

# Coronatine Promotes *Pseudomonas syringae* Virulence in Plants by Activating a Signaling Cascade that Inhibits Salicylic Acid Accumulation

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DOI 10.1016/j.chom.2012.04.014

## SUMMARY

Phytopathogens can manipulate plant hormone signaling to access nutrients and counteract defense responses. *Pseudomonas syringae* produces coronatine, a toxin that mimics the plant hormone jasmonic acid isoleucine and promotes opening of stomata for bacterial entry, bacterial growth in the apoplast, systemic susceptibility, and disease symptoms. We examined the mechanisms underlying coronatine-mediated virulence and show that coronatine activates three homologous NAC transcription factor (TF) genes, *ANAC019*, *ANAC055*, and *ANAC072*, through direct activity of the TF, MYC2. Genetic characterization of NAC TF mutants demonstrates that these TFs mediate coronatine-induced stomatal reopening and bacterial propagation in both local and systemic tissues by inhibiting the accumulation of the key plant immune signal salicylic acid (SA). These NAC TFs exert this inhibitory effect by repressing *ICS1* and activating *BSMT1*, genes involved in SA biosynthesis and metabolism, respectively. Thus, a signaling cascade by which coronatine confers its multiple virulence activities has been elucidated.

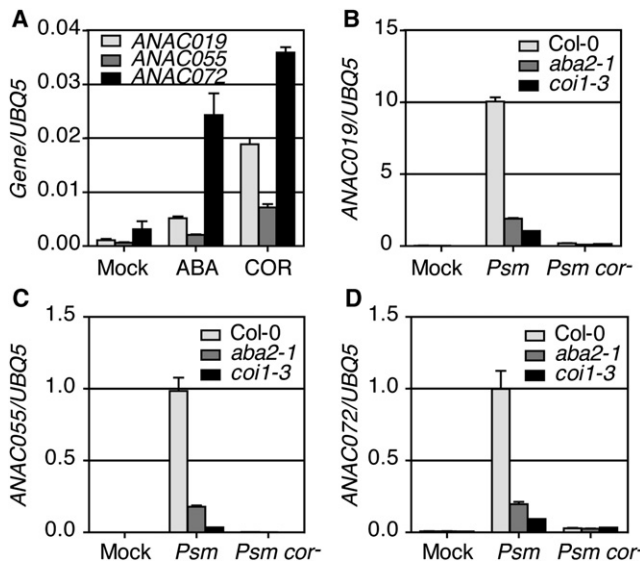
## INTRODUCTION

In the constant battle for survival, pathogens evolved diverse mechanisms to facilitate their virulence in the hosts. Many phytopathogens are able to manipulate plant hormone signaling pathways to gain access to nutrients or to counteract plant defense responses. The bacterial pathogen *Agrobacterium tumefaciens*, through T-DNA-mediated production of the plant hormones auxin and cytokinin, is known to induce the formation of crown galls, where the plant primary metabolites are used to produce

opines serving as carbon and nitrogen sources for the pathogen (Escobar and Dandekar, 2003). Another well-known example of hormone manipulation involves the phytotoxin coronatine (COR), which is produced by various strains of *Pseudomonas syringae* (Mittal and Davis, 1995). COR enhances bacterial growth and disease symptom development and promotes systemic susceptibility (Brooks et al., 2005; Cui et al., 2005; Mittal and Davis, 1995). Structurally similar to the plant hormone jasmonic acid isoleucine (JA-Ile), it has been used as a JA analog in making many essential discoveries in the JA signaling pathway (Browse, 2009).

The *Arabidopsis* JA perception mutant *coronatine insensitive 1* (*coi1*) was initially identified in a screen for insensitivity to COR (Feys et al., 1994). The *coi1* mutant is defective in many JA-induced responses such as root elongation inhibition and wound response. *COI1* encodes an F box protein of the Skp/Cullin/F box complex (SCF<sup>COI1</sup>), which is involved in ubiquitin-mediated degradation of substrates, the JAZ (jasmonate ZIM domain) proteins (Chini et al., 2007; Thines et al., 2007; Xie et al., 1998). The COI1-JAZ receptor-substrate complex formation is facilitated by JA-Ile or COR (Katsir et al., 2008; Yan et al., 2009). Since JAZ proteins are normally associated with the JA signaling transcription factors (TFs) such as MYC2, degradation of JAZ proteins leads to the release of these TFs and transcription activation of JA/COR-responsive genes (Katsir et al., 2008; Melotto et al., 2008).

For defense against *Pseudomonas syringae*, the plant immune signal salicylic acid (SA) plays an essential role. However, some plant hormones, such as JA, can counteract the SA-mediated defense to fine-tune the immune response through signaling crosstalk (Grant and Jones, 2009; Kunkel and Brooks, 2002; Spoel and Dong, 2008). In the COR-insensitive *coi1* mutant, SA levels are elevated, and resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326) is enhanced (Kloek et al., 2001). The JA signaling mutant, *myc2/jin1*, which is less sensitive to COR, also exhibits a similar phenotype (Laurie-Berry et al., 2006; Nickstadt et al., 2004). Since COR can activate the JA signaling pathway, it is hypothesized to enhance bacterial



**Figure 1. *Psm* ES4326 Induces the Expression of *ANAC019*, *ANAC055*, and *ANAC072* through the ABA- and JA-Signaling Pathways as well as through COR**

(A) *ANAC019*, *ANAC055*, and *ANAC072* transcripts were measured in wild-type (Col-0) treated with either ABA, COR, or mock.

(B–D) Col-0 plants, the ABA-deficient mutant *aba2-1*, and the JA-signaling mutant *coi1-3* were pressure infiltrated with the mock solution (Mock), *Psm* ES4326 (*Psm*), or *Psm* ES4326 *cor*<sup>-</sup> (*Psm cor*<sup>-</sup>), and samples were collected 24 hr later. Error bars represent the standard deviation of three technical replicates. Each experiment was repeated at least three times with similar results. See also Figure S1.

virulence by suppressing SA-mediated defense through cross-talk. Supporting this hypothesis, virulence can be restored to the COR-deficient *Pst* DC3000 mutant (*Pst* DC3000 *cor*<sup>-</sup>) when inoculated into the *Arabidopsis* mutant plants defective in SA production (Brooks et al., 2005). However, the downstream signal transduction pathway by which COR suppresses SA-mediated immunity remains largely unknown. Block et al. showed that wild-type *Arabidopsis* plants did not accumulate more SA in response to *Pst* DC3000 *cor*<sup>-</sup> compared to *Pst* DC3000 (Block et al., 2005), whereas in other studies COR was found to suppress SA accumulation (de Torres Zabala et al., 2009; Uppalapati et al., 2007). Moreover, COR targets more than one cellular function. Melotto et al. discovered that COR was essential for the reopening of the stomata, which were closed upon *Pst* DC3000 infection, to facilitate bacterial entry into the apoplast (Melotto et al., 2006). However, even when infiltrated directly into the apoplast, the *Pst* DC3000 *cor*<sup>-</sup> mutant still exhibited a reduced growth rate compared to the toxin-producing strain, revealing a role of COR in inhibiting plant defense in the apoplast (Brooks et al., 2004; Zeng and He, 2010). Besides affecting bacterial growth, COR contributes to the chlorotic (yellow) disease symptom development that is independent of bacterial growth (Mittal and Davis, 1995). While *Pst* DC3000 *cor*<sup>-</sup> grew to a similar level as *Pst* DC3000 in the SA-deficient mutant, the former induced a less chlorotic symptom than the latter (Brooks et al., 2005). Moreover, COR not only functions locally in the infected tissue but also en-

hances bacterial virulence systemically (Cui et al., 2005). Despite these studies, it is still largely unknown what downstream host signaling components are targeted by the COR-COI1-JAZ-MYC2 module and whether there is a common signaling pathway leading to these various COR effects.

