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ORIGINAL ARTICLE

Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses

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Jasmonic acid (JA) is an important phytohormone that regulates plant defense responses against herbivore attack, pathogen infection and mechanical wounding. In this report, we provided biochemical and genetic evidence to show that the *Arabidopsis thaliana* NAC family proteins ANAC019 and ANAC055 might function as transcription activators to regulate JA-induced expression of defense genes. The role of the two *NAC* genes in JA signaling was examined with the *anac019 anac055* double mutant and with transgenic plants overexpressing *ANAC019* or *ANAC055*. The *anac019 anac055* double mutant plants showed attenuated JA-induced *VEGETATIVE STORAGE PROTEIN1 (VSP1)* and *LIPOXYGENASE2 (LOX2)* expression, whereas transgenic plants overexpressing the two *NAC* genes showed enhanced JA-induced *VSP1* and *LOX2* expression. That the JA-induced expression of the two *NAC* genes depends on the function of COI1 and AtMYC2, together with the finding that overexpression of *ANAC019* partially rescued the JA-related phenotype of the *atmyc2-2* mutant, has led us to a hypothesis that the two NAC proteins act downstream of AtMYC2 to regulate JA-signaled defense responses. Further evidence to substantiate this idea comes from the observation that the response of the *anac019 anac055* double mutant to a necrotrophic fungus showed high similarity to that of the *atmyc2-2* mutant.

***Keywords*:** *Arabidopsis thaliana*, ANAC019 and ANAC055, transcription factor, jasmonic acid signaling, defense response, pathogen infection

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Introduction

The jasmonate family of oxylipins, including jasmonic acid (JA), methyl jasmonic acid (MeJA) and other bioactive derivatives of JA are important signaling molecules in the plant kingdom. JAs are perhaps best known for their role in regulating defense responses against biotic stresses such as herbivore attack and necrotrophic pathogen infection [1-6]. In addition to biotic stresses, JAs are also involved in the control of plant responses to a range of abiotic stresses [7-12].

Molecular genetic studies, mainly conducted in the *Arabidopsis thaliana* model system, have identified several important players in the JA signal transduction pathway. The *coronatine insensitive 1* (*coi1*) mutant in *Arabidopsis* is fully insensitive to JA in both root growth inhibition and defense gene expression [13]. Molecular characterization of *COI1* has indicated that this gene encodes an F-box protein, which suggests the involvement of a ubiquitin-mediated protein-degradation process in JA signaling [14]. Support for this hypothesis comes from the demonstration that COI1 interacts with Skp1 and Cullin1 to assemble a functional SCF^{COI1} ubiquitin ligase complex *in vivo* [15, 16]. Compared with *coi1*, the

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jasmonate resistance1 (jar1) [17] and *jasmonate insensitive1 (jin1)* [18, 19] mutants exhibit a relatively weak phenotype in JA-induced inhibition of root growth. *JAR1* encodes an enzyme that has JA adenylation activity to form JA-amino acid conjugates, especially JA-isoleucine (JA-Ile), which suggests that JA-Ile, rather than JA itself, might be the active hormone [20]. *JIN1* encodes a nuclear-localized basic helix-loop-helix (bHLH)-type transcription factor known as AtMYC2 [19]. Genome-wide transcriptional profiling demonstrated that AtMYC2 acts as both activator and repressor to regulate diverse aspects of JA responses [21]. Recently, a family of *Arabidopsis* proteins named JAZ (jasmonate ZIM domain) was identified as a target of the SCF^{COI1} ubiquitin ligase complex in JA signaling [22, 23]. Significantly, JA-Ile promotes the binding of the SCF^{COI1} ubiquitin ligase to and subsequent degradation of the JAZ proteins, which suggests that the SCF^{COI1}-JAZ complex may be the site of JA-Ile perception [23]. Furthermore, JAZ proteins interact and negatively regulate AtMYC2, a central regulator of JA-induced gene expression. Therefore, the JAZ family proteins represent the molecular link between SCF^{COI1}-mediated protein degradation and transcriptional activation of jasmonate responses [22].

Among the identified components of JA signaling, the role of JIN1/AtMYC2 (henceforth referred to as AtMYC2) is interesting. As mentioned above, AtMYC2 differentially regulates two types of JA signaling defense responses [19, 24, 25]. One of these types, which is positively regulated by AtMYC2, induces the expression of genes that are involved in the response to wounding (mechanical or biotic). The other type, which is negatively regulated by AtMYC2, represses the expression of pathogen defense-related genes. Therefore, while the *jin1*/*atmyc2* mutants showed decreased expression of wound responsive genes such as *VEGETATIVE STORAGE PROTEIN1* (*VSP1*) [18] and *LIPOXYGENASE2* (*LOX2*) [26], they showed increased expression of a group of pathogen defensive genes that includes *PDF1.2* [27] and *PATHOGENESIS RELATED1* (*PR-1*) [19, 24, 25]. However, less is known about the JA signaling components that function downstream of AtMYC2.

NAC (NAM/ATAF1, 2/CUC2) proteins are a family of plant-specific transcription factors with diverse biological functions [28]. Accumulating evidence indicates that NAC family proteins play an important role in different aspects of plant development [29-32]. In addition, several NAC proteins in *Arabidopsis* are believed to be important in plant responses to abiotic stresses. For example, it was shown that three dehydration-inducible *NAC* genes (*ANAC019*, *ANAC055* and *ANAC072*) were associated with drought tolerance [33] and that *AtNAC2/ANAC092* was associated with salt-stress responses [34]. A role for NAC proteins in biotic stress responses was recently exemplified by the demonstration that the *Arabidopsis* NAC protein ATAF2 [29] functions as a repressor of pathogenesis-related genes [35]. However, the biological functions for most of the members of the NAC-family transcription factors remain to be determined [28].

ANAC019 and ANAC055 are closely related NAC family proteins in *Arabidopsis* [33, 36]. In this report, we provided evidence to show that ANAC019 and ANAC055 may function as transcription activators to regulate JA-induced expression of defense genes. The role of the two *NAC* genes in JA signaling was examined with the *anac019 anac055* double mutant and transgenic plants overexpressing *ANAC019* or *ANAC055*. Our data support a hypothesis that the two NAC proteins act downstream of AtMYC2 to regulate JA-signaled defense responses.

Results

ANAC019 and ANAC055 are JA-inducible genes encoding NAC family proteins

We identified At1g52890 and At3g15500 as JA-inducible genes in our microarray analyses using the *Arabidopsis* whole genome chip (Affymetrix) [37]. Both At1g52890 and At3g15500 encode proteins of 317 amino acids and their N-terminal regions contain an NAC domain that is highly similar to all members of the plant-specific NAC family proteins [36]. At1g52890 and At3g15500 were therefore re-named ANAC019 and ANAC055, respectively, according to the nomenclature that has been established for the family of *Arabidopsis* NAC proteins [36]. At the amino-acid level, ANAC019 showed 72% sequence similarity to ANAC055 and the regions of homology were observed not only in the NAC domain but also in the C-terminus [33], which suggests that the two members may have similar or overlapping biological functions.

