of defense genes such as *pathogenesis-related protein 1* (*PR1*) (9–13).

Constitutive activation of defense is detrimental to the normal growth of plants (14–17). Therefore, negative regulation of defense responses is very important. Negative regulators of the *PR1* expression and resistance to *P. syringae* include SNI1 (*suppressor of npr1-1, inducible1*), *NIM1-interacting1* and several WRKY

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transcription factors such as WRKY7, WRKY11 and WRKY17 (18–21). SNI1 was identified in a screen for suppressors of *npr1* (18). SNI1 encodes a protein with structural similarity to Armadillo-repeat proteins that are involved in scaffolding or protein–protein interactions. The mechanism by which NPR1 and SNI1 interact to control *PR1* expression is not clear. SA-inducible *PR* gene expression and resistance are restored in the *npr1 sni1* double mutant suggesting that there is an NPR1-independent pathway of SA activation of *PR1* transcription and that NPR1 blocks SNI1 activity. The deoxyribonucleic acid (DNA) recombination protein RAD51 seems to be involved in the regulation of *PR1* expression by the NPR1-independent pathway (22). Both SNI1 and RAD51D were found to play roles in *PR* gene transcription and DNA recombination (22). Histone modifications are involved in SNI1-mediated repression (23).

Calcium signaling is another component of the defense response. Calcium signals are transduced in many ways including the binding of calcium to calmodulins (CaMs) or CaM-like proteins (24). The Ca<sup>2+</sup>/CaM complex modulates immune responses by repressing or activating transcription. Transcription of genes involved in SA biosynthesis is modulated by Ca<sup>2+</sup>/CaM (25). TGA and WRKY transcription factors are involved in controlling *PR1* expression, and some members of these families of transcription factors are known to bind Ca<sup>2+</sup>/CaM. Details of CaM regulation of defense gene expression are not well-understood.

We show here a novel connection between SNI1 and Ca<sup>2+</sup>/CaM control of *PR1* expression. We demonstrate that a previously identified CaM-binding NAC transcription repressor designated CBNAC (26) binds to *cis*-elements on the *PR1* promoter that contain a GCTT core sequence and also interacts physically with SNI1. Genetic analyses showed that CBNAC functions as a negative regulator of pathogen-induced *PR1* expression and basal resistance to a virulent strain of *P. syringae*. CBNAC and SNI1 were found to function synergistically as negative regulators of both *PR1* expression and disease resistance.

## MATERIALS AND METHODS

### Plant and bacterial materials

All *Arabidopsis* plants used in this study were of the Columbia (Col-0) ecotype. The virulent bacterial pathogen, *P. syringae* pv. *tomato* (*Pst*) DC3000 was used for disease response tests. *Escherichia coli* BL21 (DE3) pLysS was used to express and produce recombinant GST-CBNAC protein. *Arabidopsis* transformation was performed as described previously (27).

### Generation of transgenic plants

To generate transgenic plants, *CBNAC* complementary DNA (cDNA) with or without the FLAG tag was placed under the control of the *CaMV 35S* promoter. These constructs were cloned into pCAMBIA 1300 and transformed into *Agrobacterium tumefaciens* GV3101.

*Arabidopsis* wild-type plants were transformed with the 35S:Flag-CBNAC construct according to a published protocol (27), and T3 progeny lines overexpressing *CBNAC* were selected for experiments. The 35S:CBNAC construct was used to transform *cbnac1* plants and T3 progeny lines (*cbnac1/CBNAC*) expressing approximately the same level of *CBNAC* as wild-type plants in 1 mM SA-treated leaves were selected for experiments. MS medium containing 40 µg/ml hygromycin was used for selection of transformants.

### Plant growth conditions

*Arabidopsis thaliana* plants were grown in growth chambers at 22°C and under 120 µEm<sup>-2</sup>s<sup>-1</sup> light intensity and 16-h-light/8-h-dark photoperiod.

### Isolation of the *cbnac1* and *cbnac1 sni1* mutant lines

The *cbnac1* (Salk\_065051) T-DNA insertion mutant was identified from the Salk *Arabidopsis* T-DNA population (28). The T-DNA insertion was confirmed by polymerase chain reaction (PCR) using a T-DNA-specific primer (T-DNA) and a *CBNAC*-specific primer (CBNAC-S). A homozygous *cbnac1* line was identified by PCR using a pair of primers corresponding to T-DNA flanking sequences (F1 and F2). The *sni1* mutant was provided by Dr Xinnian Dong. The *cbnac1 sni1* double mutant was obtained by crossing *cbnac1* and *sni1*, selfing the F1 progeny and screening of the F2 population. The F2 population was screened for an absence of both genes by gene-specific PCR using the following primers: *SNI1*-specific primers (SNI1-S1 and SNI1-S2) and *CBNAC*-specific primers (F1 and F2). The primers used for PCR are listed in Supplementary Table S1.

