The AP2/ERF Domain Transcription Factor ORA59 Integrates Jasmonic Acid and Ethylene Signals in Plant Defense[1][W]

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Plant defense against pathogens depends on the action of several endogenously produced hormones, including jasmonic acid (JA) and ethylene. In certain defense responses, JA and ethylene signaling pathways synergize to activate a specific set of defense genes. Here, we describe the role of the Arabidopsis (*Arabidopsis thaliana*) APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain transcription factor ORA59 in JA and ethylene signaling and in defense. JA- and ethylene-responsive expression of several defense genes, including *PLANT DEFENSIN1.2* (*PDF1.2*), depended on ORA59. As a result, overexpression of *ORA59* caused increased resistance against the fungus *Botrytis cinerea*, whereas *ORA59*-silenced plants were more susceptible. Several AP2/ERF domain transcription factors have been suggested to be positive regulators of *PDF1.2* gene expression based on overexpression in stably transformed plants. Using two different transient overexpression approaches, we found that only ORA59 and ERF1 were able to activate *PDF1.2* gene expression, in contrast to the related proteins AtERF1 and AtERF2. Our results demonstrate that ORA59 is an essential integrator of the JA and ethylene signal transduction pathways and thereby provide new insight into the nature of the molecular components involved in the cross talk between these two hormones.

Plant fitness and survival are dependent on the ability to mount fast and highly adapted responses to diverse environmental stress conditions. Perception of stress signals results in the production of one or more of the secondary signaling molecules jasmonic acid (JA), ethylene, and salicylic acid.

JA is a major intermediate signaling molecule in defense against wounding, herbivores, and certain pathogens (Turner et al., 2002). Several studies revealed complex cross talk relationships between JA, ethylene, and salicylic acid, which can act synergistically or antagonistically, in order to fine-tune the defense response (Kunkel and Brooks, 2002). Arabidopsis (*Arabidopsis thaliana*) plants impaired in JA or ethylene signaling pathways showed enhanced susceptibility to the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* (Penninckx et al., 1996; Thomma et al., 1998, 1999a), demonstrating that JA and ethylene are important signal molecules for resistance against these pathogens.

Several components of the corresponding signal transduction pathways have been characterized. The *coronatine insensitive1* (*coi1*) mutant is affected in a gene encoding an F-box protein that is important for all known JA responses, including defense against necrotrophic pathogens (Thomma et al., 1998; Xie et al., 1998). Loss-of-function mutations in the *ETHYLENE INSENSITIVE2* (*EIN2*) gene cause complete ethylene insensitivity, indicating that EIN2 is a positive component essential for ethylene responses (Alonso et al., 1999).

A crucial step in JA- and ethylene-dependent defense responses is the rapid transcription of genes coding for antimicrobial proteins or for enzymes involved in the biosynthesis of protective secondary metabolites. Therefore, studying the mechanisms whereby the expression of these defense-related genes is regulated is of major importance to understand signal transduction pathways and plant responses to environmental stress. In the past several years, a number of transcription factors regulating defense-related genes have been functionally characterized. Several of these regulatory proteins belong to a subgroup, known as the ERF family, of the large plant-specific APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily (Nakano et al., 2006). Many *ERF* genes have been shown to be regulated by a variety of stress-related stimuli, such as wounding, JA, ethylene, salicylic acid, or infection by different types of pathogens (Gutterson and Reuber, 2004; McGrath et al., 2005).

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The transcription factor ERF1 (At3g23240) was suggested to act as an integrator of JA and ethylene signaling pathways in Arabidopsis (Lorenzo et al., 2003). Constitutive overexpression of *ERF1* activates the expression of several defense-related genes, including *PLANT DEFENSIN1.2* (*PDF1.2*) and *BASIC CHITINASE* (*ChiB*; Solano et al., 1998; Lorenzo et al., 2003), and was shown to confer resistance to several fungi, including *B. cinerea* (Berrocal-Lobo et al., 2002). Constitutive overexpression of *AtERF2* (At5g47220; Brown et al., 2003; McGrath et al., 2005) or *AtERF14* (At1g04370; Oñate-Sánchez et al., 2007), encoding other ERF transcription factors, was also reported to cause high levels of *PDF1.2* and *ChiB* gene expression in transgenic Arabidopsis plants. In contrast, the ERF transcription factor AtERF4 (At3g15210) negatively regulates the expression of *PDF1.2* (McGrath et al., 2005). This suggests that several members of the ERF family control the expression of these defense genes both negatively and positively.

Here, we report the functional characterization of the transcription factor ORA59 (At1g06160), another member of the ERF family. Our findings show that ORA59 integrates JA and ethylene signals to regulate the expression of defense genes such as *PDF1.2* and *ChiB*, providing important new insights into the nature of the molecular components involved in JA-responsive gene expression and in the cross talk between JA and ethylene.