ANAC019 and ANAC055 expression is induced by MeJA in a COI1- and AtMYC2-dependent manner

The MeJA-induced expression of *ANAC019* and *ANAC055* was verified using RNA gel blot analysis. As shown in Figure 1A, transcripts of the two *NAC* genes were undetectable in the absence of MeJA but were induced upon MeJA treatment. An increase in the number of *ANAC019* and *ANAC055* transcripts was detected as early as 15 min after applying MeJA, and this increase in transcript number was maintained up to 6 h after treatment.

The MeJA-induced expression of the two *NAC* genes

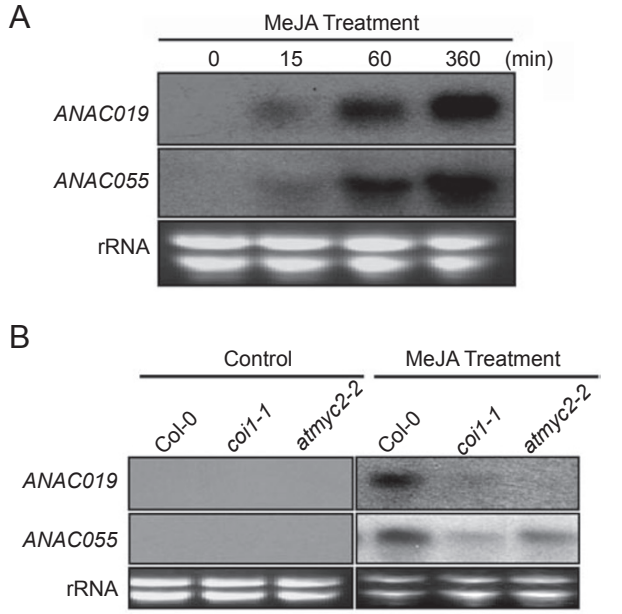

Figure 1 MeJA-induced expression of *ANAC019* and *ANAC055*. **(A)** MeJA-induced expression of the two *NAC* genes in wild-type plants. **(B)** MeJA-induced expression of the two *NAC* genes in different mutant backgrounds. Two-week-old plants were treated with 50 μM MeJA and tissues were collected at the indicated times for RNA extraction. In all, 30 μg of total RNA was loaded per lane, and the blot was hybridized with the indicated probes. A duplicated gel stained with EtBr was used as a loading control.

was compared among the wild-type and two JA-related mutants, *coi1-1* [14] and *atmyc2-2* [25]. As shown in Figure 1B, the MeJA-induced expression levels of *ANAC019* and *ANAC055* were significantly reduced in *coi1-1* and *atmyc2-2*. These results show that the MeJA-induced activation of *ANAC019* and *ANAC055* expression requires the function of the COI1 and AtMYC2 proteins, two of the known essential components of JA signaling in *Arabidopsis*.

ANAC019 transcription factor binds directly to the VSP1 promoter

Tran *et al*. [33] demonstrated that ANAC019 and ANAC055 act as transcription factors to activate the expression of drought-inducible genes. Furthermore, they determined the complete DNA-binding sequence that is recognized by the two NAC proteins, which include the so-called NAC recognition sequence (NACRS) CATGT and the core-binding site CACG. As shown in Supplementary information, Figure S1, our sequence examination revealed two CATGT motifs and six CACG motifs in the promoter region of *VSP1*, a well-recognized marker gene for JA-induced responses in *Arabidopsis* [18]. To verify the possibility that ANAC019 might bind the promoter of *VSP1*, we tested the binding activity of ANAC019 to a 10-bp DNA segment (CATGTCCACG) at positions –369 to –360 relative to the predicted translation start codon of *VSP1* [38]. The 10-bp *cis*-element CATGTCCACG contains a NACRS and a core-binding site, which are organized as a tandem array spaced by a C (Figure 2A and Supplementary information, Figure S1). Purified His-ANAC019 fusion protein was incu-

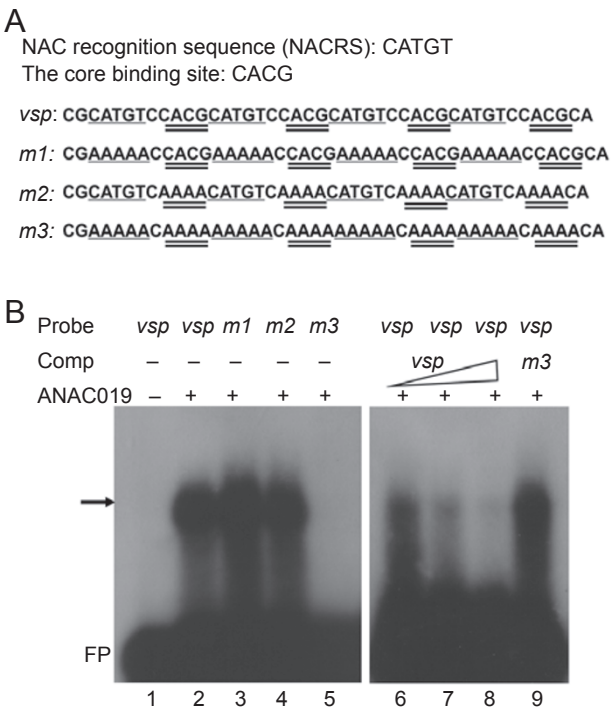

Figure 2 *In vitro* binding of His-ANAC019 to the CATGT and CACG motifs in the promoter of the *Arabidopsis VSP1* gene. **(A)** Oligonucleotides used in the gel mobility shift assay. The *vsp* probe contains four tandem repeats of a *cis*-element containing a NAC recognition site CATGT (underlined) and a core-binding site CACG (double underlined), which present in the promoter region of *VSP1*. To demonstrate the specificity of ANAC019 binding to the *VSP1* promoter, three mutant versions of the *vsp* probe were used: *m1*, CATGT was substituted by AAAAA; *m2*, CACG was substituted by AAAA; *m3*, both CATGT and CACG were mutated. **(B)** A gel mobility shift assay to test the binding of His-ANAC019 to the CATGT and CACG motifs in the promoter of the *VSP1* gene. Protein-DNA complexes were detected when His-ANAC019 fusions were incubated with *vsp* (lane 2), *m1* (lane 3), *m2* (lane 4), but not with *m3* (lane 5). *m3* lost its ability to compete with the binding of His-ANAC019 to *vsp* (lane 9). Binding reactions contained approximately 300 ng of His-ANAC019 fusion protein and 0.05 pmol/μL of labeled probe. No protein was added in lane 1. The triangle indicates the addition of 10× (lane 6), 50× (lane 7) and 100× (lane 8) molar excess amounts of unlabeled *vsp* competitors (Comp.). The arrow indicates the protein-DNA complex. FP, free probe.