### *Pseudomonas* infection

*Pseudomonas* infection was carried out as described previously (29). *Pseudomonas syringae* DC3000 (*Pst*DC3000) carrying empty vector (pVSP61) was grown at 28°C on King's agar plates supplemented with 50 µg/ml rifampicin and 50 µg/ml kanamycin. In brief, bacteria were suspended in 10 mM MgCl<sub>2</sub>, adjusted to optical density (OD)<sub>600</sub> = 0.001 and pressure infiltrated into leaves using a needleless syringe. Leaf discs from four independent plants were combined, ground in 10 mM MgCl<sub>2</sub>, serial-diluted 1:10 and plated onto King's B medium containing the appropriate antibiotics. Plates were incubated at 28°C for 2 or 4 d, after which the colonies were counted.

### Quantitative PCR and ribonucleic acid gel blot analysis

Total ribonucleic acid (RNA) was isolated using the guanidinium thiocyanate-phenol-chloroform extraction method with subsequent ultracentrifugation (29). *Arabidopsis* RNA was extracted using LiCl method, and cDNA was synthesized using the SuperScript<sup>TM</sup> II RNase-Reverse Transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed using the SsoFast EvaGreen Supermix (Bio-Rad) in a CFX96<sup>TM</sup> Real-Time PCR System (Bio-Rad). The primers used for qPCR are listed in Supplementary Table S2. Expression

of *CBNAC* was detected by RNA gel blot analysis. RNA was separated on 1.5% agarose-formaldehyde gels and transferred to nylon membranes. Membranes were incubated with an ( $\alpha$ - $^{32}$ P)dATP-labeled gene-specific probe at 65°C overnight and washed under high stringency conditions as described (29).

#### Electrophoretic mobility shift assays

For mapping of the *CBNAC*-binding promoter region of the *PRI* gene, DNA probes were generated by PCR amplification with a Klenow fragment polymerase,  $\alpha$ - $^{32}$ P-ATP (6000 Ci/mmol; Amersham) and the following primers, for E0, E1, E2, E3, E4, E5, E6, E0-1, E0-2, E0-3, E0-4, E3-1, E3-2, E4-1, E4-2, E4-3, E5-1, E5-2, E6-1 and E6-2 and by end labeling with polynucleotide kinase,  $\gamma$ - $^{32}$ P-ATP and the following primers, for E0-1-1, E0-1-2, E0-4-1, E0-4-2, E3-1-1, E3-1-2, E3-1-3, E4-1-1, E4-1-2, E5-3-1, E5-3-2, E6-1-1 and E6-1-2 (Supplementary Table S2). DNA-binding reactions were conducted at 25°C for 20 min in binding buffer [20 mM HEPES/KOH (pH 7.9), 0.5 mM DTT, 0.1 mM ethylenediaminetetraacetic acid (EDTA)], 50 mM KCl, 15% glycerol, 1  $\mu$ g poly(dI-dC) and 0.5  $\mu$ g bacterially produced fusion protein purified with glutathione-Sepharose.  $^{32}$ P-labeled DNA probes (40 000 cpm) were added and incubated with the mixture at 25°C for 30 min. The reactions were separated on an 8% polyacrylamide gel in 0.5 $\times$  tris-borate-EDTA buffer at 80 V for 3 h. The gel was dried, mounted for autoradiography with intensifying screens and exposed at -70°C.

#### Chromatin immunoprecipitation assay

Chromatin samples were prepared as described previously (30). Wild-type and *35S:Flag-CBNAC* overexpression lines were fixed with 1% formaldehyde for 10 min. The eluted DNA was analyzed by PCR using the following specific primers: E0, E3, E4, E5 and E6 (Supplementary Table S2). The amplified bands were visualized on a 2% agarose gel.

#### Yeast two-hybrid assay

*CBNAC* cDNA was digested with EcoRI and XhoI and ligated into the pAS2-1 plasmid (bait vector), which contains the *Trp1* selection marker. The *SNII* cDNA was cloned into the pGAD424 plasmid (prey vector), which harbors the *Leu2* selection marker. For mapping, the interacting domain deletions of *CBNAC* were PCR amplified using gene-specific primers and cloned in the pAS2-1 plasmid. Prey and different bait plasmids were co-transformed in the pJ69-4A (31) strain of yeast. Two-hybrid assays were performed as described in CLONTECH's Yeast Protocols. Positive interactions were verified by the  $\beta$ -galactosidase assay.

#### Luciferase complementation imaging assay

Luciferase (Luc) complementation imaging (LCI) assay was carried out as described previously (32). *CBNAC* was fused with the C-terminal fragment of firefly Luc in pCAMBIA NLuc vector (CLuc-*CBNAC*). *SNII* was

fused with the N-terminal fragment of Luc in pCAMBIA NLuc vector (*SNII*-NLuc). *STG1a*-NLuc and *CLuc*-*RAR1* constructs described previously were used as positive interaction controls (32). The constructs were each introduced into *A. tumefaciens* strain GV3101. Each bacterial strain was grown overnight in LB medium at 30°C, collected by centrifugation, then washed two times with infiltration buffer (10 mM MgCl<sub>2</sub>, 10 mM MES and 100  $\mu$ M acetosyringone) and re-suspended in the same buffer. Equal volumes of bacterial suspensions of a CLuc and an NLuc construct were mixed and co-infiltrated into fully expanded leaves of the 3-week-old *Nicotiana benthamiana* plants using a needleless syringe. After infiltration, plants were placed at 23°C for 48 h. *Pst*DC3000 (OD<sub>600</sub> = 0.001 in 10 mM MgCl<sub>2</sub>) was treated after 24 h as Agro infiltration. The leaves were sprayed with luciferin solution (100  $\mu$ M luciferin, 0.1% Triton X-100) and kept in the dark for 4 h to quench fluorescence. Luc activity (luminescence) was observed with a low-light cooled CCD imaging apparatus (Andor iXon; Andor).