RESULTS

ORA59 Gene Expression Is Controlled by the JA and Ethylene Signal Transduction Pathways

In a family-wide screening, Atallah (2005) previously characterized 14 genes encoding AP2/ERF domain proteins that were rapidly induced by JA treatment in young Arabidopsis seedlings. The JA-induced expression of these genes, named *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF* (*ORA*) genes, was severely reduced in the JA-insensitive *coi1* mutant (Atallah, 2005), consistent with a possible role of ORA proteins in JA signaling. Among these *ORA* genes, the protein encoded by the *ORA59* gene showed high sequence similarity to ERF1 (40% amino acid identity over their entire lengths). Expression of *ERF1* in response to JA or ethylene treatments requires intact JA and ethylene signaling pathways (Lorenzo et al., 2003). To establish whether *ORA59* expression was similar to *ERF1* expression in this respect, we analyzed the induction of *ORA59* gene expression after treatment with JA, ethephon (an ethylene-releasing agent), or a combination of both in wild-type plants and mutants impaired in jasmonate or ethylene signaling (*coi1-1* and *ein2-1*, respectively; Guzman and Ecker, 1990; Feys et al., 1994). In accordance with our previous data (Atallah, 2005), the results shown in Figure 1 indicate that the induction of *ORA59* gene expression by JA is transient and requires both intact JA and ethylene signaling pathways. Moreover, a combined treatment with JA and ethephon led to a prolonged superinduction of *ORA59* gene expression. In response to JA and ethephon, *ORA59* gene expression was strongly reduced in both mutants compared with the wild type (Fig. 1). The defense gene *PDF1.2* is a well-characterized marker of the JA and ethylene signaling pathways, and *PDF1.2* expression was monitored as a control for hormone treatments in the different genetic backgrounds. Expression of *PDF1.2* in response to JA and/or ethylene was similar to *ORA59* gene expression. These results indicate that the induction of *ORA59* gene expression by JA, ethylene, or a combination of both requires intact JA as well as ethylene signaling pathways for full responsiveness.

Genome-Wide Identification of Putative ORA59 Target Genes

To characterize the genes regulated by ORA59, we performed a genome-wide transcriptome analysis of transgenic XVE-*ORA59* plants overexpressing *ORA59* in an estradiol-inducible manner from the XVE expression module. The full Arabidopsis genome was

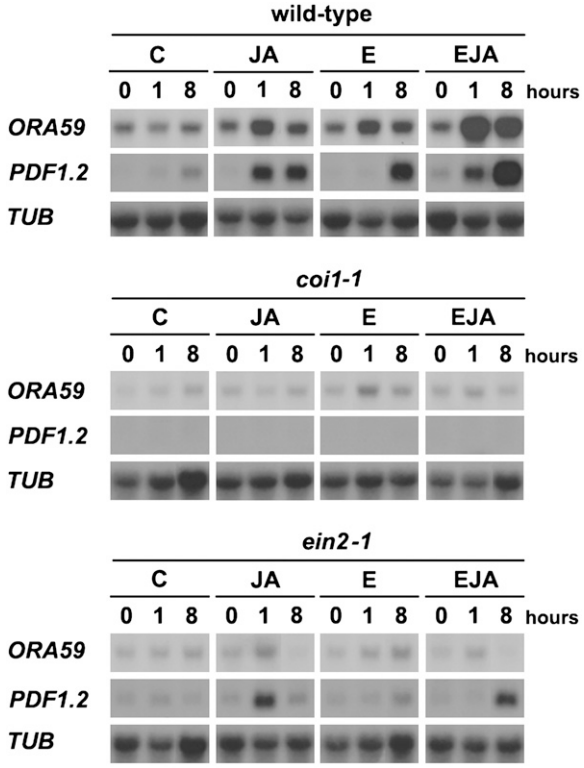

Figure 1. *ORA59* gene expression is controlled by the JA and ethylene signal transduction pathways. RNA was extracted from 14-d-old wild-type or mutant Arabidopsis seedlings treated with 50 μM JA, 1 mM ethephon (E; an ethylene releaser), a combination of both (EJA), or with the solvents (C) for the number of hours indicated. The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading. All panels for each probe were on the same blot and exposed to film for the same time, allowing direct comparison of expression levels.

covered using the Agilent Arabidopsis 3 Oligo microarray platform. Microarray data analyses revealed that 86 genes showed significantly increased expression levels of at least 2-fold in plants treated for 16 h with 2 μM estradiol to overexpress the *ORA59* transgene. Many of these genes are known to be involved in defense against biotic or abiotic stress, signaling, or primary or secondary metabolism (Supplemental Table S1). Several defense-related genes, such as *PDF1.2*, *HEL*, and *ChiB*, were highly expressed in plants overexpressing the *ORA59* gene. The expression of these genes is also induced by JA or ethylene and is superinduced by a combination of both (Potter et al., 1993; Penninckx et al., 1998; Thomma et al., 1998; Figs. 1 and 4). To get an indication of the role of ORA59 in the regulation of JA and/or ethylene-responsive genes, expression profiles of XVE-*ORA59* plants were compared with those of wild-type plants treated with JA or JA in combination with ethephon for 8 or 24 h (Supplemental Fig. S1). From the 86 genes up-regulated in XVE-*ORA59* plants, 13% (11 genes) were up-regulated in wild-type plants treated with JA for 8 h while 40% (34 genes) were up-regulated in wild-type plants treated with JA for 24 h. For plants treated simultaneously with JA and ethylene, the proportions were even higher, with 66% (57 genes) and 48% (41 genes) of common genes up-regulated after 8 and 24 h of treatment, respectively. These results show that a large number of ORA59-up-regulated genes are responsive to JA alone or in combination with ethephon at the selected time points. The microarray data were confirmed by RNA-blot analyses for a selected set of genes (Supplemental Fig. S2).