bated with a labeled DNA probe containing four tandem repeats of the 10-bp *cis*-element (named as *vsp*, Figure 2A) and incubation mixtures were then analyzed using a gel mobility shift assay. A high-affinity DNA-protein complex was detected along with the free probe (Figure 2B, lane 2), and the DNA-binding activity was reduced by the addition of an excess of unlabeled *vsp* oligonucleotide probe (Figure 2B, lanes 6-8). To define the sequence specificity of the DNA-binding activities, we analyzed the binding activities of the ANAC019 fusion protein to three mutant versions of the *vsp* probe (named as *m1*, *m2* and *m3*, respectively, Figure 2A). When DNA probes with mutations in only CATGT (substituted by AAAAA) or CACG (substituted by AAAA) were tested, no apparent reduction in binding activity was observed (Figure 2B, lanes 3 and 4). By contrast, when both CATGT and CACG were mutated, ANAC019 lost its binding activity (Figure 2B, lane 5). Furthermore, unlabeled mutant probe in which both CATGT and CACG were substituted lost its ability to compete with the wild-type *vsp* probe for interaction with the ANAC019 fusion protein (Figure 2B, lane 9). These results suggest that ANAC019 can interact directly with the promoter of *VSP1*. Given the high sequence similarity of ANAC055 with that of ANAC019, it is reasonable to predict that ANAC055 also can bind directly to the *VSP1* promoter.

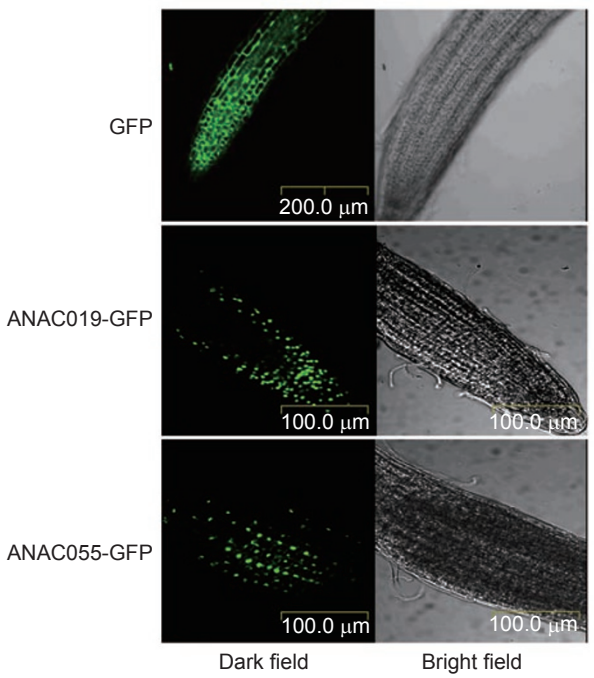

Figure 3 Subcellular localization of the NAC-GFP fusion proteins. Root tips of transgenic plants containing the *35S:GFP* (upper panel), *35S:ANAC019-GFP* (middle panel) and *35S:ANAC055-GFP* (bottom panel) fusion genes were observed and photographed using laser-scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany).

Nuclear localization of ANAC019 and ANAC055

To investigate the subcellular localization of the two NAC proteins, we generated transgenic plants that contained full-length cDNA of *ANAC019* or *ANAC055* fused with the cDNA of the green fluorescent protein (GFP) under the control of the 35S promoter. Homozygous *35S:ANAC019-GFP* and *35S:ANAC055-GFP* plants showed increased MeJA-induced *VSP1* expression compared to *35S:GFP* plants (data not shown), which suggests that the ANAC019-GFP and ANAC055-GFP fusions are functional. Root tips of the transgenic seedlings were examined for localization of GFP fluorescence. Root tips of transgenic plants containing the 35S:GFP vector control were found to display a broad distribution of GFP fluorescence throughout the cells (Figure 3, upper panel). By contrast, the fusion proteins ANAC019-GFP (Figure 3, middle panel) and ANAC055-GFP (Figure 3, bottom panel) were found to target the nuclei. These results suggest that ANAC019 and ANAC055 can be localized to the nucleus.

ANAC019 and ANAC055 C-terminal domains have transactivational activity

Until now it was generally believed that the C-terminal domain of NAC family proteins had transactivational activity [32-36]. Here, we tested for the presence of transactivation domains in ANAC019 and ANAC055 using yeast as an assay system. Yeast assays were conducted using the previously described yeast strain HF7c, which contains the two reporter genes *His3* and *LacZ* [32]. The C-terminal domains (amino acids 148-317) of ANAC019 and ANAC055, respectively, were fused to the GAL4 DNA-binding domain to examine their ability to activate transcription from the GAL4 upstream activation sequence (UAS) and thereby promote yeast growth. The yeast cells containing pBD-ANAC019C, pBD-ANAC055C and control plasmid pBD all grew well on YPDA medium (Figure 4B), while on SD medium without histidine and tryptophan only yeast cells containing pBD-ANAC019C or pBD-ANAC055C could grow (Figure 4C), which indicates that ANAC019C and ANAC055C have transcription activation activity and therefore could promote yeast growth without histidine and trytophan. In the β-gal activity assay, the yeast cells containing pBD-ANAC019C or pBD-ANAC055C also turned blue, indicating the activation of another reporter gene *LacZ* (Figure 4D). These results indicate that the C-terminal domains of ANAC019 and ANAC055 have transactivational activity in yeast.

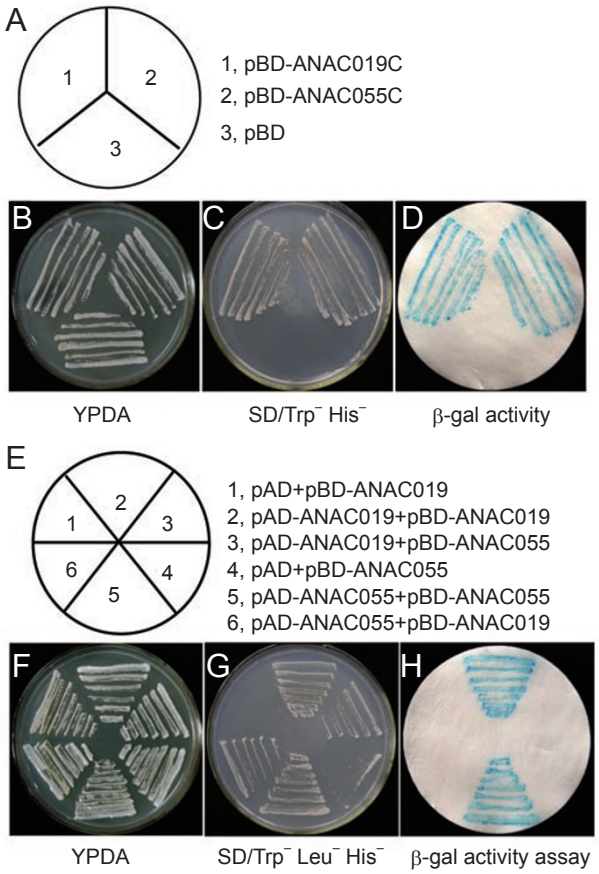

Figure 4 Yeast assays showing the transcriptional activity and dimerization of ANAC019 and ANAC055. **(A-D)** The C-terminal domains of ANAC019 and ANAC055 showed transcription activation activity. Yeast cells were transformed with the indicated plasmids **(A)**. The transformants were streaked on YPDA **(B)** and SD/Trp$^-$His$^-$ medium to examine growth **(C)** and assayed for β-galactosidase activity on replica filters **(D)**. **(E-H)** Dimerization of ANAC019 and ANAC055 analyzed using yeast two-hybrid assays. Yeast cells were co-transformed with the indicated plasmids **(E)**. The transformants were streaked on YPDA **(F)** and SD/Trp$^-$Leu$^-$His$^-$ medium to examine growth **(G)** and assayed for β-galactosidase activity on replica filters **(H)**.