## RESULTS

### *CBNAC* transcripts are induced by pathogen and SA treatment

To identify the biological function of *CBNAC*, we investigated the gene expression of *CBNAC* in response to environmental stresses. The transcript level of *CBNAC* was examined after exposure to several biotic and abiotic stresses including bacterial pathogen, SA, jasmonic acid (JA), ABA, drought and NaCl. Interestingly, *CBNAC* transcript levels were increased in leaves of wild-type *Arabidopsis* plants after exposure to the virulent bacterial pathogen *Pst*DC3000 or SA (Figure 1). *CBNAC* transcripts were undetectable in untreated leaves and remained undetectable over a 48-h period in leaves after infiltration with 10 mM MgCl<sub>2</sub>. In leaves infiltrated with *Pst*DC3000, significant accumulation of *CBNAC* transcripts was observed at 6–12 h post-inoculation, and it returned to an undetectable level by 24 h (Figure 1A). Expression of *CBNAC* was also induced by SA, the inducer of systemic acquired resistance (Figure 1B). In SA-treated leaves, *CBNAC* transcripts reached maximum levels at 6 h, persisted for 24 h and declined to basal levels 48 h after treatment. These results suggested that *CBNAC* may be involved in SA-mediated pathogen resistance signaling in plants.

### *CBNAC* is a negative regulator of the plant defense

The following genetic resources were developed for investigation of *CBNAC* function *in vivo*. A Salk line (Salk\_065051) carrying a T-DNA insertion in *CBNAC* (*cbnac1*) was identified and homozygous F2 progeny derived from this line were used for analyses. Location of the T-DNA insertion at the third exon of the *CBNAC* gene was confirmed by PCR analyses of genomic DNA (Supplementary Figure S1A). There was no detectable accumulation of *CBNAC* transcripts in untreated leaves of the wild-type and *cbnac1* mutant plants. Complete loss of

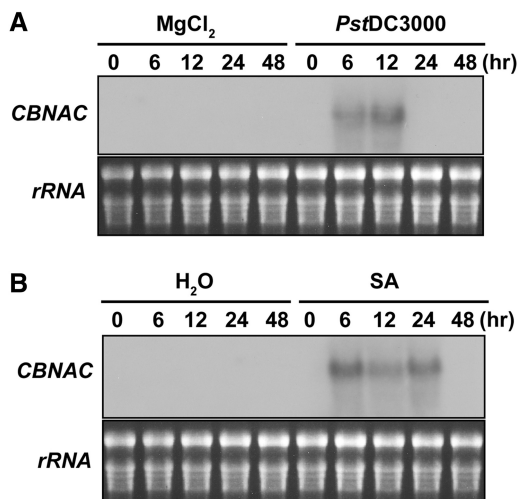


*CBNAC* expression was revealed by comparing the transcript abundance in SA-treated leaves of wild-type and *cbnac1* plants (Supplementary Figure S1B). Attempts to identify additional independent *cbnac* mutants were unsuccessful. Thus, a *35S:CBNAC* construct was used to transform the *cbnac1* mutant, and transgenic lines (*cbnac1/CBNAC*) exhibiting *CBNAC* expression levels similar to wild-type plants were chosen for complementation analysis (Supplementary Figure S2A). Transgenic lines constitutively overexpressing *CBNAC*

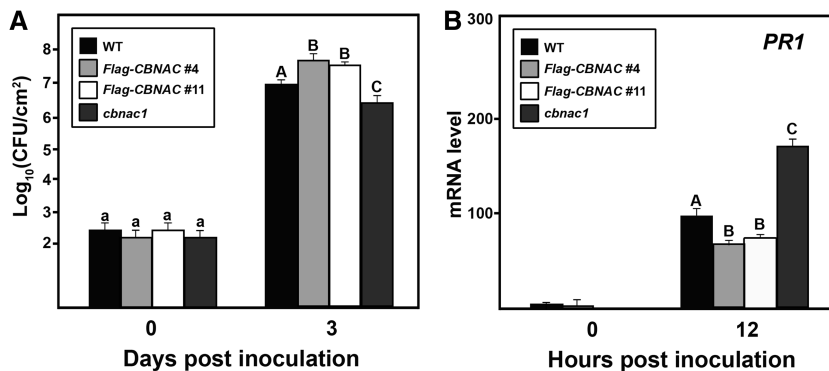
(*35S:Flag-CBNAC*) were generated in the wild-type background. Western blot analysis using anti-FLAG antibody revealed several transgenic plants contained elevated levels of *CBNAC* protein regardless of SA treatment (Supplementary Figure S1C).