ORA59 Functions Downstream of COI1

The expression studies indicated that only a small proportion of genes induced by JA or JA and ethephon (around 5%–10%) were regulated by ORA59 (Supplemental Table S1; Supplemental Fig. S1). We hypothesized that, for these genes, ORA59 forms the terminal component of the ethylene and jasmonate signal transduction pathways and that it serves as the integrator of the JA and ethylene signal inputs, thereby determining the final expression output for this set of defense genes. To test whether ORA59 functions downstream of the JA signal transduction component COI1, we investigated whether *ORA59* overexpression would lead to target gene expression without requiring COI1. We introduced the XVE-*ORA59* expression module in the *coi1-1* mutant background and analyzed the expression of *PDF1.2*, *HEL*, and *ChiB* after treatment with the inducer estradiol. As shown in Figure 2, estradiol-induced expression of *ORA59* resulted in high expression levels of the target genes *PDF1.2* and *HEL* in a COI1-independent manner. In contrast, estradiol-induced expression of *ORA59* in the *coi1-1* mutant background did not lead to high expression of the *ChiB* gene, indicating that regulation of the *ChiB* gene by ORA59 is controlled by COI1-dependent molecular mechanisms. As expected, treatment with JA and ethephon induced the expression of all defense genes tested in the wild type but not in the *coi1-1* mutant background.

These results demonstrate that for some genes, ORA59 functions downstream from COI1, which is compatible with the hypothesis that ORA59 is the terminal integrator of the JA and ethylene signal inputs for a subset of JA- and ethylene-responsive genes, including *PDF1.2* and *HEL*.

Silencing of the *ORA59* Gene Compromises JA- and Ethylene-Induced Expression of Several Defense-Related Genes

To assess the role of ORA59 in a loss-of-function approach, *ORA59* gene expression was silenced using the RNA interference (RNAi) technique. Two copies of the *ORA59* full-length open reading frame were cloned in an inverted repeat orientation in front of the cauliflower mosaic virus (CaMV) 35S promoter, and the construct was transformed to plants. Under normal growth conditions, all transgenic *ORA59*-silenced lines displayed no visible aberrant phenotype compared with wild-type plants. *ORA59* mRNA levels in 10-d-old seedlings from different silenced lines were monitored by RNA-blot analyses using a probe corresponding to the 3′ untranslated region of *ORA59*. In most lines, *ORA59* mRNA was undetectable after treatment with JA for 30 min (Fig. 3A). In contrast, transgenic line 5 showed wild-type *ORA59* mRNA accumulation in response to JA, whereas line 13 showed a reduced *ORA59* mRNA level. Induction of the *PDF1.2* and *HEL* genes in response to 8 h of JA treatment was severely compromised in those *ORA59*-silenced lines with undetectable *ORA59* mRNA (Fig. 3B). Furthermore, the reduction in *PDF1.2* and *HEL* transcript abundance was inversely correlated with the *ORA59* mRNA level in individual lines, indicating that the JA-induced expression of these defense genes was dependent on ORA59.

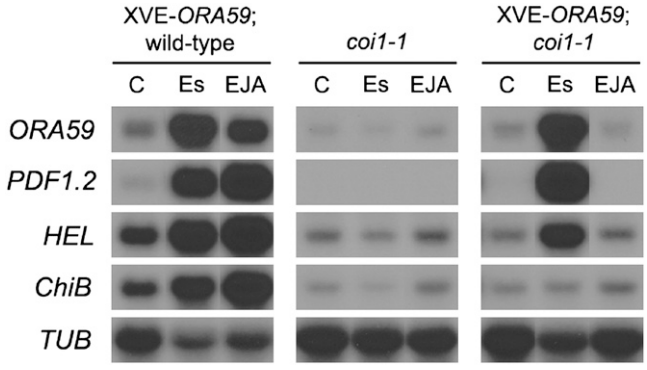

Figure 2. *ORA59* overexpression activates the expression of several putative target genes without requiring COI1. Two-week-old *coi1-1* mutant plants, and plants containing the XVE-*ORA59* expression module in the wild-type and *coi1-1* backgrounds, were treated for 8 h with 50 μM JA and 1 mM ethephon (EJA), 5 μM estradiol (Es), or the solvents (C). The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