Here we report the identification of a signaling cascade by which COR suppresses SA accumulation. We discovered three homologous NAC (petunia *NAM* and *Arabidopsis* *ATAF1*, *ATAF2*, and *CUC2*) TFs, the expression of which is induced by COR directly through MYC2. These three NAC TFs function not only in COR-mediated stomatal reopening but also in promoting bacterial growth in the apoplast and promoting systemic susceptibility. We further demonstrate that these TFs suppress SA accumulation by regulating genes involved in SA synthesis and metabolism. We propose that the same signaling cascade is responsible for the virulence effects of COR in stomata, apoplast, and systemic tissue.

## RESULTS

### ***Pseudomonas syringae* Induces the Expression of Three NAC TF Genes through the JA and ABA Signaling Pathways and through COR Production**

To identify the signaling cascade responsible for *Pseudomonas* virulence promoted by the phytotoxin COR, we first searched for TF genes that are inducible by both bacterial infection and treatment of the plant hormone JA, as COR is a known mimic of JA-Ile. We found three homologous NAC family TF genes, *ANAC019* (At1G52890), *ANAC055* (At3G15500), and *ANAC072* (At4G27410), as plausible candidates, since they were induced by *Pst* DC3000 infection as well as by methyl-JA treatment (de Torres-Zabala et al., 2007; Ooka et al., 2003; Tran et al., 2004). These NAC genes were also induced in our “*Pseudomonas* half leaf injection” microarray experiment (see Figure S1A available online; NASCARRAYS-168), in which the virulent *Psm* ES4326 bacteria was infiltrated into one half of the *Arabidopsis* leaf and gene expression was examined in the uninfected half. Since COR can trigger systemic susceptibility in plants (Cui et al., 2005), the induction of NAC TFs in the uninfected tissue suggests that they are possible host targets for this COR function. Interestingly, treatment with another plant hormone, abscisic acid (ABA), can also increase the expression of these three NAC TF genes (Tran et al., 2004). Biosynthesis of ABA and ABA-mediated gene expression are rapidly induced by *Pst* DC3000 through effector proteins delivered into the host cells by the type III secretion system (de Torres-Zabala et al., 2007), suggesting that the three NAC TF genes could be targets of not only COR but also type III effectors.

The microarray data were further supported by qRT-PCR analysis. As shown in Figure 1, COR and ABA treatment as well as *Psm* ES4326 infection all increased the expression of these NAC genes. The *Psm* ES4326-induced expression was diminished in the JA receptor mutant *coi1-3* and the ABA biosynthesis mutant *aba2-1* (Figures 1B–1D), indicating that *Psm* ES4326 induces the three NAC genes through the JA and ABA signaling pathways. In wild-type plants, the COR-deficient mutant *Psm* ES4326 *cor*<sup>-</sup> (Cui et al., 2005) caused little increase in transcription of the NAC genes, suggesting that *Psm* ES4326 induces these genes mainly through COR.

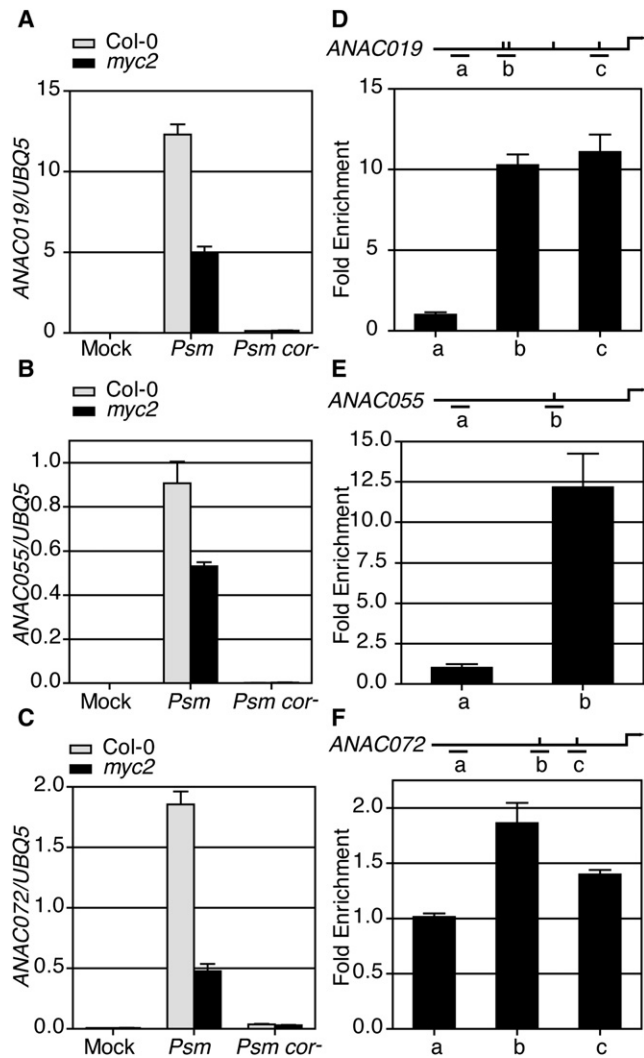
### Pathogen-Produced COR Upregulates the Expression of *ANAC019*, *ANAC055*, and *ANAC072* Directly through *MYC2*

To study the mechanism by which *Psm* ES4326-generated COR induces *ANAC019*, *ANAC055*, and *ANAC072*, we analyzed the time course expression patterns of these three genes upon *Psm* ES4326 infection and found them to be highly correlated using linear regression (Figures S1B and S1C). This suggests a common regulatory mechanism that controls the three NAC genes. The promoters of these three NAC genes were then searched for conserved *cis*-elements. Based on the Athena online analysis tool (O'Connor et al., 2005), the CACGTG G box element was enriched in these three promoters ( $p$  value = 0.0061). Since this G box element is the preferential binding site of the TF *MYC2* (Dombrecht et al., 2007), the three NACs might be regulated by *MYC2*. This is consistent with the observation that induction of *ANAC019* and *ANAC055* by both MeJA and ABA was dependent on *MYC2* (Bu et al., 2008). To test whether induction of *ANAC019*, *ANAC055*, and *ANAC072* by *Psm* ES4326 is also *MYC2* dependent, we performed the infection experiment in the *myc2* mutant and found significant reduction in expression of all three genes (Figures 2A–2C). These results suggest that *MYC2*, a known TF released after COR-triggered degradation of JAZ, is involved in *Psm* ES4326-induced *ANAC019*, *ANAC055*, and *ANAC072* expression. However, this induction was not completely abolished in the *myc2* mutant, probably due to the intact functions of *MYC3* and *MYC4*, which are also JAZ-bound TFs that are activated in response to JA (Fernandez-Calvo et al., 2011).