There were no obvious differences in growth or development characteristics of the wild-type, *cbnac1* or *35S:Flag-CBNAC* plants. The effect of *CBNAC* expression level on susceptibility to the virulent bacterial pathogen *PstDC3000* was then examined. Three days after inoculation, bacterial growth in infiltrated leaves was slightly lower in the null *cbnac1* mutant and slightly higher in *35S:Flag-CBNAC* overexpression lines compared with the wild type (Figure 2A). There was no significant difference in bacterial growth in leaves of the wild-type plant and a *cbnac1/CBNAC* complementation line (Supplementary Figure S2B). As loss of *CBNAC* function is associated with resistance and overexpression of *CBNAC* is associated with susceptibility in un-induced plants, *CBNAC* is a negative regulator of basal defense against the bacterial pathogen *PstDC3000*.

Induction of *PR1* is a marker of SA-mediated defense signaling that leads to *Arabidopsis* resistance to bacterial pathogens (2). *PR1* transcript accumulation was not observed in leaves of wild-type, *cbnac1*, *cbnac1/CBNAC* or *35S:Flag-CBNAC* plants before inoculation with *PstDC3000* (Figure 2B and Supplementary Figure S2C). At 12 h post-inoculation when the expression of *CBNAC* was expected to peak in wild-type plants (Figure 1A), accumulation of *PR1* transcript was significantly suppressed in *35S:Flag-CBNAC* plants, induced in the *cbnac1* mutant and unchanged in the *cbnac1/CBNAC* complementation line in comparison with wild type (Figure 2B and Supplementary Figure S2C). Thus, the level of *CBNAC* expression correlates inversely with resistance to pathogen infection and *PR1* expression, suggesting that *PR1* may be a direct target of *CBNAC*.



**Figure 1.** *CBNAC* expression is induced by pathogen- and SA. (A) Induction of *CBNAC* gene expression by *PstDC3000*. Leaves of 4-week-old *Arabidopsis* plants (Col-0) were infiltrated with a bacterial suspension ( $OD_{600} = 0.001$  in 10 mM  $MgCl_2$ ). Infiltrated leaves were harvested at the indicated times after inoculation. The gel blot analysis of total RNA that was performed with a  $^{32}P$ -labeled *CBNAC* probe is shown. Ethidium bromide-stained *rRNA* is shown as loading control. (B) Induction of *CBNAC* gene expression by SA. Leaves of 4-week-old *Arabidopsis* plants (Col-0) were treated with 1 mM SA. Leaf collection, RNA isolation and RNA gel blot analysis was performed as in (A).



**Figure 2.** *CBNAC* negatively regulates resistance to *PstDC3000* and *PR1* expression. Leaves of wild type (WT), *35S:Flag-CBNAC* and *cbnac1* plants were inoculated with a bacterial suspension ( $OD_{600} = 0.001$  in 10 mM  $MgCl_2$ ). (A) Growth of *PstDC3000* in inoculated leaves at 0 and 3 dpi. Mean bacterial densities  $\pm$  SE were calculated from six to eight replicate plants are shown. Significant differences as calculated by Student's *t* test ( $P < 0.05$ ) are indicated by unique letters. The experiment was repeated at least three times with similar results. (B) qRT-PCR analysis of *PR1* expression in inoculated leaves. Values were normalized using the expression level of *Tubulin 2* and expressed relative to the expression level in WT at 12 hpi, which is arbitrarily set at 100. Mean relative expression values  $\pm$  SE from three independent experiments are shown. Data were analyzed by Student's *t* test. Different letters indicate statistically significant differences between genotypes ( $P < 0.05$ ).

### CBNAC binds to the *PR1* promoter by a GCTT core element

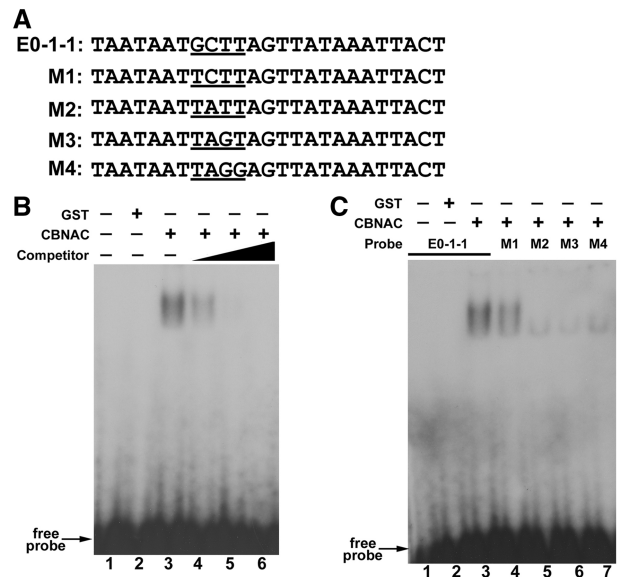
In *cbnac1* knockout plants, the levels of *PR1* transcripts were higher compared with those in wild-type plants. To examine whether this effect was attributable to direct binding of the CBNAC to the *PR1* promoter, we investigate the direct interaction of CBNAC to the PR promoter by electrophoretic mobility shift assays (EMSA). EMSA using recombinant GST-CBNAC fusion protein and *PR1* promoter fragments up to 1041-bp upstream of the *PR1* start codon were used to demonstrate that CBNAC protein can bind to the *PR1* promoter. Using seven ~110-bp overlapping fragments of the *PR1* promoter sequence (Supplementary Figure S3A), five regions (E0, E3, E4, E5 and E6) were found to bind to CBNAC (Supplementary Figure S3B).