Dimerization of ANAC019 and ANAC055

To test the ability of ANAC019 and ANAC055 to dimerize using a yeast two-hybrid assay, yeast cells were co-transformed with different combinations of constructs (Figure 4E). The results indicate that, whereas all the yeast lines grew well on YPDA medium (Figure 4F), only the yeast lines containing the construct combinations of pAD-ANAC019+pBD-ANAC019 and pAD-ANAC055+pBD-ANAC055 could grow well on SD/His$^-$ medium plus 10 mM amino-1, 2, 4-triazole (3-AT) and turned blue during a β-gal activity assay (Figure 4G-4H). This suggests that ANAC019 or ANAC055 interacts with itself in yeast. However, and on the contrary, yeast lines containing the construct combinations of pAD-ANAC019+pBD-ANAC055 and pAD-ANAC055+pBD-ANAC019 could grow fewer clones on the SD/His$^-$ medium plus 10 mM 3-AT (Figure 4G), and these clones failed to show β-gal activity (Figure 4H). This suggests that ANAC019 and ANAC055 cannot interact in yeast. As a control, yeast lines containing the pAD+pBD-ANAC019 or the pAD+pBD-ANAC055 construct could not grow (Figure 4G-4H). Collectively, the results from the yeast assays suggest that, for ANAC019 and ANAC055, homodimeric complexes are formed preferentially over heterodimeric complexes. This dimerization assay may help to explain why the *anac019 anac055* double mutant shows stronger JA-related phenotypes than the single mutants (see below).

The anac019 anac055 double mutant shows reduced expression of MeJA-induced VSP1 and LOX2

In order to explore the physiological functions of the two *NAC* genes in JA signaling, we identified and analyzed the *Arabidopsis* T-DNA insertion lines *anac019* and *anac055* (see Materials and Methods). The *anac019* allele (SALK_096295) contains a T-DNA insertion in the third exon of *ANAC019* (Figure 5A). The *anac055* allele (SALK_014331) contains a T-DNA insertion in the third exon of *ANAC055* (Figure 5A). However, compared with the wild type, neither *anac019* nor *anac055* showed altered JA-induced responses in either root growth or defense gene expression (data not shown). Therefore, *anac019* and *anac055* were crossed and an F2 population that was segregated according to the two T-DNA insertions was obtained. Through PCR-based genotyping of 266 individuals from this F2 population, we obtained 15 (close to 1/16) *anac019 anac055* double mutant plants. These results demonstrate that each of the T-DNAs had segregated as a single locus, and that both *anac019* and *anac055* are single-locus T-DNA insertion mutants. RNA gel blot analysis indicated that the double mutants simultaneously disrupted *ANAC019* and *ANAC055* expression (Figure 5B).

The MeJA-induced response of the *anac019 anac055* double mutant was first examined using a root growth inhibition assay. As shown in Figure 5C, in the absence or presence of a range of concentrations of MeJA, the root length of the *anac019 anac055* double mutant was comparable with that of the wild type. To further study the effects of the *anac019 anac055* double mutant on JA-regulated gene expression, we analyzed the expression levels of *VSP1* and *LOX2*, which are two marker genes for JA-regulated defense responses in *Arabidopsis*.

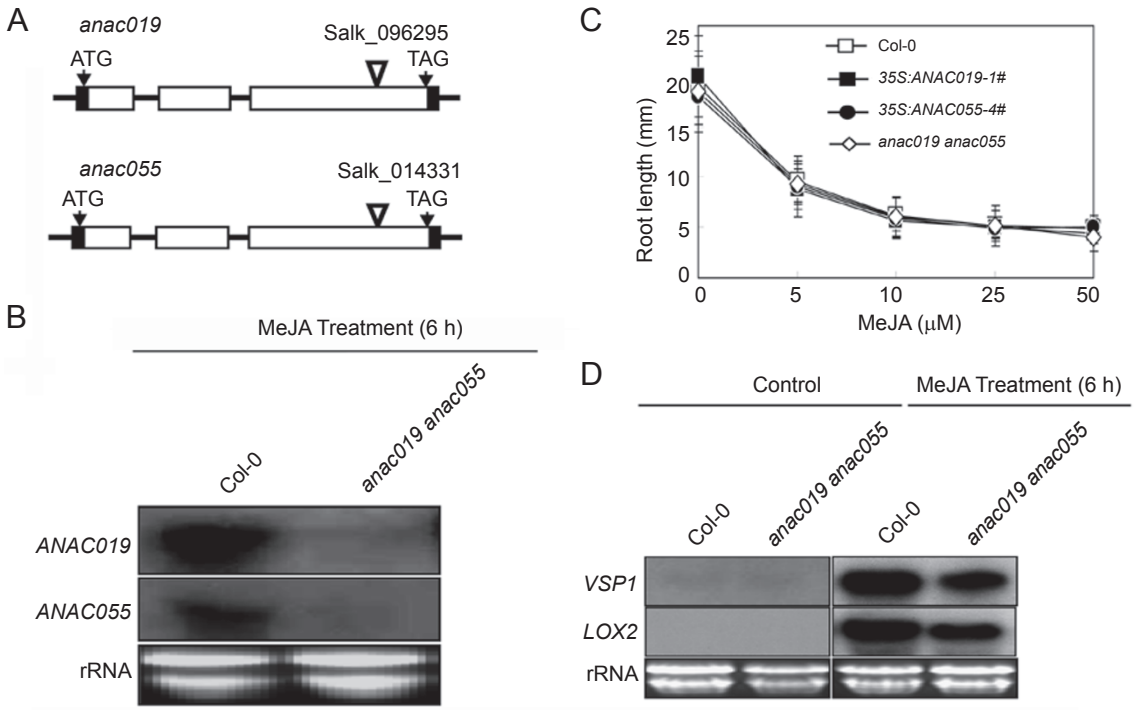

Figure 5 Reduced expression of *VSP1* and *LOX2* in response to MeJA in the *anac019 anac055* double mutant. **(A)** Diagrams of *ANAC019* and *ANAC055* showing the positions of the T-DNA insertions. **(B)** RNA gel blot analysis showing disrupted expression of the *NAC* genes in the *anac019 anac055* double mutant. Two-week-old plants were treated with 50 μM MeJA and tissues were collected at the indicated times for RNA extraction. In all, 30 μg of total RNA was loaded per lane. A duplicated gel stained with EtBr was used as a loading control. **(C)** Comparison of seedling root length of the indicated genotypes in different concentrations of MeJA. Each data point represents the mean ± SD of 20 plants per genotype. Three independent experiments were conducted and similar results were obtained. **(D)** Comparison of MeJA-induced expression of *VSP1* and *LOX2* in the *anac019 anac055* double mutant and the wild type. Two-week-old seedlings were treated with 50 μM MeJA and tissues were collected at the indicated times for RNA extraction. In all, 10 μg of total RNA was loaded per lane. A duplicated gel stained with EtBr was used as a loading control.