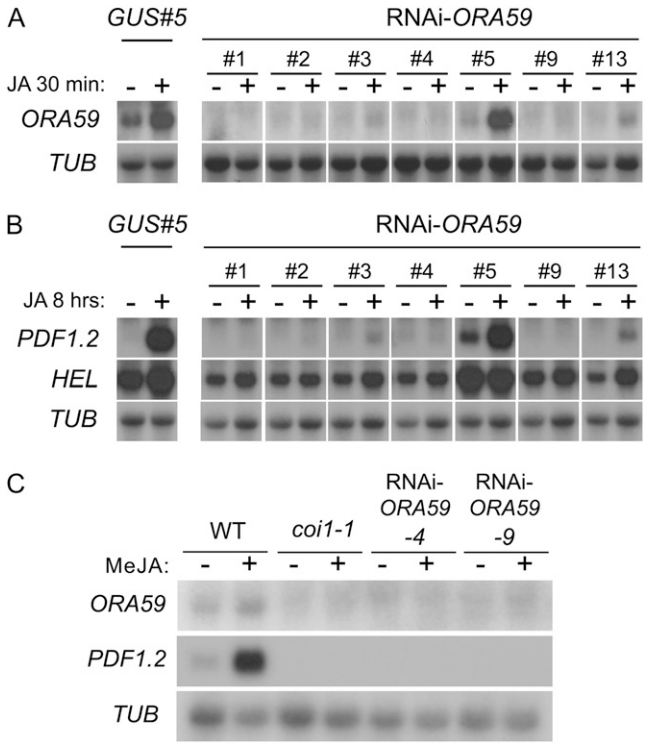

Figure 3. The *ORA59* gene is silenced in the majority of independent transgenic RNAi-*ORA59* lines. Ten-day-old seedlings from control line *GUS* 5 or seedlings from several independent RNAi-*ORA59* transgenic lines (indicated by numbers) were treated for 30 min (A) or for 8 h (B) with 50 μM JA (+) or with the solvent DMSO (−). In C, 5-week-old soil-grown wild-type (WT) plants, *coi1-1* mutant plants, and RNAi-*ORA59* lines 4 and 9 were dipped in 50 μM methyl jasmonate (MeJA) solution (+) or water (−) and harvested after 6 h. The RNA gel blots were hybridized with the indicated probes. *ORA59* expression was detected using a 150-bp fragment corresponding to the 3′ untranslated region of the *ORA59* gene, which was not present in the silencing construct. The *TUB* probe was used to verify RNA loading.

JA-responsive expression of *PDF1.2* was also impaired in 5-week-old RNAi-*ORA59* plants grown in soil treated with methyl jasmonate for 6 h (Fig. 3C).

Gene expression in response to hormone treatments was further analyzed using the transgenic line RNAi-*ORA59* 9. In agreement with previous findings, RNA-blot analyses using a probe corresponding to a gene-specific part of the *ORA59* coding region showed that treatments with JA and/or ethephon did not induce the accumulation of *ORA59* mRNA in 2-week-old seedlings of the RNAi-*ORA59* 9 line compared with the control line (Fig. 4A). Instead, a smeary signal was observed when hybridizing with this probe. This autoradiographic pattern observed in the *ORA59*-silenced lines is likely due to the detection of the complete inverted repeat intron RNA transcribed from the silencing construct and its degradation products, based on similar hybridization patterns with the *PDK* intron separating the two inverted *ORA59* repeats (data not shown). In the *ORA59*-silenced plants, the JA- and ethephon-induced expression of several genes identified by the microarray analysis, including *PDF1.2*, *AN5-AT*, *ChiB*, and *HEL* genes, was dramatically reduced compared with the control line (Fig. 4A). In contrast, the defense gene *VSP1*, which was not a target gene of ORA59 according to the microarray analysis, was induced to similar levels in response to JA in both *ORA59*-silenced and control plants.

To verify that the silencing effects of the RNAi construct on defense gene expression were specific for ORA59, the mRNA levels of *ERF1* and *AtERF2*, encoding two possibly functionally equivalent transcription factors, were determined (Fig. 4A). The JA- and ethephon-induced gene expression levels of *ERF1* and *AtERF2* in the *ORA59*-silenced line were similar to those observed in the control line. ERF1 and ORA59 form a small subgroup within group IX of the ERF family, which includes a third protein, AtERF15, encoded by gene *At2g31230* (Nakano et al., 2006). AtERF15 shares 63% and 42% amino acid identity over the entire protein lengths with ORA59 and ERF1, respectively. At the nucleotide level, the *ORA59* and *AtERF15* coding regions share 69% identity, making it difficult to design a specific probe for hybridization or even to design specific primers for reverse transcription-PCR. Therefore, we analyzed gene expression using gene-specific primers corresponding to the 3′ untranslated regions. The results in Figure 4B show that, in the *GUS* control lines, the *AtERF15* mRNA level was induced about 3-fold by treatment with JA combined with ethephon for 6 h, compared with 10-fold induction of *ORA59* mRNA accumulation. The *ORA59* mRNA level after hormone treatment of the silenced lines was lower than the uninduced level in the GUS control lines. The *AtERF15* mRNA level was reduced 1.4-fold in the silenced lines compared with the GUS control lines, indicating that the *ORA59* silencing construct had some effect on the *AtERF15* mRNA level. However, *AtERF15* mRNA was still detectable and there was still an approximately 3-fold increase in response to hormone treatment.

Taken together, these results demonstrate that ORA59 is responsible for the activation of a subset of JA- and ethylene-responsive genes, including *PDF1.2*, *ChiB*, and *HEL*, and that ORA59 is an essential node of convergence of the concomitant activation of the JA and ethylene signal transduction pathways, which is absolutely required for the expression of these genes.

ORA59 Controls Resistance against the Necrotrophic Fungus *B. cinerea*

In Arabidopsis, the JA and ethylene signal transduction pathways are involved in resistance against the necrotrophic fungus *B. cinerea* (Thomma et al., 1998, 1999a). The regulation by ORA59 of JA- and ethylene-responsive defense-related genes prompted us to test whether constitutive expression or silencing of *ORA59* would affect resistance to this pathogen.