Within the five regions, smaller overlapping fragments (~52 bp) were used to define the specific target of CBNAC (Supplementary Figure S4A). Six regions (E0-1, E0-4, E3-1, E4-1, E5-3 and E6-1) demonstrated binding to CBNAC (Supplementary Figure S4B). Overlapping oligonucleotides within the E0-1, E0-4, E3-1, E4-1, E5-3 and E6-1 fragments were used to further delineate CBNAC-binding sequences (Supplementary Figure S5A). CBNAC bound six *PR1* promoter fragments (Supplementary Figure S5B and Table 1). Of these, E0-1-1 exhibited very strong CBNAC binding. E0-4-2, E3-1-2 and E5-3-1 had moderate affinity. The two remaining elements, E4-1-1 and E6-1-1, bound very weakly. The E0-1-1 and E0-4-2 fragments contain the GCTT core sequence that was previously identified as a CBNAC-binding sequence by the random binding site selection method (26). E3-1-2 has been previously identified as a negative regulatory element on the *PR1* promoter (12).

Interactions between CBNAC and the E0-1-1 element were further analyzed. Figure 3A depicts the four mutant E0-1-1 elements (M1–M4) used in the EMSA. As shown in Figure 3B, CBNAC bound strongly and specifically with radiolabeled E0-1-1. A mobility shift was not observed on incubation of the labeled E0-1-1 element with GST alone. Furthermore, addition of unlabeled E0-1-1 element inhibited binding of labeled E0-1-1 to CBNAC in a concentration-dependent manner (Figure 3B). Compared with the interaction of CBNAC with E0-1-1, its binding with M1 was weaker and was almost negligible with M2, M3 and M4 (Figure 3C). These results

indicated that the GCTT core sequence of E0-1-1 is critical for CBNAC binding.

Chromatin immunoprecipitation (ChIP) experiments were then used to determine whether CBNAC binds the *PR1* promoter *in vivo*. In these experiments, cross-linked chromatin from leaves of wild-type and *35S:Flag-CBNAC* plants were incubated with FLAG-specific monoclonal antibodies enriching for CBNAC and *PR1* promoter complexes. The five *PR1* promoter regions shown to bind CBNAC *in vitro* (E0, E3, E4, E5 and E6) were then amplified with specific primers. Three independent ChIP experiments were performed, and representative results from a single assay are shown (Supplementary Figure S5C). PCR analyses of input chromatin samples verified that similar quantities of ChIP starting materials were



**Figure 3.** CBNAC interacts with the E0-1-1 element of the *PR1* promoter. (A) Nucleotide sequence of the native and mutated (M1–M4) E0-1-1 elements used in EMSA. (B) Analysis of binding specificity. EMSA was performed using  $^{32}$ P-labeled native E0-1-1 as probe as above except that GST-CBNAC protein was preincubated with 50- (lane 4), 100- (lane 5) or 200- (lane 6) fold molar excess of cold native E0-1-1 (competitor) before addition of probe. (C) EMSA of CBNAC binding.  $^{32}$ P-labeled native (lanes 1–3) and mutated (lanes 4–7) E0-1-1 probes were incubated with equal amounts of *E. coli*-expressed GST-CBNAC (lanes 3 to 7) or GST alone (lanes 1 and 2) before electrophoresis.

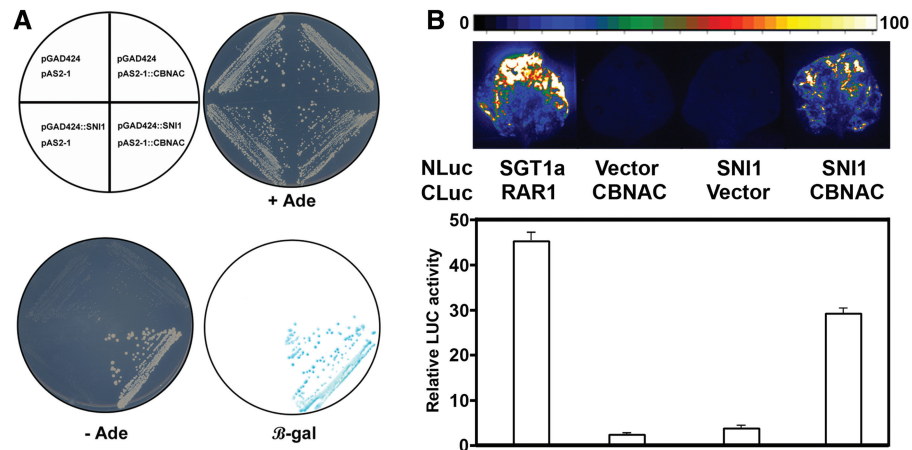
**Table 1.** Putative CBNAC-binding *cis*-acting elements in the *PR1* promoter

<i>cis</i> element	Sequence <sup>a</sup>	Position <sup>b</sup>	Binding affinity <sup>c</sup>
E0-1-1	TAATAAT <u>GCTT</u> AGTTATAAATTACT	(–) 1209~(–) 1185	+++
E0-4-2	TGTTATT <u>GCTT</u> AGAATCACAGATTC	(–) 994~(–) 970	++
E3-1-2	CTATTGACTGTTTCTCTACGTCACTATT	(–) 715~(–) 688	++
E4-1-1	ATACTCATATGCATGAAACACTAAGAAAC	(–) 618~(–) 590	++
E5-3-1	ATATACAATGTTTCTTAATAAACTTCATTT	(–) 340~(–) 311	++
E6-1-1	AAAAAAATATATCAACAATGGCAAAGCT	(–) 288~(–) 261	+

<sup>a</sup>Sequences are indicated from 5' to 3'. GCTT core sequence is underlined.