We next tested whether *MYC2* TF directly control transcription of the three NAC genes using chromatin immunoprecipitation (ChIP) experiments performed in a transgenic *Arabidopsis* line constitutively expressing the *MYC2*-GFP fusion protein (Figure S2). For the promoter of each NAC gene, several DNA regions were analyzed to cover all, but one (1,234 bp upstream of the transcription start site of *ANAC019*), potential *MYC2*-binding sites (Table S1). In addition, promoter regions without the binding sites were used as negative controls. As shown in Figures 2D and 2E, all DNA segments containing the potential *MYC2*-binding sites were enriched in the *ANAC019* and *ANAC055* promoters. For the *ANAC072* promoter, enrichment was found in one of the two potential binding sites (Figure 2F). These results show that *MYC2* activates expression of the three NAC genes by direct interaction.

### *ANAC019*, *ANAC055*, and *ANAC072* Are Required for COR-Regulated Stomatal Reopening, but Not ABA-Mediated Stomatal Closure

To further investigate the functions of *ANAC019*, *ANAC055* and *ANAC072* in plant defense, homologous T-DNA insertion mutants for each gene were isolated. The *anac019* mutant (SALK\_096295) has an insertion in the second exon of *ANAC019*, *anac055* (SALK\_014331) in the third exon of *ANAC055*, and *anac072* (SALK\_083756) in the first exon of *ANAC072*. Since *ANAC019*, *ANAC055*, and *ANAC072* have high amino acid sequence similarities and are able to bind to the same *cis*-element (Tran et al., 2004), they are likely to have functional redundancy. In anticipation of this possibility, we generated double mutants of all combinations and a triple



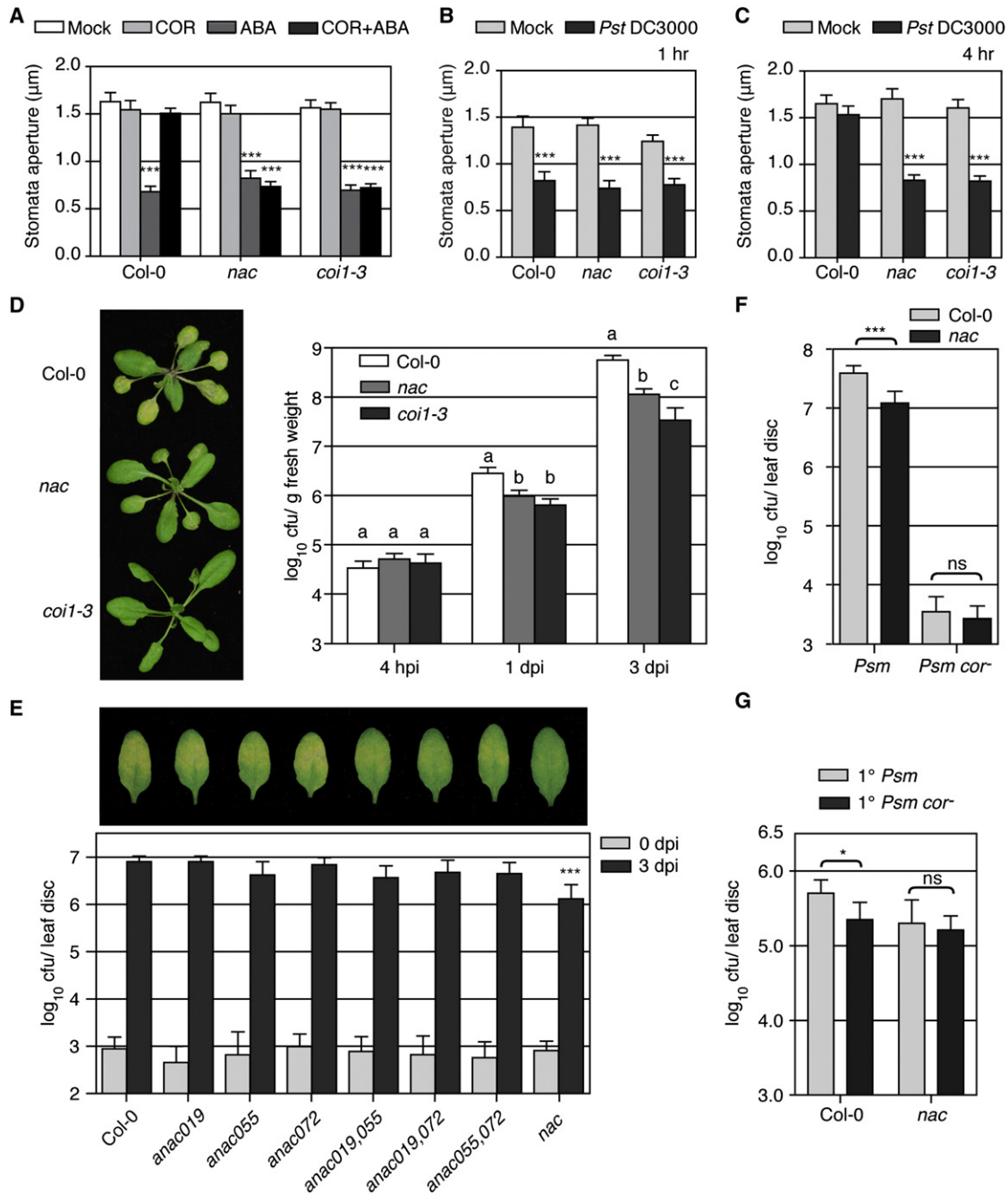
**Figure 2. *MYC2* Induces the Expression of *ANAC019*, *ANAC055*, and *ANAC072* by Directly Interacting with Their Promoters**

(A–C) Transcript levels of *ANAC019*, *ANAC055*, and *ANAC072* were measured in Col-0 and *myc2* in response to mock, *Psm* ES4326, or *Psm* ES4326 *cor*<sup>−</sup>. Error bars represent standard deviation of three technical replicates.