RNA gel blot analyses indicated that, in the absence of MeJA induction, the transcripts of the two genes were barely detectable (Figure 5D). MeJA treatment induced the expression of the two genes in both the wild-type and the *anac019 anac055* double mutant, and the transcript accumulation levels of *VSP1* and *LOX2* were found to be lower in the double mutant than in the wild type (Figure 5D). These results indicate that the disruption of *ANAC019* and *ANAC055* expression leads to the attenuation of MeJA-induced *VSP1* and *LOX2* expression and suggest that there is a significant functional redundancy between ANAC019 and ANAC055.

Overexpression of ANAC019 and ANAC055 enhances MeJA-induced expression of VSP1 and LOX2

The effects of the *NAC* genes on JA signaling were also examined in transgenic *Arabidopsis* plants that overexpressed the full-length cDNAs of *ANAC019* or *ANAC055* under the control of the 35S promoter (see Materials and Methods). Homozygous transgenic lines were examined by RNA gel blot analysis for elevated expression of the *ANAC019* (Figure 6A) or *ANAC055* (Figure 6C) transcripts. Representative lines named *35S:ANAC019-1#* and *35S:ANAC055-4#*, respectively, were selected to compare their responses to MeJA with those of the wild type.

In the absence or presence of a range of concentrations of MeJA, *35S:ANAC019-1#* and *35S:ANAC055-4#* seedlings showed a root phenotype that was indistinguishable from that of wild-type plants (Figure 5C). The two transgenic lines were then compared with the wild type for MeJA-induced defense gene expression. As shown in Figure 6B and 6D, RNA gel blot analysis indicates that the overexpression of *ANAC019* or *ANAC055* does not lead to constitutive expression of *VSP1* and *LOX2*. However, the MeJA-induced expression levels of these transcripts were found to be significantly higher in the transgenic lines than in the wild-type plants. Together, these

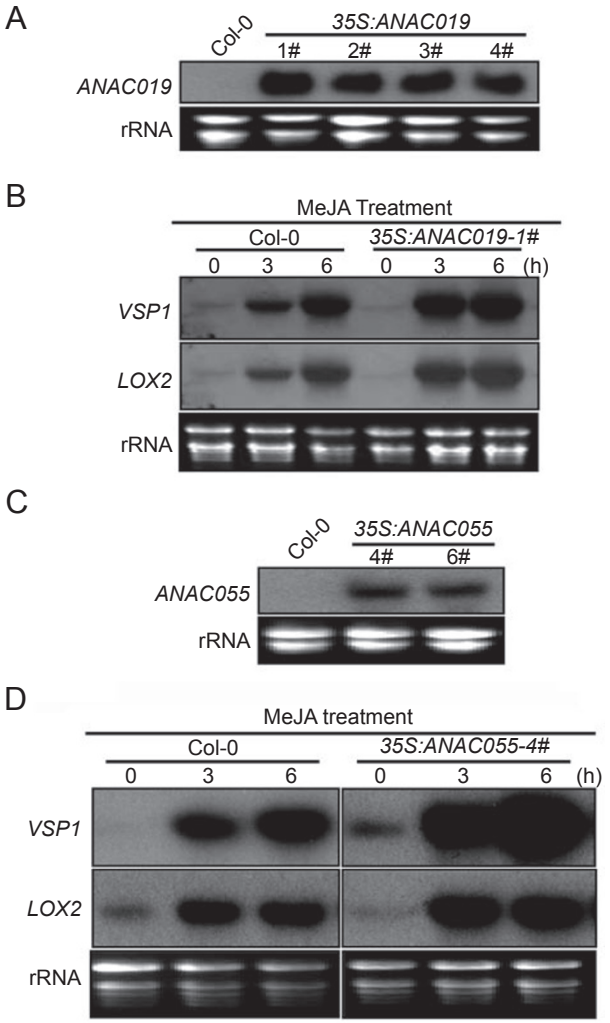

Figure 6 Increased expression of *VSP1* and *LOX2* in response to MeJA, due to overexpression of the two *NAC* genes. **(A)** RNA gel blot analysis showing enhanced expression of *ANAC019* in different *ANAC019*-overexpressing lines. Tissues of 2-week-old seedlings (untreated) were used for RNA extraction and 10 μg of RNA was loaded per lane. **(B)** Comparison of MeJA-induced *VSP1* and *LOX2* expression between *35S:ANAC019-1#* and wild-type plants. **(C)** RNA gel blot analysis showing enhanced expression of *ANAC055* in different *ANAC055*-overexpressing lines. Tissues of 2-week-old seedlings (untreated) were used for RNA extraction and 10 μg of RNA was loaded per lane. **(D)** Comparison of MeJA-induced *VSP1* and *LOX2* expression between *35S:ANAC055-4#* and wild-type plants. For **(B** and **D)**, 2-week-old plants were treated with 50 μM MeJA and tissues were collected at the indicated times for RNA extraction. In all, 10 μg of total RNA was loaded per lane. Duplicated gels stained with EtBr were used as loading controls.

results demonstrate that the overexpression of *ANAC019* or *ANAC055* enhances the responses of plants to MeJA in defense gene expression.