For disease resistance tests, transgenic lines constitutively overexpressing the *ORA59* gene from the

CaMV 35S promoter were constructed. In general, independent 35S:*ORA59* lines showed a severe dwarf phenotype under normal growth conditions (Supplemental Fig. S3), similar to plants overexpressing *ERF1* (Solano et al., 1998). Mature leaves of soil-grown wild-type plants, JA-insensitive *coi1-1* mutant plants, and transgenic 35S:*ORA59* and RNAi-*ORA59* plants at comparable developmental stages were inoculated at the same time with *B. cinerea*. In order to reach a plant size similar to the other genotypes, the 35S:*ORA59* plants were allowed to grow for 7 weeks before inoculation, instead of 5 weeks for all other genotypes, allowing a more reliable comparison of disease symptoms among plants with similar leaf sizes (Supplemental Fig. S3). Percentages of disease severity classes for each genotype at 4 d after inoculation are presented in Figure 5A. Infected wild-type leaves were relatively tolerant of *B. cinerea* at the applied inoculum density, while *coi1-1* plants showed increased susceptibility, with a large percentage of leaves with spreading necrotic lesions, consistent with previous findings (Thomma et al., 1998). Interestingly, 35S:*ORA59* plants showed enhanced resistance to *B. cinerea*, with a majority of leaves with no or mild symptoms, whereas infected leaves from *ORA59*-silenced plants mainly developed spreading necrotic lesions (Fig. 5A). These results demonstrate that ORA59 plays an important role in resistance to *B. cinerea* and that overexpression of *ORA59* increases resistance to this fungus. For gene expression analyses, RNA was extracted from primary infected (local) and distal (systemic) leaves collected 2 and 4 d after inoculation. Inoculation of wild-type plants resulted in an increase in the *ORA59* mRNA level both locally and systemically (Fig. 5B). In the *coi1-1* mutant, *B. cinerea* infection did not result in the induction of *ORA59*, showing that fungus-induced *ORA59* expression was mediated by the JA signal transduction pathway. Also, the expression of the ORA59 target genes was either not induced (*PDF1.2* and *ChiB*) or strongly reduced (*HEL*) in *coi1-1* plants. In the *ORA59*-silenced lines, which showed enhanced susceptibility to *B. cinerea*, expression of these defense genes was strongly reduced in response to *B. cinerea*, whereas in 35S:*ORA59* plants, *PDF1.2*, *HEL*, and *ChiB* mRNA levels were constitutively high. The *actin* (*actA*) gene from *B. cinerea* was used as a molecular marker of disease progression. As shown in Figure 5B, elevated levels of *actA* mRNA were detected in locally infected leaves of the susceptible *coi1-1* and RNAi-*ORA59* plants after 4 d, indicative for growth of the pathogen *B. cinerea*. Hybridization with the *ROC* gene, encoding a cytosolic cyclophilin, showed equal loading of RNA. The infection experiment was repeated three times with similar results. To rule out the possibility that the differences in resistance between plants overexpressing ORA59 and other plant genotypes may be due to differences in plant age (7 versus 5 weeks) instead of *ORA59* expression levels, we also performed the disease resistance assay with plants that were all 5 weeks old. Although the small size of ORA59-overexpressing plants made scoring of large spreading lesions difficult, the results were essentially the same as those shown in Figure 5A (Supplemental Fig. S3D). These results show that there is a tight correlation between the *ORA59* expression level, defense gene expression, and resistance against *B. cinerea*.

To test whether ORA59 also controls defense gene expression in response to infection with other fungi, 5-week-old wild-type plants, *ORA59*-silenced plants, *pad3-1* mutant plants impaired in camalexin biosynthesis (Glazebrook and Ausubel, 1994), and JA-insensitive *coi1-1* mutant plants were inoculated with *A. brassicicola*. RNA-blot analyses of infected and systemic leaves revealed that *A. brassicicola* infection induced *ORA59* gene expression in wild-type plants both locally and systemically (Fig. 5C). In *coi1-1* mutant plants and *ORA59*-silenced plants, the expression levels of *ORA59* and its target gene *PDF1.2* in response to *A. brassicicola* were strongly reduced (Fig. 5C). Disease ratings were assessed at 7 d after inoculation. In comparison with wild-type plants, the *pad3-1* and *coi1-1* mutant plants developed severe symptoms upon *A. brassicicola* inoculation (data not shown), confirming previous findings showing that these mutants have enhanced susceptibility toward this pathogen (Thomma et al., 1998, 1999b). In contrast to these mutants, the level of basal resistance against *A. brassicicola* in *ORA59*-silenced plants did not differ from that of wild-type plants (data not shown).

Thus, although ORA59 is required for the expression of *PDF1.2* and presumably of other ORA59 target genes after *A. brassicicola* infection, ORA59 and its target genes do not play an important role in the resistance against this pathogen under these experimental conditions.

Which AP2/ERF Domain Transcription Factors Regulate *PDF1.2*?

Our results show that ORA59 is a crucial regulator of several defense-related genes, including *PDF1.2*. *ORA59* loss-of-function studies revealed that no other AP2/ERF domain transcription factor or member of another class of transcriptional regulators was able to activate the expression of *PDF1.2* in response to JA (Fig. 3) or JA + ethephon treatments (Fig. 4) or to infection with *B. cinerea* or *A. brassicicola* (Fig. 5). These results appear to contradict a previous report showing that constitutive overexpression of the AP2/ERF domain transcription factor *ERF1* gave rise to increased *PDF1.2* gene expression (Solano et al., 1998). Constitutive overexpression of another AP2/ERF domain transcription factor, *AtERF2*, was also reported to lead to an increase in *PDF1.2* gene expression (Brown et al., 2003; McGrath et al., 2005). Similarly, constitutive overexpression of *AtERF1*, a close homolog of *AtERF2*, led to high *PDF1.2* expression levels (Fig. 6A). One possible explanation for the apparent discrepancy with our ORA59 results is that overexpression of *ERF1*, *AtERF1*, or *AtERF2* causes a stress condition,