<sup>b</sup>Positions of the *cis* elements with respect to the translation start site (ATG).

<sup>c</sup>The relative binding affinity (+) was determined by densitometry of autoradiograms of DNA-bound CBNAC.



**Figure 4.** CBNAC interacts with SNI1. (A) Yeast two-hybrid analysis. Transformants of yeast strain pJ69-4A were grown as indicated (upper left) on minimal medium with (+Ade) or without (–Ade) selection. Adenine prototrophy indicates positive interaction.  $\beta$ -Galactosidase activity in the colonies grown in +Ade medium was determined by filter-lift assay (LacZ). (B) LCI assay for detecting interaction *in planta*. Tobacco leaves were transformed by *Agrobacterium* infiltration using a needleless syringe. The indicated NLuc and CLuc construct pairs were used for transformation. Shown are luminescence images (upper panel) and quantitative luminescence measurements (lower panel) depicting luciferase activity in inoculated leaves at 48 hpi.

used for each primer pair. As expected, immunoprecipitation reactions lacking anti-FLAG antibody did not result in the recovery of chromatin fragments containing the *PR1* promoter (Supplementary Figure S5C, No Ab). Only the E0 (–1209 to –970 bp) region was amplified in immunoprecipitates obtained with anti-FLAG antibody (Supplementary Figure S5C,  $\alpha$ -Flag Ab), confirming that CBNAC directly binds to a region of the *PR1* promoter that contains the GCTT core sequence.

### CBNAC physically interacts with SNI1

Similar to CBNAC, SNI1 is a negative regulator of *PR1* expression. However, unlike CBNAC, SNI1 does not have a DNA-binding domain. Therefore, the possibility of interaction between CBNAC and SNI1 was examined in a yeast two-hybrid assay. The bait construct (pAS2-1::CBNAC) contained full-length CBNAC cDNA fused to the GAL4 DNA-binding domain. The prey construct (pGAD424::SNI1) contained full-length SNI1 cDNA fused to the GAL4 activation domain. As shown in Figure 4A, yeast cells expressing either bait or prey construct alone were not able to grow on selection media. Growth on selective medium and expression of the LacZ reporter gene was observed only if yeast contained both pAS2-1::CBNAC and pGAD424::SNI1, indicating specific interaction between CBNAC and SNI1.

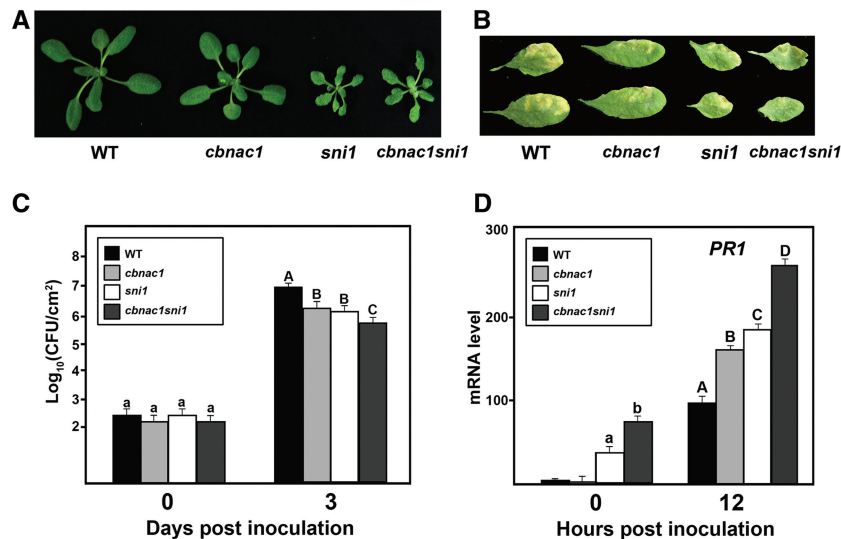
Interaction of CBNAC with SNI1 *in planta* was then examined using the LCI assay in *N. benthamiana* leaves using transient expression (32). The positive control combination of STG1a-NLuc and CLuc-RAR1 resulted in strong Luc activity as previously reported (32) (Figure 4B). The negative control combinations of CLuc-CBNAC/NLuc vector and SNI1-NLuc/CLuc vector did not show Luc activity (Figure 4B). Luc activity was detected with the combination of SNI1-NLuc/CLuc-CBNAC showing that CBNAC interacts with SNI1 *in planta*.

Additional yeast two-hybrid assays were performed to define the CBNAC domain responsible for interaction with SNI1. A schematic diagram of the CBNAC deletion constructs used for these assays is shown in Supplementary Figure S6A. The NAC domain of CBNAC was not required for interaction with SNI1 (Supplementary Figure S6B). The C-terminal region (301–512 amino acids) of CBNAC interacted with SNI1. The interaction between CBNAC and SNI1 required the CaM-binding domain as well as other sequences.