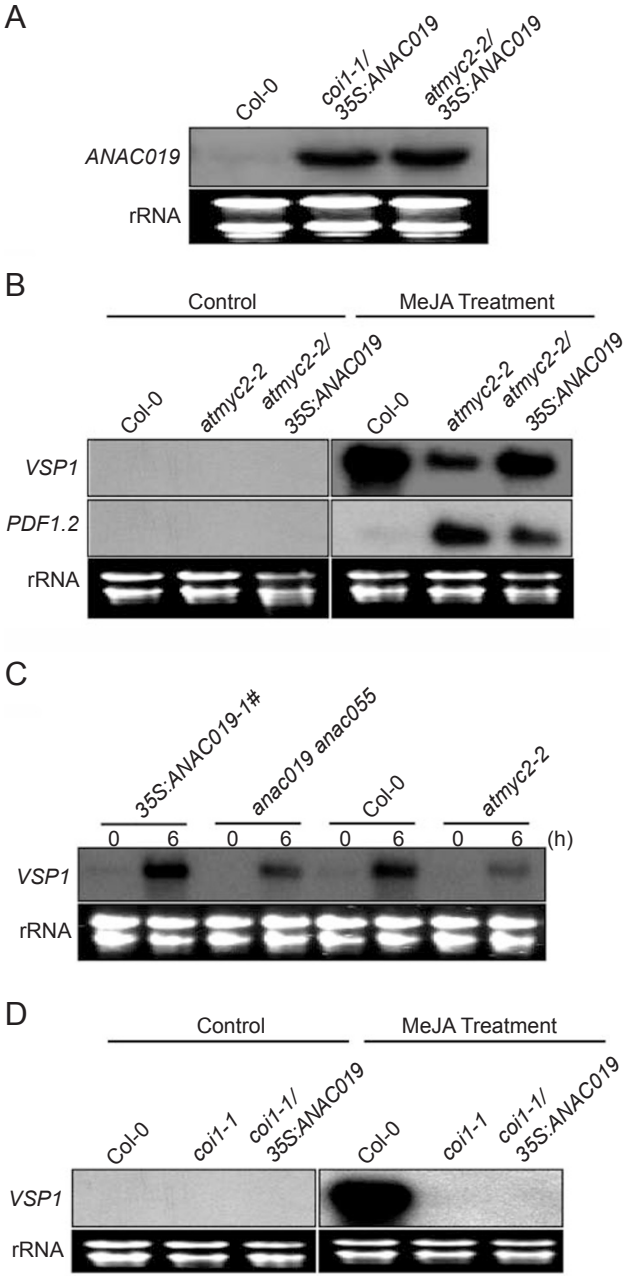

Figure 7 Overexpression of *ANAC019* in the *coi1-1* and *atmyc2-2* mutant backgrounds. **(A)** RNA gel blot analysis of *ANAC019* expression in the various backgrounds as indicated. In all, 10 μg of total RNA from 2-week-old untreated plants was loaded per lane. A duplicated gel stained with EtBr was used as a loading control. **(B)** RNA gel blot analysis of MeJA-induced *VSP1* and *PDF1.2* expression in different genotypes. **(C)** RNA gel blot analysis of MeJA-induced *VSP1* expression in the indicated genotypes. **(D)** RNA gel blot analysis of MeJA-induced *VSP1* expression in wild-type, *coi1-1* and *coi1-1/35S:ANAC019* transgenic plants. For **B** to **D**, 2-week-old plants were treated with 50 μM MeJA for 6 h and tissues were collected for RNA extraction. In all, 10 μg of total RNA was loaded per lane. A duplicated gel stained with EtBr was used as a loading control.

Overexpression of ANAC019 partially rescues alterations of MeJA-induced defense gene expression in the atmyc2-2 mutant

To study the action sites of the two *NAC* genes in the JA signaling, *ANAC019* was overexpressed in the genetic background of the JA-insensitive mutant *atmyc2-2* (Figure 7A). It has previously been shown that AtMYC2 differentially regulates two branches of JA-induced defensive genes and, therefore, the *atmyc2-2* mutant exhibits decreased expression of JA-induced *VSP1* versus elevated expression of JA-induced *PDF1.2* [19, 25]. In agreement with this, our results indicate that the overexpression of *ANAC019* in the *atmyc2-2* background does not lead to the constant expression of *VSP1* or *PDF1.2* in the absence of MeJA; it merely rescues, at least partially, the alterations of MeJA-induced *VSP1* and *PDF1.2* expression of the *atmyc2-2* mutant (Figure 7B). These results suggest that constitutive expression of *ANAC019* bypasses *AtMYC2* for JA-induced defense gene expression and support the idea that ANAC019 probably acts downstream of AtMYC2 in the JA signaling. Further evidence to support this idea came from the finding that the reduction of MeJA-induced *VSP1* expression in *anac019 anac055* was not as severe as in *atmyc2-2* (Figure 7C).

Given that elegant studies have demonstrated that AtMYC2 functions downstream of COI1 to regulate JA-induced defense gene expression [19, 25], it is reasonable to predict that the action site of ANAC019 localizes downstream of COI1 and AtMYC2. To test this, we crossed the above-described *35S:ANAC019-1#* with *coi1-1* and identified plants that had overexpression of *ANAC019* in the genetic background of *coi1-1* (Figure 7A). However, overexpression of *ANAC019* failed to rescue the MeJA-induced *VSP1* expression of the *coi1-1* mutant (Figure 7D), suggesting that the function of COI1 is still required for MeJA-induced *VSP1* expression even in transgenic plants that constitutively express *ANAC019*. This result is consistent with the fact that COI1 is required for the sensitivity of plants to JA. Supporting evidence to this idea came from the recent finding that the SCF^{COI1}-JAZ complex might be the site of JA-Ile perception [22, 23].

Knockout or overexpression of the two NAC genes affects plant responses to pathogen attack

Given the established roles of JA in regulating plant defense responses against pathogens, especially necrotrophic pathogens [3, 39], we were interested in finding out whether the two *NAC* genes are involved in these processes. Indeed, our RNA gel blot analysis indicated that the expression of *ANAC019* and *ANAC055* was induced by pathogen inoculation (Figure 8A). We then examined the performance of *anac019 anac055, 35S:ANAC019-1#* and *35S:ANAC055-4#* against a necrotrophic fungus, *Botrytis cinerea*. As a control, wild-type plants and the *atmyc2-2* mutant were also included in these experiments. Four-week-old plants were challenged with *B. cinerea* and symptoms of infection were scored. In agreement with previous studies, *atmyc2-2* showed an increased resistance to this pathogen. Like *atmyc2-2*, the *anac019 anac055* double mutant also showed increased resistance to *B. cinerea* compared with the wild type, as indicated by fewer symptoms of infection in the infected plants (Figure 8B). By contrast, transgenic lines overexpressing *ANAC019* or *ANAC055* showed decreased resistance to this pathogen (Figure 8B).

