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 (PstDC3000), 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 PstDC3000 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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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

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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 RAD15 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\(^{2+}\)/CaM complex modulates immune responses by repressing or activating transcription. Transcription of genes involved in SA biosynthesis is modulated by Ca\(^{2+}\)/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\(^{2+}\)/CaM. Details of CaM regulation of defense gene expression are not well-understood.

We show here a novel connection between SNI1 and Ca\(^{2+}\)/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 \(\mu\)g/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 \(\mu\)Em \(^{-2}\) s\(^{-1}\) 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, selling the F1 progeny and screening 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 (PstDC3000) carrying empty vector (pVSP61) was grown at 28°C on King’s agar plates supplemented with 50 \(\mu\)g/ml rifampicin and 50 \(\mu\)g/ml kanamycin. In brief, bacteria were suspended in 10 mM MgCl\(_2\), adjusted to optical density (OD)\(_{600}\) = 0.001 and pressure infiltrated into leaves using a needleless syringe. Leaf discs from four independent plants were combined, ground in 10 mM MgCl\(_2\), 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™ II RNase-Reverse Transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed using the SsoFast EvaGreen Supermix (Bio-Rad) in a CFX96™ 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 ($\alpha$-32P)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, $\gamma$-32P-ATP (6000 Ci/mol; 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, $\gamma$-32P-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 µg poly(dI-dC) and 0.5 µg bacterially produced fusion protein purified with glutathione-Sepharose. 32P-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× 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 SN1 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 into the pGAD424 plasmid (prey vector), which harbors the 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). SN1 was fused with the N-terminal fragment of Luc in pCAMBIA NLuc vector (SN1-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 MgCl2, 10 mM MES and 100 µM acetoxyserine) 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. PstDC3000 (OD600 = 0.001 in 10 mM MgCl2) was treated after 24 h as Agro infiltration. The leaves were sprayed with luciferin solution (100 µM 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 (AndorXon; 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 PstDC3000 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 MgCl2. In leaves infiltrated with PstDC3000, 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.
CBNAC binds to the PRI promoter by a GCTT core element

In cbnac1 knockout plants, the levels of PRI 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 PRI promoter, we investigate the direct interaction of CBNAC to the PRI promoter by electrophoretic mobility shift assays (EMSA). EMSA using recombinant GST-CBNAC fusion protein and PRI promoter fragments up to 1041-bp upstream of the PRI start codon were used to demonstrate that CBNAC protein can bind to the PRI promoter. Using seven ~110-bp overlapping fragments of the PRI 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 PRI 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). E0-1-1 element inhibited binding of labeled E0-1-1 to EMSA was performed using 32P-labeled native E0-1-1 as probe as shown on incubation of the labeled E0-1-1 element 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).

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

| cis element | Sequence | Position | Binding affinity |
|-------------|----------|----------|-----------------|
| E0-1-1      | TAATAATGCTTAGTTATAAATTACT | (-) 1209 to (-) 1185 | +++ |
| E0-4-2      | TTATATGCTTAGTTATAAATTACT | (-) 994 to (-) 970 | ++ |
| E3-1-2      | CTTTTGCTTAGTTATAAATTACT | (-) 715 to (-) 688 | ++ |
| E4-1-1      | ATATATGCTTAGTTATAAATTACT | (-) 618 to (-) 590 | + |
| E5-3-1      | ATATATGCTTAGTTATAAATTACT | (-) 340 to (-) 311 | + |
| E6-1-1      | ATATATGCTTAGTTATAAATTACT | (-) 288 to (-) 261 | + |

sequences are indicated from 5' to 3'. GCTT core sequence is underlined.

The relative binding affinity (+) was determined by densitometry of autoradiograms of DNA-bound CBNAC.
used for each primer pair. As expected, immunoprecipitation reactions lacking anti-FLAG antibody did not result in the recovery of chromatin fragments containing the \textit{PRI} 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, \textit{a-Flag Ab}), confirming that CBNAC directly binds to a region of the \textit{PRI} promoter that contains the GCTT core sequence.

CBNAC physically interacts with SNI1

Similar to CBNAC, SNI1 is a negative regulator of \textit{PRI} 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: \textit{CBNAC}) contained full-length \textit{CBNAC} cDNA fused to the GAL4 DNA-binding domain. The prey construct (pGAD424::\textit{SNI1}) contained full-length \textit{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 \textit{LacZ} reporter gene was observed only if yeast contained both pAS2-1::\textit{CBNAC} and pGAD424::\textit{SNI1}, indicating specific interaction between CBNAC and SNI1.