### CBNAC and SNI1 function synergistically as negative regulators of disease resistance

A *cbnac1 sni1* double mutant was generated through genetic crossing and analyzed for potential interactions between CBNAC and SNI1. Morphological phenotypes of the *sni1* mutant resembled those of the *sni1* mutant rather than the *cbnac1* mutant. Both *sni1* and *cbnac1 sni1* plants had smaller rosettes compared with the wild type (Figure 5A). Similar to the *sni1* mutant, *cbnac1 sni1* plants exhibited pleiotropic phenotypes, including decreased leaf size and altered leaf texture (Figure 5A and B). In contrast, the *cbnac1* plants showed no differences in growth, development or morphology in comparison with the wild type (Figure 5A and B). As shown in Figure 5C, the *cbnac1* and *sni1* mutants had similar levels of basal resistance to *PstDC3000* (Figure 5C). Bacterial counts in inoculated leaves of both lines were slightly lower than in leaves of the wild type 3 days post-inoculation. Disease symptoms were also less severe in the *cbnac1* and *sni1* mutants than in wild-type leaves (Figure 5B). The *cbnac1 sni1* double mutant was markedly more resistant to *PstDC3000* than either the *cbnac1* or *sni1* single mutants (Figure 5C). Bacterial counts in inoculated leaves of the *cbnac1 sni1* mutant were 10-fold lower than in leaves of the wild type at 3 days post-inoculation. This marked reduction in bacterial growth





**Figure 5.** Altered responses of the *cbnac1 sni1* double mutant to *PstDC3000*. (A) Morphology of 5-week-old wild-type (WT), *cbnac1*, *sni1* and *cbnac1 sni1* plants grown on MS agar plates. (B–D) Disease resistance responses in leaves inoculated with bacterial suspension as in Figure 3. Disease symptoms in inoculated leaves at 5 dpi are depicted (B). Bacterial growth in inoculated leaves at 0 and 3 dpi are compared (C). Mean bacterial densities  $\pm$  SE were calculated from six to eight replicate plants. Significant differences as calculated by Student's *t* test ( $P < 0.05$ ) are indicated by unique letters. The experiment was repeated at least three times with similar results. *PR1* expression was monitored in inoculated leaves by qRT-PCR (D). Values were normalized using the expression level of *Tubulin 2* and expressed relative to the expression level in WT at 12hpi, which is arbitrarily set at 100. Mean relative expression values  $\pm$  SE values from three independent experiments are shown. Data were analyzed by Student's *t* test. Different letters indicate statistically significant differences between genotypes ( $P < 0.05$ ).

in inoculated leaves of the *cbnac1 sni1* mutant compared with the *cbnac1* or *sni1* mutants was accompanied by the substantially reduced disease symptom development (Figure 5B).

To investigate the enhanced basal resistance of the double mutant, *PR1* expression was analyzed following bacterial pathogen infection. The level of *PR1* mRNA was comparable in uninoculated leaves of wild type and *cbnac1* plants (Figure 5D). *SNI1* has been reported to repress basal *PR1* expression (18). Accordingly, the level of *PR1* mRNA was higher in uninoculated leaves of the *sni1* mutant than in wild type. At 12h post-inoculation, *PR1* transcript abundance was elevated in the *cbnac1* and *sni1* mutants compared with wild type, as expected if they function as transcriptional repressors. The *PR1* levels in uninoculated and inoculated leaves of the *cbnac1 sni1* double mutant were significantly higher than in leaves of *cbnac1* or *sni1* single mutants under the same condition, suggesting a synergistic effect of the combined *cbnac1* and *sni1* mutations on *PR1* expression (Figure 5D). Together, these results show that *CBNAC* and *SNI1* function synergistically as negative regulators of basal resistance to the virulent bacterial pathogen *PstDC3000* due, in part, to their overlapping activities as negative regulators of *PR1* expression.

#### SNI1 enhances the DNA-binding activity of the CBNAC

Although *SNI1* functions as a transcriptional repressor of *PR1* expression, it lacks a DNA-binding domain (23). Therefore, the possibility that *SNI1* could influence the DNA-binding activity of *CBNAC* toward the *PR1*

promoter *cis*-element (E0-1-1) was investigated because *SNI1* interacts with *CBNAC*.

The *PR1* promoter element (E0-1-1), previously identified by EMSA, contains the preferred GCTT core binding sequence for *CBNAC* (Figure 6A). Incubation of *SNI1* with the E0-1-1 probe did not result in a retarded band in an EMSA (Figure 6B, lane 3) indicating that *SNI1* does not directly bind to this element. Addition of *SNI1* to the *CBNAC*-binding mixture increased *CBNAC* binding to the E0-1-1 element without producing a supershift band, indicating that the DNA-protein complex contained *CBNAC* but not *SNI1* (Figure 6B, lanes 5 and 4). These results suggested that *SNI1* enhances the binding of *CBNAC* to the E0-1-1 element.