(D–F) 35S:*MYC2*-GFP transgenic plants were used for ChIP experiments. Long horizontal lines represent the promoters. Ticks above the lines represent putative *MYC2*-binding sites. Short horizontal lines show the regions where different qPCR primers amplify. Error bars represent standard error of three technical replicates. Each experiment was repeated at least three times with similar results. See also Figure S2 and Table S1.

mutant by crossing. The *anac019*, *anac055*, *anac072* triple mutant (the *nac* triple mutant) was verified by measuring the transcripts of these three genes (Figure S3A).

It is known that ABA signaling is responsible for pathogen-triggered stomatal closure, a key step in plant immune response against *Pseudomonas syringae*, while COR produced by this pathogen can reopen stomata to facilitate pathogen entry into the plant (Melotto et al., 2006). Since *ANAC019*, *ANAC055*, and *ANAC072* are induced by both ABA and COR, we were interested in their roles in ABA-mediated stomatal closure and COR-mediated stomatal reopening. As shown in Figure 3A, in



**Figure 3. ANAC019, ANAC055, and ANAC072 Are Involved in COR-Triggered Virulence in Stomata, Apoplast, and Systemic Tissue**

(A) Stomata aperture from leaf peels of Col-0, the *nac* triple mutant, and the *coi1-3* mutant treated with mock, COR, ABA, or a combination of COR and ABA for 1 hr.

(B and C) Stomatal closure response in leaf peels of Col-0, the *nac* triple mutant, and the *coi1-3* mutant after 1 hr incubation (B) or 4 hr incubation (C) with either mock or *Pst* DC3000. Error bars represent standard error. Each experiment was repeated at least twice with similar results. Student's t test was used to compare means between mock and specific treatment of the same genotype.

(D) Col-0, the *nac* triple mutant, and the *coi1-3* mutant were dip inoculated with *Psm* ES4326. Pictures of the disease symptoms were taken at 3 dpi, and bacterial growth was measured at 4 hpi, 1 dpi, and 3 dpi. Student's t test was used to compare the log-transformed data of different genotypes at the same time point.

(E) Col-0; the single and double mutants of *ANAC019*, *ANAC055*, and *ANAC072*; and the *nac* triple mutant were pressure infiltrated with *Psm* ES4326. Photographs of the disease symptoms were taken at 3 dpi. Bacterial growth was measured at the same time. Bonferroni's multiple comparison test was performed to compare the log-transformed data of each mutant to that of Col-0.

(F) Col-0 and the *nac* triple mutant were pressure infiltrated with *Psm* ES4326 or *Psm* ES4326 *cor*<sup>-</sup> and assayed at 3 dpi.

(G) *Psm* ES4326 growth in systemic leaves with preinoculation with *Psm* ES4326 or *Psm* ES4326 *cor*<sup>-</sup>. Error bars represent 95% confidence intervals. Experiments were repeated for at least twice with similar results. ns, no significant difference. See also Figure S3.



wild-type plants (Col-0), ABA treatment induced stomatal closure, and COR inhibited this ABA effect (Melotto et al., 2006). In response to ABA, the *nac* triple mutant closed the stomata, demonstrating that these three NACs are not required for ABA-mediated stomatal closure. However, COR could not inhibit ABA-induced stomatal closure in the *nac* triple mutant, suggesting the requirement of ANAC019, ANAC055, and ANAC072 for this COR function.

To further confirm the function of ANAC019, ANAC055, and ANAC072 in stomatal regulation, we incubated *Pst* DC3000 with leaf peels from wild-type plants, the *nac* triple mutant, and the *coi1-3* mutant. Previous studies have shown that *Pst* DC3000 causes a reduction in the stomatal aperture in wild-type plants 1 hr after bacterial incubation. However, stomata reopen 4 hr after bacterial incubation, and this reopening is dependent on COR (Melotto et al., 2006; Zeng and He, 2010). We found that the presence of *Pst* DC3000 caused stomatal closure in all three genotypes (Figure 3B), but the *coi1-3* mutant and the *nac* triple mutant were compromised in the ability to reopen the stomata after 4 hr, and the stomatal apertures remained small (Figure 3C). These results suggest that the *nac* triple mutant, like the *coi1* mutant, is defective in COR-triggered stomatal reopening.

To examine the effect of the *nac* triple mutant on bacterial entry, we dip inoculated wild-type, the *nac* triple mutant, and the *coi1-3* mutant plant with *Psm* ES4326; observed symptom development; and measured the bacterial growth (Figure 3D). At 1 day postinoculation (dpi), the bacterial numbers present in the *nac* triple mutant and the *coi1-3* mutant were similarly lower than that in wild-type plants, probably due to the fact that these mutants are equally defective in early infection steps including reopening of stomata (Figures 3A and 3C). Interestingly, at 3 dpi, the *nac* triple mutant exhibited a bacterial titer that was lower than wild-type but higher than the *coi1-3* mutant, suggesting that the three NACs may play a partial role in COR-mediated *Psm* ES4326 growth in apoplast later in the infection.

### COR Promotes Virulence in the Apoplast Partly through ANAC019, ANAC055, and ANAC072

To validate our hypothesis that the NAC TFs play a role in the COR-mediated virulence in the apoplast, we pressure infiltrated *Psm* ES4326 into the *nac* single, double, and triple mutants to bypass the stomatal regulation. Though the single and double mutants developed symptoms similar to those observed in wild-type plants, the *nac* triple mutant was more resistant than the wild-type (Figure 3E). Furthermore, disease susceptibility could be restored in the *nac* triple mutant by expressing any one of the three wild-type NAC genes (Figure S3B). These results demonstrate that the three NACs are required for *Psm* ES4326 virulence in the apoplast. When the COR-deficient *Psm* ES4326 *cor*<sup>-</sup> was pressure infiltrated, wild-type plants and the *nac* triple mutant exhibited similar levels of bacterial growth (Figure 3F), supporting the notion that the virulence effect of COR is mediated by the three NACs. However, the growth difference between *Psm* ES4326 and *Psm* ES4326 *cor*<sup>-</sup> in wild-type plants was much greater than the growth difference of *Psm* ES4326 in wild-type plants and the *nac* triple mutant, suggesting the existence of other TFs mediating the COR activity in the apoplast.