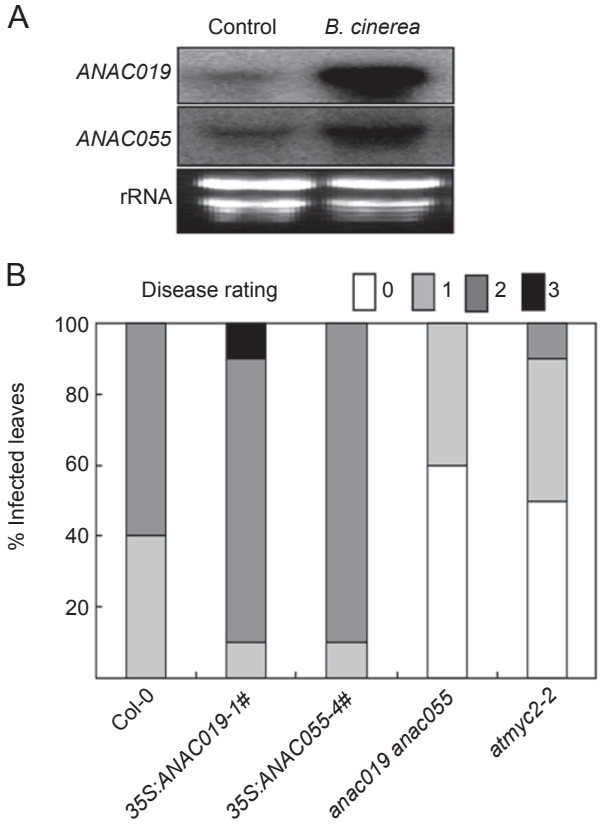

Figure 8 Knockout or overexpression of *ANAC019* and *ANAC055* affects plant responses to *B. cinerea*. **(A)** *B. cinerea* infection induced the expression of *ANAC019* and *ANAC055* in wild-type plants. Wild-type plants were inoculated with 5×10^5 spores ml^{-1} of *B. cinerea*. Leaves were collected for RNA extraction 2 days after inoculation. In all, 30 μg of total RNA was loaded per lane. **(B)** Graphical representation of disease symptoms 5 days after inoculation of the leaves infected with 5×10^5 spores ml^{-1} of *B. cinerea*. The results shown are representative of three independent experiments.

Discussion

Our data indicate that ANAC019 and ANAC055 have an important role in regulating JA-signaled defense responses. First, the expression of the two *NAC* genes was induced by MeJA in a COI1- and AtMYC2-dependent manner (Figure 1). Second, an *in vitro* protein-DNA-binding assay showed that ANAC019 interacts with a *cis*-element (CATGTCCACG) in the promoter region of *VSP1* (Figure 2). Third, the *anac019 anac055* double mutant plants showed attenuated MeJA-induced *VSP1* and *LOX2* expression (Figure 5D), whereas transgenic plants overexpressing *ANAC019* or *ANAC055* showed enhanced MeJA-induced *VSP1* and *LOX2* expression (Figure 6B-6D). Fourth, overexpression of *ANAC019* partially rescued the JA-related phenotype of *atmyc2-2* (Figure 7B), which disrupted the function of the bHLH transcription factor AtMYC2, a recently identified player involved in JA-signaling in *Arabidopsis* [19, 25]. Fifth, the susceptibility of the *anac019 anac055* double mutant to a necrotrophic fungus showed high similarity to that of the *atmyc2-2* mutant (Figure 8). Together, these data led us to a hypothesis that the two NAC proteins might act downstream of AtMYC2 to regulate JA-mediated defense responses.

An interesting aspect of the role of the two NAC transcription factors in JA-signaling concerns the phenotypes of the *anac019 anac055* double mutant, which showed decreased expression of MeJA-induced *VSP1* and *LOX2* (Figure 5D), but increased resistance to the necrotrophic pathogen *B. cinerea* (Figure 8). This observation is consistent with those from mutants with disrupted AtMYC2 function. Previous studies have showed that, in response to JA treatment, AtMYC2 promotes the expression of a group of wound response-related genes, whereas it represses the expression of a group of pathogen defense-related genes. Consistently, knockout mutations in *AtMYC2* have shown a reduced expression of *VSP1* and *LOX2* versus an increased expression of pathogen defensive *PDF1.2* and *PR-1* and, as a consequence, have exhibited a significant increase in their resistance to the necrotrophic pathogen *B. cinerea* [19, 25]. Similarity of mutant phenotype, together with our finding that overexpression of *ANAC019* (in the genetic background of *atmyc2-2*) rescues the alterations caused by MeJA-induced *VSP1* and *PDF1.2* expression in the *atmyc2-2* mutant (Figure 7B), provides supporting evidence that ANAC019 and ANAC055 act downstream of AtMYC2 in the JA signaling.

Further evidence to support the idea that ANAC019 acts downstream of AtMYC2 came from a comparison of MeJA-induced expression levels of *VSP1* among *35S:ANAC019-1#*, *anac019 anac055* and *atmyc2-2*. As shown in Figure 7C, in contrast to *35S:ANAC019-1#*, which showed higher expression levels of *VSP1* compared with the wild type, both *anac019 anac055* and *atmyc2-2* showed reduced expression levels of *VSP1* compared with the wild type. However, the reduction of MeJA-induced *VSP1* expression in *anac019 anac055* was not as severe as that in *atmyc2-2*.

In contrast to the reported alleles of the *atmyc2* mutant, which are less sensitive than the wild type to the inhibition effect of JA on root growth [19, 25], our root-growth assay indicates that the *anac019 anac055* double mutant does not show an apparent root phenotype in the presence of MeJA (Figure 5C). This may be explained by the existence of other ANAC-related proteins; for example, ANAC072 (At4g27410) shows high sequence similarity to ANAC019 and ANAC055. Further possibilities rely on the existence of unknown components (branches), which may function in parallel with the ANAC proteins to regulate JA-mediated root growth.

Materials and Methods

Plant materials and growth conditions

All *A. thaliana* lines used were on the Columbia (Col-0) background. The *anac019* (SALK_096295) and *anac055* (SALK_014331) mutants were obtained from the Arabidopsis Biological Resources Center. The JA response mutants *coi1-1* [14] and *atmyc2-2* have previously been described [25].

Arabidopsis seeds were surface-sterilized with 30% bleach and 0.001% Triton X-100 for 10 min and washed three times with sterile water. Sterilized seeds were then suspended in 0.2% agarose and plated on Murashige and Skoog medium. Plants were vernalized in darkness for 3 d at 4 °C and then transferred to a phytotrone set at 22 °C with a 16-h light/8-h dark cycle. After 2-3 weeks, seedlings were also potted in soil and placed in a growth room at 22 °C with a 16-h light/8-h dark cycle.

Identification of T-DNA insertion lines and generation of the anac019 anac055 double mutant

Putative knockout mutant lines of *anac019* (SALK_096295) and *anac055* (SALK_014331) were identified from the SALK T-DNA insertion library database (http://signal.salk.edu/cgi-bin/tdnaexpress). Seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) (Ohio State University), and seedlings were analyzed individually using PCR amplification to confirm the presence of T-DNA using the LBa1 primer (located in the T-DNA) and gene-specific primers (Supplementary information, Table S1). A SALK_096295 plant that was homozygous for the T-DNA insertion was further backcrossed to the wild type and the resulting F2 progeny were germinated on kanamycin-containing medium. An F2 population consisting of 251 individuals was scored, and the segregation ratio of kanamycin-resistant versus kanamycin-sensitive seedlings was found to be 192:59 (which is close to 3:1). These results suggest that a single T-DNA insertion was present in the homozygous SALK_096295 plant. Kanamycin-resistant

F2 seedlings were subjected to another round of PCR analysis to identify plants that were homozygous for the T-DNA insertion. F3 progeny of the identified SALK_096295 plants that were homozygous for the T-DNA insertion were then examined by RNA gel blot for a lack of *ANAC019* expression in the presence or absence of MeJA. Based on these analyses, we identified a homozygous T-DNA insertion line with disrupted expression of *ANAC019* (designated as *anac019*). Using a similar procedure, we also identified a homozygous T-DNA insertion line with disrupted expression of *ANAC055* (designated as *anac055*).

anac019 and *anac055* were crossed and an *anac019 anac055* double mutant line was identified from the resulting F2 population by PCR analysis. Linkage analysis indicated that the JA-related phenotype of the double mutant was co-segregated with the double T-DNA insertions (data not shown).