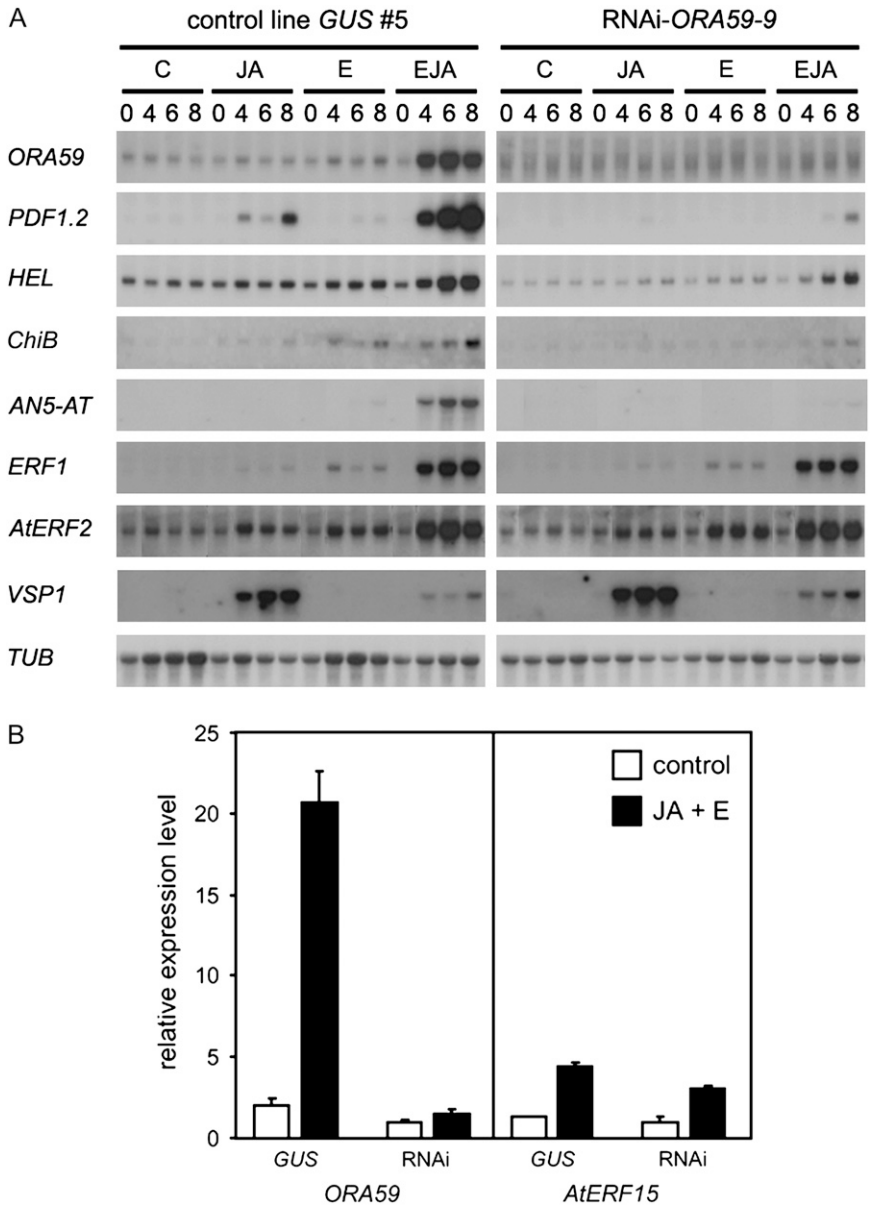

Figure 4. Specific silencing of the *ORA59* gene severely compromises the JA- and/or ethylene-responsive expression of several defense genes. A, Two-week-old seedlings from the representative RNAi-*ORA59* line 9 or from the control line *GUS* 5 were treated for the number of hours indicated with 50 μM JA, 1 mM ethephon (E), a combination of both (EJA), or the solvents (C). The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading. B, Relative mRNA levels corresponding to *ORA59* and its closest homolog *AtERF15*. Two-week-old seedlings from *GUS* control lines 5 and 6 and from RNAi-*ORA59* lines 4 and 9 were treated with 50 μM JA and 1 mM ethephon (E) for 6 h. Control treatments consisted of addition of the solvents. RNA samples were DNaseI treated, reverse transcribed, and PCR amplified for 20 cycles using primers specific for the *ORA59, AtERF15,* and *Actin7* genes. *ORA59* and *AtERF15* band intensities were related to the corresponding *Actin7* band intensities. Corresponding values from each set of two lines were averaged and expressed relative to the values from control-treated RNAi-*ORA59* lines arbitrarily set to 1.