### ANAC019, ANAC055, and ANAC072 Are Involved in COR-Triggered Systemic Susceptibility

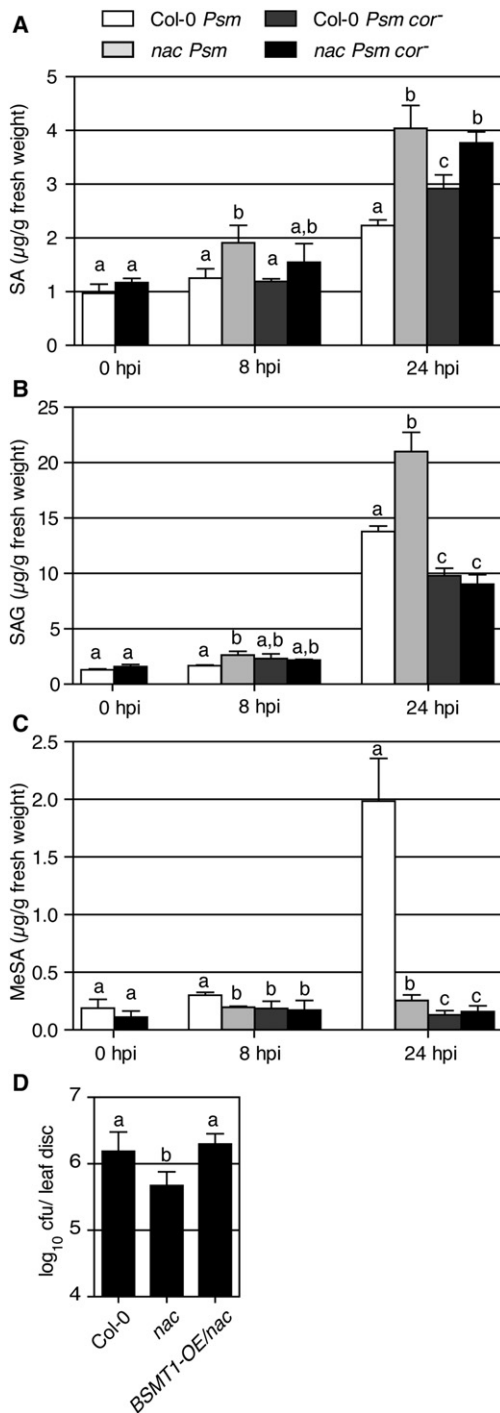
Another known function of COR in counteracting plant defense is the promotion of plant systemic susceptibility (Cui et al., 2005). The systemic induction of ANAC019, ANAC055, and ANAC072 by *Psm* ES4326 (Figure S1A) suggests that they play a role. As shown in Figure 3G, wild-type plants preinoculated with *Psm* ES4326 exhibited significantly enhanced susceptibility than those preinoculated with *Psm* ES4326 *cor*<sup>-</sup>, displaying COR-mediated systemic susceptibility. However, in the *nac* triple mutant, this induced systemic susceptibility was abolished, validating our hypothesis.

### COR Suppresses SA Accumulation through ANAC019, ANAC055, and ANAC072

To understand how COR promotes *Psm* ES4326 virulence through induction of ANAC019, ANAC055, and ANAC072, we sought their downstream targets. Since SA is an essential signal for resistance against *Pseudomonas* infection and SA levels in the COR-insensitive *coi1* mutant are elevated (Kloek et al., 2001), we first measured the SA levels in wild-type and the *nac* triple mutant in response to *Psm* ES4326 and *Psm* ES4326 *cor*<sup>-</sup> infection. As shown in Figure 4A, in wild-type plants, *Psm* ES4326 *cor*<sup>-</sup> infection induced a higher level of SA compared to *Psm* ES4326 infection at 24 hr postinoculation (hpi), displaying the suppressive effect of COR on SA accumulation. In the *nac* triple mutant, the SA level was dramatically higher than that in the wild-type after *Psm* ES4326 inoculation at both 8 hpi and 24 hpi, indicating that the NAC TFs are principally responsible for COR-mediated repression of SA accumulation. Supporting this conclusion, in the *nac* triple mutant, the wild-type *Psm* ES4326 and the COR-deficient *Psm* ES4326 *cor*<sup>-</sup> strain triggered comparable levels of SA accumulation.

### ANAC019, ANAC055, and ANAC072 Directly Regulate SA Biosynthesis and Metabolism Genes

Several *Arabidopsis* genes are known to affect SA biosynthesis and metabolism (Table S2) (Vlot et al., 2009). We tested the possibility that these genes are direct targets of the three NACs. First, we found six of these SA-related genes to contain at least one copy of the known NAC core-binding site (CACG tetramer) (Tran et al., 2004) in their promoters (Table S2). To determine whether these genes could be regulated by ANAC019, ANAC055, and ANAC072, we analyzed their expression. We found that the basal transcript level of the SA synthesis enzyme isochorismate synthase gene 1 (*ICS1*) was higher in the *nac* triple mutant than in the wild-type (Figure 5A). In contrast, the SA glucosyl transferase gene 1 (*SAGT1*), which converts SA to inactive storage forms SA glucose ester (SGE) and SA O-β-glucoside (SAG) (Dean and Delaney, 2008), had a lower transcript level in the *nac* triple mutant (Figure 5B). These results suggest the three NAC TFs may decrease SA synthesis, but increase SA storage through transcriptional repression of *ICS1* and transcriptional activation of *SAGT1*, respectively. The basal expression levels of other SA biogenesis genes were not significantly different between the wild-type and the *nac* triple mutant, implying that they are not regulated by the three NACs (Figures S5A–S5C). The basal transcript level of the SA methyl transferase 1 (*BSMT1*), which converts SA to the inactive, volatile