Transgenic manipulation of ANAC019 or ANAC055 expression in wild-type (Col-0), atmyc2-2 and coi1-1 backgrounds

The coding sequences of *ANAC019* and *ANAC055* were amplified by RT-PCR and cloned into the *Bam*HI and *Sac*I sites of the binary vector pBI121 under the control of the 35S promoter of *cauliflower mosaic virus*. The primers that were used are listed in Supplementary information, Table S1. The resulting *35S:ANAC019* and *35S:ANAC055* constructs were introduced into wild-type *Arabidopsis* plants using *Agrobacterium tumefaciens*-mediated transformation [40]. T2 seeds from each of the selected transgenic plants were plated on germination medium containing kanamycin as selection antibiotics, and the homozygous lines were selected. Homozygous T3 progeny were then examined for the expression levels of the target genes by RNA gel blot analysis. In total, 12 homozygous lines showing elevated expression of *ANAC019* and 7 lines showing elevated expression of *ANAC055* were identified. Representative lines overexpressing *ANAC019* (Figure 6A) or *ANAC055* (Figure 6C) were used for further analysis. Results from *ANAC019-1#* and *ANAC055-4#* are shown in this report.

To get *ANAC019*-overexpressing plants in the *atmyc2-2* background, the above-mentioned *ANAC019* coding sequence was cloned into the *Bam*HI and *Sac*I sites of the pCAMBIA1300-221-HA vector and the resulting plasmid was introduced into *atmyc2-2* plants. Selection of positive transformants was conducted on germination medium containing hygromycin as an antibiotic selection marker. T3 progeny of transgenic lines that were homozygous for the *ANAC019* transgene were subjected to RNA gel blot analysis for elevated levels of *ANAC019* expression (Figure 7A) and PCR analysis for the presence of the *atmyc2-2* allele [25].

To get *ANAC019*-overexpressing plants in the *coi1-1* background, *35S:ANAC019-1#*, which showed increased expression of *ANAC019* (Figure 6A), was crossed to the *coi1-1* mutant and the resulting F1 plants were selfed. Kanamycin-resistant F2 seedlings were analyzed individually with a cleaved amplified polymorphic sequence (CAPS) marker [14] to confirm the presence of the *coi1-1* mutation. The identified plants were further examined using northern blot techniques to verify the overexpression of *ANAC019* (Figure 7A).

Physiological assays

To carry out a root growth inhibition assay, *Arabidopsis* seeds were germinated on MS medium containing specific concentrations of MeJA (Sigma, St Louis, MO) and the root length was scored after 7 days of growth on vertical plates. To carry out a JA-induced defense gene expression assay, 2-week-old seedlings that were grown on MS medium were sprayed evenly with solutions containing specific concentrations of MeJA and then incubated in a growth chamber under continuous light. Tissues were harvested at specific time intervals for RNA extraction.

Plant infection with pathogens

Four-week-old *Arabidopsis* plants were used for pathogen inoculation based on published methods [25] with minor modifications. For inoculation with *B. cinerea*, fungal progression and infection symptoms were monitored for 10 days, and infection ratings from 0 to 3 were assigned to the inoculated plants (0, no infection/necrosis; 1, leaves showing some necrosis; 2, leaves showing severe necrosis; 3, dead/decayed leaves), based on a previously described method [25]. In each experiment, at least 15 plants per genotype were inoculated and 4 leaves from each plant were scored for symptom development. Experiments were repeated at least three times with similar results.

RNA gel blot analysis

Total RNA extraction and northern blot analysis were conducted according to a published method [37]. RNA gel blots were probed with PCR-amplified DNA fragments using gene-specific primers (Supplementary information, Table S1).

Subcellular localization of ANAC019 and ANAC055

The cDNA of *ANAC019* was amplified by PCR, digested by *Sal*I and *Spe*I, and fused in frame with *GFP* to a pBA-GFP vector, which contains a 35S promoter. The PCR-amplified cDNA of *ANAC055* was digested with *Spe*I and fused in frame with *GFP* to the pBA-GFP vector. After sequencing confirmation, the *35S:ANAC019-GFP* and *35S:ANAC055-GFP* constructs, as well as the *35S:GFP* vector control, were introduced into wild-type plants as described above and homozygous T3 transgenic plants were obtained based on antibiotic selection. The root tips of 7-day-old transgenic seedlings were visualized with a laser scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany).

Yeast assays

Yeast assays were conducted using the previously described yeast strain HF7c, which contains the two reporter genes *His3* and *LacZ* [32]. To test the dimerization of the two NAC proteins, the PCR-derived full-length coding sequences of *ANAC019* and *ANAC055* were cloned into the pGBKT7 vector containing the GAL4 DNA-binding domain (pBD), and the pGADT7 vector containing the GAL4 activation domain (pAD). The resulting plasmids were used in a yeast two-hybrid assay [32]. To test the transcription activation activity of ANAC019 and ANAC055, their C-terminal fragments (amino acids 148-317), named ANAC019C and ANAC055C, respectively, were cloned into the pGBKT7 vector (pBD) and transformed into yeast. The transcription activation activity of each protein was evaluated based on a published protocol [32]. The primers that were used are shown in Supplementary information, Table S1.

Production of His-ANAC019 fusion proteins and in vitro DNA-binding assay

The pET28a vector (Novagen) was used to construct a plasmid

expressing His-tagged fusions of ANAC019. The primers that were used are shown in Supplementary information, Table S1. Production and purification of the His-tag fusion protein was performed according to the manufacturer's instructions. The wild-type and mutant oligonucleotide *vsp* probes used (see legend of Figure 2) were designed according to the 10-bp element 'CATGTCCACG', which presents in the promoter region of *VSP1*. Oligonucleotide probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Pharmacia). The DNA-binding assays were performed in the absence or presence of a non-radiolabeled competitor at room temperature in a final volume of 20 μL with a binding buffer of 15 mM Hepes, pH 7.5, 35 mM KCl, 1 mM EDTA, 6% glycerol, 1 mM DTT, 1 mM $MgCl_2$, and 2 μg of poly(dI-dC). The samples were incubated at room temperature for 30 min and then separated on 2.0% agarose gel. After drying, the gels were autoradiographed.

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(**Supplementary information** is linked to the online version of the paper on the Cell Research website.)