manifested also by the dwarf phenotype, that leads indirectly to *PDF1.2* expression. To address the question of which AP2/ERF domain proteins directly control the activity of the *PDF1.2* promoter, we used two different approaches. In one approach, we measured the expression of the endogenous *PDF1.2* gene in stably transformed Arabidopsis plants expressing *ORA59*, *ERF1*, *AtERF1*, or *AtERF2* in an estradiol-inducible manner. Under noninduced conditions, the *ERF* transgene is silent. We reasoned that the relatively short period of transgene expression in response to estradiol treatment is unlikely to cause a general stress condition and that, therefore, nonspecific activation of *PDF1.2* gene expression is less likely to occur in these plants. As shown in Figure 6B, estradiol treatment effectively induced the expression of the *ORA59*, *ERF1*, *AtERF1*, and *AtERF2* transgenes in the different lines to essentially similar levels. The XVE-*ORA59-TAP* and XVE-*ERF1-TAP* lines carry an inducible expression module of the *ORA59* and *ERF1* genes, respectively, fused to a *TAP* tag (Puig et al., 2001). Expression of the *PDF1.2* gene was only induced by estradiol in XVE-*ORA59*, XVE-*ORA59-TAP*, and XVE-*ERF1-TAP* lines. The transcript level of the *PDF1.2* gene in the estradiol-treated XVE-*ORA59-TAP* line was similar to that in the XVE-*ORA59* line, indicating that ORA59 activity was not significantly affected by the fusion with a TAP tag. Similarly, ERF1 activity appeared to be preserved in the TAP fusion protein. Two to three independent lines per construct were tested with essentially similar results (data not shown). In general, we observed that the *PDF1.2* gene expression level was slightly higher in XVE-*ORA59-TAP* lines compared with XVE-*ERF1-TAP* lines. In a second approach, we analyzed the ability of

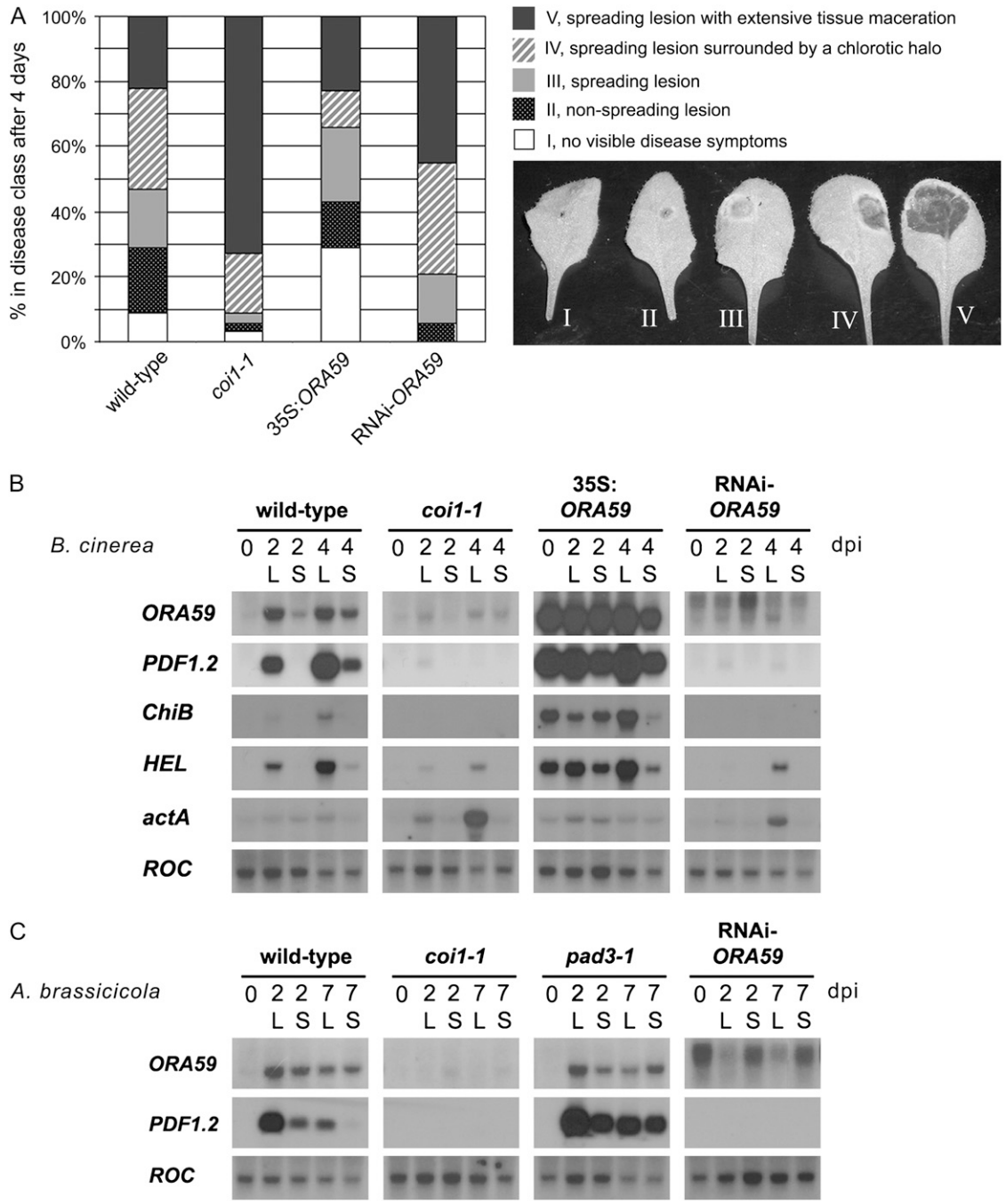

Figure 5. ORA59 is involved in resistance against *B. cinerea*. A, Distribution of disease severity classes in wild-type plants, *coi1-1* mutant plants, and transgenic plants overexpressing (35S:*ORA59*) or silencing (RNAi-*ORA59*) the *ORA59* gene. Disease rating was scored on plants with comparable leaf sizes grown as described in "Materials and Methods," with phenotypes as shown in Supplemental Figure S3C for ORA59-overexpressing plants and in Supplemental Figure S3B for the other genotypes, at 4 d after inoculation with *B. cinerea*. Disease rating is expressed as the percentage of leaves falling in the disease severity classes as follows: I, no visible disease symptoms; II, nonspreading lesion; III, spreading lesion; IV, spreading lesion surrounded by a chlorotic halo; and V, spreading lesion with extensive tissue maceration and sporulation by the pathogen. Data were collected from 60 to 100 leaves derived from 15 to 20 plants per genotype. B, Infected local (L) and noninfected systemic (S) leaves from several inoculated plants of each genotype were collected at days 0, 2, and 4 after inoculation (dpi) with *B. cinerea* and RNA was extracted. C, Infected local and noninfected systemic leaves from several inoculated plants of each genotype were collected at days 0, 2, and 7 after inoculation with *A. brassicicola* and RNA was extracted. The RNA gel blots were hybridized with the indicated probes. The *ROC* probe was used to verify RNA loading.