**Figure 4. COR Suppresses SA Accumulation through ANAC019, ANAC055, and ANAC072 to Promote Virulence**

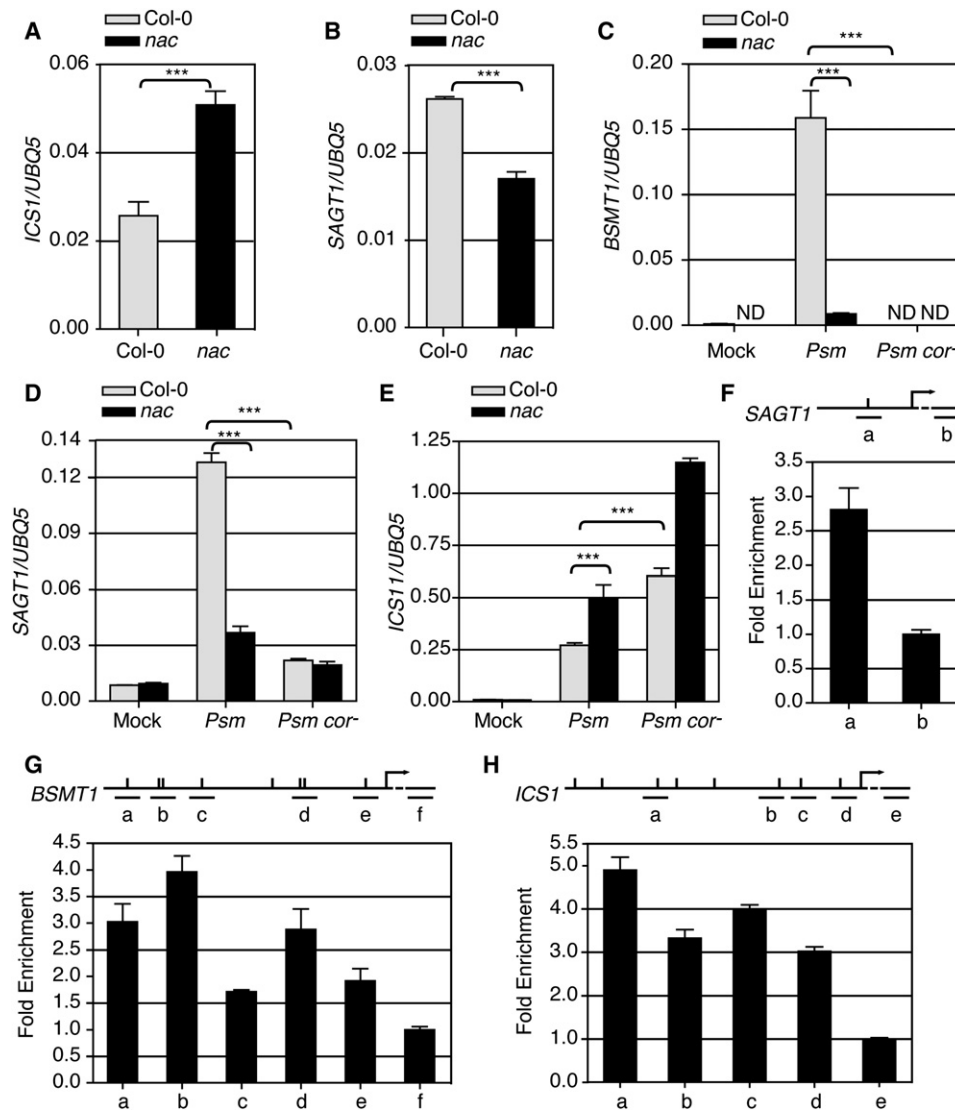
Col-0 and the *nac* triple mutant were pressure infiltrated with *Psm* ES4326 or *Psm* ES4326 *cor*<sup>-</sup>. Samples were collected at specified time points to measure the levels of SA (A), SAG (B), and MeSA (C). Error bars represent standard deviation of three replicates. Student's t test was used to compare two means at the same time point. (D) *Psm* ES4326 growth was measured in Col-0, the *nac* triple mutant, and the *nac* triple mutant overexpressing *BSMT1* (*BSMT1-OE/nac*) as described in Figure 3E. Error bars represent 95% confidence intervals. Experiments were repeated at least twice with similar results. See also Figure S4 and Table S2.

methyl SA (MeSA) (Chen et al., 2003), was too low for accurate measurement.

We then analyzed the potential target gene expression after *Psm* ES4326 induction to determine whether they are regulated by COR through the NACs. As shown in Figure 5C, while *BSMT1* was induced by *Psm* ES4326 in wild-type, this induction was dramatically compromised in the *nac* triple mutant. Similarly, induction of *SAGT1* by *Psm* ES4326 was also blocked in the *nac* triple mutant (Figure 5D). Consistent with our hypothesis that COR represses SA accumulation through NACs, transcript reductions were observed for *SAGT1* and *BSMT1* in wild-type plants when the COR-deficient *Psm* ES4326 *cor*<sup>-</sup> was inoculated instead of *Psm* ES4326 (Figures 5C and 5D). In contrast to the SA metabolic enzyme genes, the expression of the SA synthesis gene *ICS1* was further induced in the *nac* triple mutant and by *Psm* ES4326 *cor*<sup>-</sup> compared to *Psm* ES4326 (Figure 5E), consistent with our hypothesis that COR suppresses SA accumulation through NAC-mediated repression of *ICS1* expression. However, we could only consistently observe elevated *ICS1* expression in the *nac* triple mutant when a higher number of bacteria were infiltrated. This was probably due to the fact that *Psm* ES4326 not only produces COR, which negatively regulates the *ICS1* gene expression, but also carries other signals that positively regulate the plant defense machinery, including the *ICS1* gene.

To demonstrate that the three NACs regulate *SAGT1*, *BSMT1*, and *ICS1* through direct interaction, ChIP experiments were performed. We constructed a transgenic *Arabidopsis* line that constitutively expresses the ANAC019-GFP fusion protein (Figure S5D) for these experiments. For each promoter, primers were designed to cover the NAC core-binding sites, except for regions where no appropriate primers could be found. Since the NAC core-binding sites are scattered in the entire promoters (Table S3), a region in the coding sequence where there is no NAC core-binding site within a 500 bp stretch of sequence was included to measure the background binding. As shown in Figures 5F–5H, the DNA samples precipitated by ANAC019-GFP were enriched in some NAC core-binding sites in the *SAGT1*, *BSMT1*, and *ICS1* promoters, suggesting a direct role of ANAC019 in regulating expression of these genes. Since ANAC019, ANAC055, and ANAC072 bound to the same *cis*-element in an *Arabidopsis* protoplast assay (Tran et al., 2004), it is reasonable to extrapolate that ANAC055 and ANAC072 could also bind to the promoters of *SAGT1*, *BSMT1*, and *ICS1* and regulate their expression. The binding of these three NACs might lead to induction of the SA metabolism genes *SAGT1* and *BSMT1* and repression of the SA synthesis gene *ICS1*, resulting in an overall reduction of the defense signal SA accumulation and enhancement of bacterial virulence.

To further validate the aforementioned hypothesis, we measured the SAG and MeSA levels (the products of *SAGT1* and *BSMT1* enzymatic activities, respectively) in wild-type and the *nac* triple mutant. In wild-type plant, *Psm* ES4326 *cor*<sup>-</sup> induced lower SAG and MeSA levels than *Psm* ES4326 at 24 hpi (Figures 4B and 4C), consistent with the lower *SAGT1* and *BSMT1* transcript levels found in the wild-type in response to *Psm* ES4326 *cor*<sup>-</sup> (Figures 5C and 5D). However, the *nac* triple mutant accumulated more SAG than the wild-type in response to *Psm* ES4326 (Figure 4B), despite the lower *SAGT1* transcript



**Figure 5. ANAC019, ANAC055, and ANAC072 Differentially Regulate *ICS1*, *BSMT1*, and *SAGT1* through Direct Interaction with Their Promoters**

(A–E) Basal transcript levels of *ICS1* (A) and *SAGT1* (B) were measured in 3-week-old leaves. Col-0 and the *nac* triple mutant were pressure infiltrated with 10 mM MgCl<sub>2</sub>, *Psm* ES4326, or *Psm* ES4326 *cor*<sup>-</sup> and were collected after 24 hr to measure the expression of *BSMT1* (C), *SAGT1* (D), and *ICS1* (E). For *ICS1*, a higher dose of pathogen (OD<sub>600nm</sub> = 0.02) was used. ND, no detectable signal. Error bars represent standard deviation of three technical replicates.