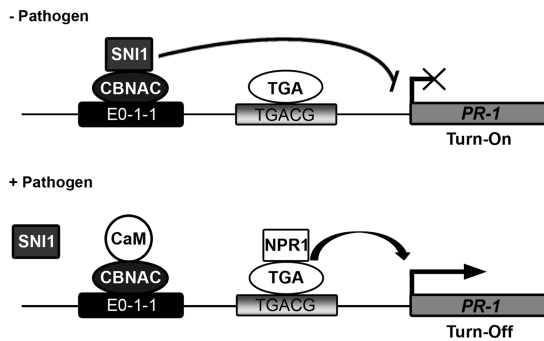
## DISCUSSION

### CBNAC acts as a negative regulator of plant defense responses

Discrepancies exist in several studies that had reported on the biochemical properties of *CBNAC/NTL9* (26,33). First, *CBNAC/NTL9* had been reported to contain a C-terminal transmembrane domain and that *CBNAC/NTL9* was localized in the plasma membrane (33). However, the predicted C-terminal transmembrane domain of *CBNAC/NTL9* was identified as a CaM-binding domain (26). Furthermore, the GFP-tagged *CBNAC/NTL9* protein was considered to be dominantly localized in nuclei (26). Finally, on a different level of function, *CBNAC/NTL9* was reported to be involved in regulating signaling during leaf senescence (33). The biological function of *CBNAC/NTL9* was based on







**Figure 7.** Model for the regulation of *PR1* by the CBNAC-SNI1 complex. In non-induced conditions (–Pathogen), because SNI1 does not contain a known DNA-binding domain, we postulate that SNI1 binds to CBNAC and is thereby recruited to the E0-1-1 element of *PR1* promoter. SNI1 enhances the DNA-binding activity of CBNAC and somehow this enhances repression of *PR1* by SNI1. In the presence of inducer (+Pathogen), *PR1* gene expression is induced by the translocation of a large amount of active NPR1 to the nucleus and its interaction with TGA transcription factors. The SNI1/CBNAC protein complex can be removed by NPR1, CaM or other unknown mechanisms.

NPR1 stimulates the DNA binding of TGA factors to the LS7 element (10). The tobacco ankyrin repeat protein ANK1 inhibits the binding of the bZIP factor BZI-1 to its cognate promoter element in EMSA without altering the complex mobility (38). Similarly, SNI1 enhanced the binding of CBNAC to its cognate promoter element in EMSA without altering the complex mobility (Figure 6B). There are two possible explanations. First, SNI1 may have been released from the complex by a conformational change resulting from CBNAC binding to DNA. Second, other interacting proteins may be required for a stable interaction between CBNAC and SNI1.

### Model for *PR1* regulation

Based on the information generated in this and previous studies, the following model is proposed (Figure 7). The *PR1* promoter contains both positive TGA-binding (TGACG) and negative CBNAC-binding (E0-1-1) elements. Genetic analyses showed that *CBNAC* interacts synergistically with *SNI1* as a transcriptional repressor of *PR1* gene expression and basal resistance to *PstDC3000* (Figure 5C and D). Unlike *cbnac1* plants, *snil* and *cbnac1 snil* plants exhibit constitutive *PR* gene expression and other pleiotropic phenotypes (Figure 5A). Therefore, in absence of pathogen induction, SNI1 is proposed to function as the major negative regulator of *PR* gene expression whose activity is governed, in part, by its interaction with the transcriptional repressor CBNAC at the E0-1-1 element. The constitutive binding of transcriptional activators (such as TGA1, TGA3 and TGA6) to the positive element in a non-induced state is still a possibility. However, this binding would not lead to gene expression in the presence of CBNAC and SNI1.

On pathogen infection, transcription of *PR1* is activated and repression is terminated. Transcriptional activation of *PR1* by NPR1 is well characterized and is mediated by its

association with the positive TGA transcription factors. However, mechanisms by which repression of *PR1* expression are terminated following pathogen infection are poorly understood. On the basis of the data presented herein, three possibilities can be considered. First, CBNAC and SNI1 could be degraded by a pathogen signal, but there is no strong evidence in support of this hypothesis. Second, CBNAC and SNI1 could be removed by the binding of other regulator proteins or by covalent modifications because the interaction between CBNAC and SNI1 is reduced or removed by pathogen treatment (Supplementary Figure S9). CaM could also be involved in the termination of transcriptional repression through the *PR1* promoter E0-1-1 element because CaM interacts with CBNAC (26), and the CaM-binding domain of CBNAC is required for interaction with SNI1 (Supplementary Figure S6). This scenario is rendered likely because influx of  $Ca^{2+}$  followed by the activation of  $Ca^{2+}$ /CaM signaling processes represents early and essential events in the response to pathogen infection (39,40). Several CaM-binding proteins have already been shown to be involved in plant defense responses (41,42). Third, CBNAC and SNI1 repression is removed by NPR1. It has been proposed that the role of NPR1 in a wild-type plant is to inactivate SNI1-mediated transcriptional repression of *PR* genes (18). As such, *PR* gene expression is restored and is inducible in the *snil npr1-1* double mutant. However, a physical interaction between SNI1 and NPR1 has never been demonstrated so it is unclear whether NPR1 regulates the SNI1 repression of *PR* genes. Further molecular and genetic studies are required to precisely characterize the regulatory mechanisms that converge on the *PR1* gene, resulting in the precise control of *PR1* expression in response to environmental stimuli.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–9 and Supplementary Tables 1 and 2.

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