Interaction of CBNAC with SNI1 \textit{in planta} was then examined using the LCI assay in \textit{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 SN1-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 \textit{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 \textit{cbnac1 sni1} double mutant was generated through genetic crossing and analyzed for potential interactions between \textit{CBNAC} and \textit{SNI1}. Morphological phenotypes of the \textit{cbnac1 sni1} mutant resembled those of the \textit{sni1} mutant rather than the \textit{cbnac1} mutant. Both \textit{sni1} and \textit{cbnac1 sni1} plants had smaller rosettes compared with the wild type (Figure 5A). Similar to the \textit{sni1} mutant, \textit{cbnac1 sni1} plants exhibited pleiotropic phenotypes, including decreased leaf size and altered leaf texture (Figure 5A and B). In contrast, the \textit{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 \textit{cbnac1} and \textit{sni1} mutants had similar levels of basal resistance to \textit{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 \textit{cbnac1} and \textit{sni1} mutants than in wild-type leaves (Figure 5B). The \textit{cbnac1 sni1} double mutant was markedly more resistant to \textit{PstDC3000} than either the \textit{cbnac1} or \textit{sni1} single mutants (Figure 5C). Bacterial counts in inoculated leaves of the \textit{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.
in inoculated leaves of the cbnac1 snl1 mutant compared with the cbnac1 or snl1 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 snl1 mutant than in wild type. At 12 h post-inoculation, PR1 transcript abundance was elevated in the cbnac1 and snl1 mutants compared with wild type, as expected if they function as transcriptional repressors. The PR1 levels in uninoculated and inoculated leaves of the cbnac1 snl1 double mutant were significantly higher than in leaves of cbnac1 or snl1 single mutants under the same condition, suggesting a synergistic effect of the combined cbnac1 and snl1 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.

**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 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.
CBNAC (lanes 4 and 5) and SNI1 (lane 5). Equal amounts of CBNAC without (lane 1) or with the addition GST (lane 2; negative control), EMSA was performed using 32P-labeled E0-1-1 element (lanes 1 to 5), the binding sequence (TGACG) for TGA transcription factors and TGA2 independently binds the LS3 and LS7 promoter elements in vitro (10). The LS3 element seems to contribute to the negative regulation of PR1 expression both in the absence and presence of SA, whereas LS7 is required for SA-mediated PR1 induction (34). The GCTT cis-element for CBNAC binding to the PR1 promoter represents a novel PR1 regulatory module. Interestingly, CBNAC is recruited to the PR1 promoter in both non-treated and SA-treated tissues (Supplementary Figures S5C and S7). SA-independent binding of CBNAC to the PR1 promoter suggests that the DNA-binding activity of CBNAC is not altered by SA.

Both PstDC3000 and SA induced CBNAC quickly in a transient way (Figure 1). The induction of the negative regulator during the early stages of infection might serve as a mechanism to prevent gratuitous or even harmful overactivation of pathogen-induced defense mechanisms when the population of the pathogen remains at relatively low levels (35). As pathogen growth increases, enhanced defense mechanisms would be necessary, and this could be achieved at least partially by suppressed expression and inactivation of negative regulators such as CBNAC and WRKY factors.

Pathogen-induced expression of negative defense regulators could be explained by their involvement in the antagonistic crosstalk of distinct signaling pathways against various types of microbial pathogens. SA-mediated signaling activates defense mechanisms effective against biotrophic pathogens but can suppress ET/JA-mediated signaling in defense against necrotrophic pathogens (3). For example, overexpression of the transcription factor WRKY33 enhances susceptibility to the biotrophic pathogen PstDC3000 and increases resistance to necrotrophic fungal pathogens (36). cbnac1 plants showed enhanced resistance to biotrophic pathogen PstDC3000 but no difference to necrotrophic fungal pathogens. Consistently, the transcription levels of JA-responsive marker genes, PDF1.2 and LOX2, were similar in cbnac1 mutant and wild-type plants (Supplementary Figure S8). These results suggest that CBNAC is not involved in the antagonistic crosstalk of defense signaling pathways against biotrophic and necrotrophic pathogens. It is possible that CBNAC is involved in crosstalk between SA- and NPR1-mediated defense signaling and as yet unidentified environmental stress signaling.

CBNAC acts synergistically with SNI1 as a transcriptional repressor of the PR1 gene

SNI1, a transcriptional repressor of PR gene expression, has no apparent DNA-binding domain and is predicted to bind to DNA indirectly by interactions with DNA-binding proteins (23). CBNAC has been identified as one DNA-binding protein that interacts with SNI1 (Figure 5). SNI1 enhances the DNA-binding activity of CBNAC to the PR1 promoter element (Figure 6B). There are several reported instances where an interaction partner enhances or inhibits DNA-binding activity of a transcription factor. The human viral protein TAX stimulates the DNA binding of bZIP proteins (37), whereas Arabidopsis
PR1 and repression is terminated. Transcriptional activation of expression in the presence of CBNAC and SNI1.

However, this binding would not lead to gene expression whose activity is governed, in part, by its interaction with the transcriptional repressor CBNAC at the 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 Pst DC3000 (Figure 5C and D). Unlike cnbacs1 plants, sni1 and cnbacs1 sni1 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 CBNAC 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 Ca2+ followed by the activation of Ca2+/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 sni1 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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