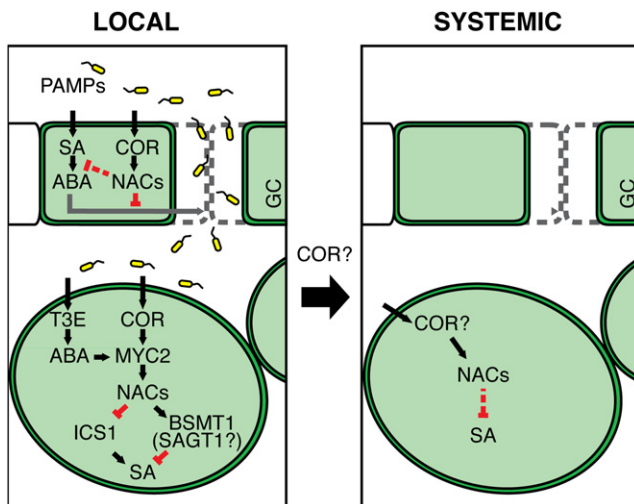
(F–H) ChIP experiments were performed using the *Psm* ES4326-infected 35S:*ANAC019-GFP* transgenic plants. Long horizontal lines represent the promoters of *SAGT1* (F), *BSMT1* (G), and *ICS1* (H). Ticks above the lines represent NAC core-binding sites. Short horizontal lines show the regions where different qPCR primers amplify. Error bars represent standard error. Experiments were repeated three times with similar results. See also Figure S5 and Table S3.

level in the *nac* triple mutant (Figure 5D). This result indicates that the amount of *SAGT1* transcript or enzyme might not be a limiting factor in the *nac* triple mutant in converting SA to SAG and that *SAGT1* expression is not a significant contributor to the inhibitory effect of the NAC TFs on SA accumulation. In contrast, the MeSA levels in the *nac* triple mutant were significantly lower than those in the wild-type at both 8 and 24 hpi (Figure 4C), suggesting a more significant role of *BSMT1* expression regulation in affecting SA level. This conclusion is further supported by the observation that overexpression of *BSMT1* in the *nac* triple mutant (*BSMT1-OE/nac*) abolished the enhanced resistance of the *nac* triple mutant (Figure 4D) and restored

symptom development (Figure S4). Therefore, COR suppresses SA level through the three NAC TFs mainly by activation of *BSMT1* and repression of *ICS1* (Figure 6).

## DISCUSSION

The bacterial toxin COR is known to cause multiple physiological effects in plants (Brooks et al., 2005; Cui et al., 2005; Melotto et al., 2006; Mittal and Davis, 1995). Our study identified three NAC TFs as targets of COR by which COR exerts its virulence in stomata, apoplast, and systemic tissue with different levels of dependency. The three NACs appear to be essential for



**Figure 6. A Model of the Signaling Cascade Mediating the Virulence Effects of COR**

In stomata, PAMPs from the pathogen trigger stomatal closure through SA and ABA. COR produced by *Pseudomonas* inhibits this effect and reopens the stomata through ANAC019, ANAC055, and ANAC072 (NACs). In apoplast, COR induces the ANAC019, ANAC055, and ANAC072 by the MYC2 TF. Type III effectors (T3E) might also induce the NACs through ABA signaling. These TFs then repress *ICS1* and induce *BSMT1* and possibly *SAGT1* to inhibit SA accumulation and promote bacterial virulence. In systemic tissue, ANAC019, ANAC055, and ANAC072 are induced possibly by translocated COR or a COR-generated signal. GC, guard cell.

COR to function in reopening stomata and systemic induced susceptibility but only partially responsible in COR-mediated bacterial growth in the apoplast. Even though we did not specifically examine the effect of the NAC TFs on COR-dependent chlorosis symptom development, we found that restoration of chlorotic symptom in the *BSMT1-OE/nac* transgenic plant (Figure S4B) correlated with an increase in bacterial growth (Figure 4D). This suggests that NACs may not be required for the symptom promotion function of COR as described by Brooks and coworkers (Brooks et al., 2005).

Our study not only identified three TF targets of COR but also elucidated a complete COR-activated signaling cascade. As shown in Figure 6, COR produced by *Pseudomonas syringae* triggers the SCF<sup>COI1</sup>-mediated degradation of the JAZ protein, resulting in the release of the MYC2 TF. MYC2 directly activates the expression of ANAC019, ANAC055, and ANAC072, which then differentially regulate the expression of *ICS1*, *BSMT1*, and *SAGT1*. Repression of the SA biosynthesis gene *ICS1* and activation of the SA metabolism gene *BSMT1* result in a net reduction in the accumulation of SA and compromised resistance to the pathogen. Though previous studies showed that ANAC019, ANAC055, and ANAC072 had transactivation activity (Bu et al., 2008; Tran et al., 2004), our study suggests that they may also function as transcriptional suppressors, binding to the *ICS1* promoter and repressing its expression. This dual transcriptional capability may be achieved by recruiting or interacting with different transcriptional partners.

Our study clearly shows that COR can suppress host defense by inhibiting SA accumulation. We believe that the contradicting

data obtained in previous studies resulted from the interplay between pathogen virulence involving COR and induction of host defense during infection (Block et al., 2005). Using dip inoculation, as reported by Block et al., fewer COR-deficient mutant bacteria could enter the apoplast than the wild-type *Pst* DC3000 to induce SA production, obscuring the effect of COR. Pressure infiltration used in our study ensured that the same number of wild-type and COR-deficient mutant bacteria were delivered into the apoplast to induce SA production, thus providing a better comparison. This COR-triggered SA suppression, which is mediated by the three NACs, may be the molecular mechanism for COR-mediated virulence in stomata, local, and systemic tissue (Figure 6). Zeng and He have shown that SA biosynthesis is required and acts upstream of ABA signaling to trigger stomatal closure (Zeng and He, 2010). However, it has yet to be determined whether NACs promote stomatal reopening by reducing SA accumulation or through a different mechanism. A method for measuring SA levels specifically in the stomata needs to be developed.

The roles of *BSMT1* and *SAGT1* in regulating SA level and plant defense have been studied previously. Though single mutants do not exhibit enhanced local resistance, overexpressing either one of them decreases local SA accumulation and increases plant susceptibility to *Pseudomonas syringae* infection (Liu et al., 2010; Song et al., 2008, 2009). Our study further illustrates that *BSMT1* and possibly *SAGT1* are the plant defense regulatory nodes that can be manipulated by pathogens. With a relatively low  $K_m$  (16  $\mu M$ ) (Chen et al., 2003), *BSMT1* can respond to small increases in SA levels and convert SA to MeSA. Therefore, COR-triggered *BSMT1* induction is able to effectively suppress SA accumulation and reduce host defense. Our study, however, does not rule out the possibility that COR also inhibits the expression or the activity of MeSA esterase, which converts MeSA back to SA (Fouhar et al., 2005).

Since COR is a JA-Ile mimic, the signaling cascade we identified for COR signaling may normally mediate the JA-SA crosstalk in plants. The antagonism between JA and SA has been reported, mostly in the direction of SA inhibiting JA (Bostock, 2005). The opposite direction has been shown in a few cases, such as the higher SA level observed in the *coi1* mutant (Kloek et al., 2001). This JA-SA antagonism is believed to be involved in fine-tuning the plant defense responses based on the types of pathogens encountered (Grant and Jones, 2009; Spoel et al., 2007). Despite its importance, not all signaling components underlying this crosstalk are identified. Since the three NACs can be induced by MeJA treatment, it is reasonable to predict that JA activates the same signaling cascade as COR to suppress SA accumulation, representing one mechanism underlying the JA-SA antagonism.

As mediators of the phytotoxicity of COR, ANAC019, ANAC055, and ANAC072 are hijacked by pathogen to promote virulence. What is the normal physiological function of these TFs? Tran et al. showed that overexpressing any one of them increased drought tolerance (Tran et al., 2004). Fujita et al. found that ANAC072-overexpressing plants are more sensitive to ABA in terms of growth inhibition (Fujita et al., 2004). Consistently, our own study showed that the *nac* triple mutant was less sensitive in ABA-triggered inhibition on seed germination (data not shown). Moreover, ANAC019 and ANAC055 overexpression



lines exhibited higher expression of *VSP1* and *LOX2* in response to MeJA (Bu et al., 2008), suggesting their function in JA-mediated wound response. Together these results indicate that ANAC019, ANAC055, and ANAC072 are integral parts of the ABA and JA signaling pathways, necessary for regulating plant developmental processes as well as responses to drought stress and wounding. Our study illustrates an example in which the *Pseudomonas* bacterial pathogen manipulates the plant signaling components, through COR production, to inhibit host defense and promote virulence.

## EXPERIMENTAL PROCEDURES

### Plants and Bacterial Strains

*Arabidopsis* plants were grown on soil in 22°C under 16/8 hr day/night cycle. The *myc2* (SALK\_017005), *anac019* (SALK\_096295), *anac055* (SALK\_014331), and *anac072* (SALK\_083756) mutants were obtained from the *Arabidopsis* Biological Resource Center. *Pst* DC3000, *Psm* ES4326, and a COR-deficient strain, *Psm* ES4326 *cor*<sup>-</sup>, were grown on King's B medium plates with corresponding antibiotics at 30°C for 2 days (Cui et al., 2005; Dong et al., 1991).

### Gene Expression Analysis

For chemical treatment, plants were grown on vertical MS plates for 12 days and then transferred to MS plates with either 5 μM ABA (Sigma), 1 μM COR (Sigma), or no additional chemicals as a control. The rosette leaves were collected for RNA extraction after 24 hr. For bacterial infection, 3-week-old leaves were pressure infiltrated with either 10 mM MgCl<sub>2</sub>, *Psm* ES4326, or *Psm* ES4326 *cor*<sup>-</sup>. OD<sub>600nm</sub> = 0.01 of bacteria was inoculated unless specified. Statistical analyses were performed using Student's t test of the differences between two means (\*p < 0.05;\*\*p < 0.01;\*\*\*p < 0.001). Primers used for qRT-PCR are listed in the Supplemental Information.

### Chromatin Immunoprecipitation

For MYC2-GFP ChIP, untreated 3-week-old leaves were harvested. For ANAC019-GFP ChIP, leaves were pressure infiltrated with *Psm* ES4326 (OD<sub>600nm</sub> = 0.01) and harvested after 24 hr. ChIP was performed as previously described (Busch et al., 2010), and the samples were analyzed by qPCR. The primers for qPCR are listed in the Supplemental Information. Input samples were first used to normalize the results. Fold difference was then calculated by taking ratios between normalized results from wild-type plants and from MYC2-GFP or ANAC019-GFP plants. Finally, the fold enrichment was calculated as the ratio between the probes and the corresponding negative control.

### Stomatal Analysis

Stomatal assay was performed as described previously (Zeng and He, 2010). For chemical treatment, 15 μM of ABA (Sigma) and 1 ng/μl of COR (purchased from C. Bender, Oklahoma State University) were used.

### SA, SAG, and MeSA Measurement

Three-week-old leaves were pressure infiltrated with *Psm* ES4326 or *Psm* ES4326 *cor*<sup>-</sup> (OD<sub>600nm</sub> = 0.01) and harvested at specified time points. SA and SAG were extracted and measured by HPLC as previously described (Liu et al., 2010). MeSA was extracted and measured using GC/MS as previously described (Liu et al., 2010). Three replicates were taken for each data point. Statistical analyses were performed using Student's t test of the differences between two means.

### *Pseudomonas syringae* Infection

For pressure infiltration, 3-week-old plants were pressure infiltrated using a needleless syringe with *Psm* ES4326 (OD<sub>600nm</sub> = 0.001), and eight plants were assayed for each data point. Bacterial growth was measured after 3 days as described previously (Durrant et al., 2007). For dip inoculation, 4-week-old plants were dipped into a solution of *Psm* ES4326 (OD<sub>600nm</sub> = 0.2) with 0.02% Silwet L-77 and covered with clear plastic lids for 1 day. The infected leaves were surface sterilized with 15% H<sub>2</sub>O<sub>2</sub> and washed

twice before being homogenized to assay the bacterial growth. Statistical analyses were performed on means of log-transformed data using one-way ANOVA and Bonferroni's multiple comparison tests or Student's t test (\*p < 0.05;\*\*p < 0.01;\*\*\*p < 0.001).

For the COR-induced systemic susceptibility assay, two lower leaves were pressure infiltrated with *Psm* ES4326 (OD<sub>600nm</sub> = 0.2) or *Psm* ES4326 *cor*<sup>-</sup> (OD<sub>600nm</sub> = 0.2). Three days later, the infected leaves were removed and two upper leaves were pressure infiltrated with *Psm* ES4326 (OD<sub>600nm</sub> = 0.0002). Colony-forming unit was determined 3 days later. Student's t test was used to compare the means of log-transformed data (\*p < 0.05;\*\*p < 0.01;\*\*\*p < 0.001).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found online at doi:10.1016/j.chom.2012.04.014.

## ACKNOWLEDGMENTS

We thank Dr. Hironaka Tsukagoshi for providing the MYC2-GFP over-expression plants, Dr. Chuanyou Li for sharing the *anac019 anac055* double mutant, Wei Wang for assisting the statistical analysis, and Musoki Mwimba for assisting the HPLC analysis. This work was supported by a grant from NSF (IOS - 0929226) (to X.D.), a NIH training predoctoral training grant (to N.W.S.), grants from NIH (R01AI068718) and DOE (the Chemical Sciences, Geosciences, and Biosciences Division, Office of Basic Energy Sciences, Office of Science; DE-FG02-91ER20021) (to S.Y.H.), and a grant from NSF (IOS-0820405) (to D.F.K.). X.D. and S.Y.H. are Howard Hughes Medical Institute-Gordon and Betty Moore Foundation Investigators.

Received: November 28, 2011

Revised: March 16, 2012

Accepted: April 25, 2012

Published: June 13, 2012

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