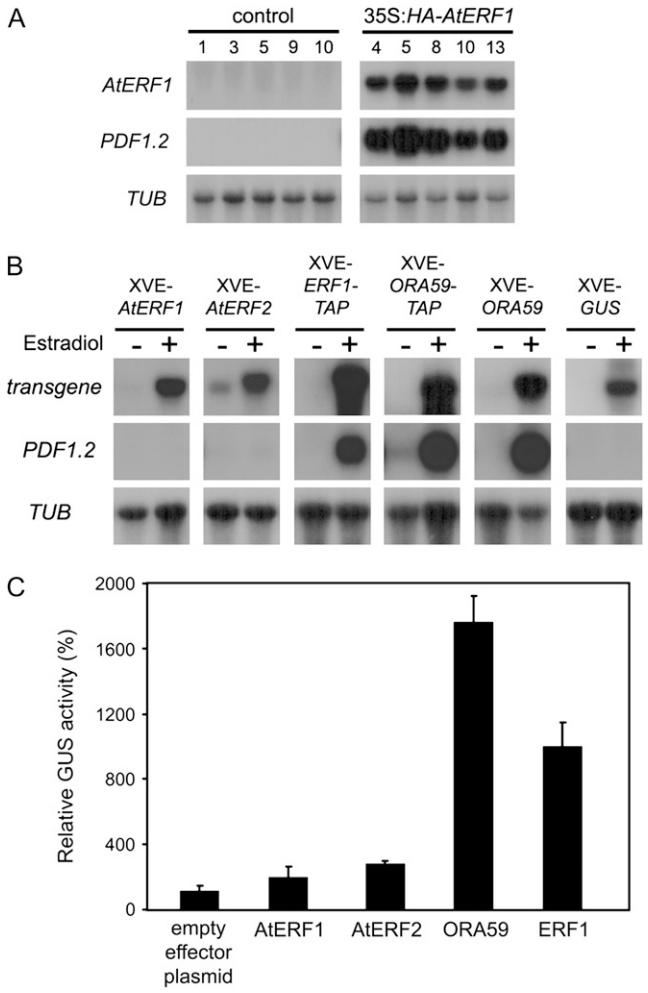

Figure 6. ORA59 and ERF1 are activators of *PDF1.2* gene expression in planta. A, *PDF1.2* gene expression in *AtERF1*-overexpressing plants. RNA was extracted from 10-d-old seedlings from several independent 35S:*HA-AtERF1* and control lines (indicated by numbers). B, Estradiol-induced *PDF1.2* gene expression in stably transformed XVE lines. Two-week-old seedlings from transgenic lines carrying the *AtERF1*, *AtERF2*, *ORA59*, *GUS*, *TAP*-tagged *ORA59*, or *TAP*-tagged *ERF1* gene under the control of the inducible XVE system were treated for 24 h with estradiol (+) or the solvent DMSO (−). The top panels were hybridized with separate *ERF* gene or *GUS* probes and exposed for 8 to 24 h. Expression levels cannot be directly compared but are similar within a 3-fold range. The *PDF1.2* and *TUB* panels were hybridized on the same blot, and expression levels can be directly compared among individual transgenic lines. The *TUB* probe was used to verify RNA loading. C, Arabidopsis protoplasts were cotransformed with a reporter plasmid carrying *PDF1.2* promoter-*GUS* and effector plasmids carrying the *AtERF1*, *AtERF2*, *ERF1*, or *ORA59* gene fused to the 35S promoter. GUS activities are shown as percentages of the empty effector plasmid value. A reference plasmid carrying the *Renilla LUC* gene fused to the 35S promoter was cotransformed to correct for transformation and protein extraction efficiencies. Values represent means ± SE of triplicate measurements.

ORA59, ERF1, AtERF1, and AtERF2 to transactivate the *PDF1.2* promoter in transient expression assays. Arabidopsis protoplasts were cotransformed with a reporter plasmid carrying the *PDF1.2* promoter fused to the *GUS* reporter gene and an effector plasmid carrying *ORA59*, *ERF1*, *AtERF1*, or *AtERF2* genes fused to the CaMV 35S promoter. *GUS* reporter gene activity was increased about 17- and 10-fold upon cotransformation with *ORA59* and *ERF1* effector plasmids, respectively, compared with the empty effector plasmid (Fig. 6C). Relatively insignificant activation of the *GUS* reporter gene was observed with effector plasmids carrying the *AtERF1* or *AtERF2* gene.

Both experimental approaches demonstrate that ORA59 and ERF1 are able to function as activators of the *PDF1.2* promoter, whereas AtERF1 and AtERF2 do not activate the *PDF1.2* promoter when inducibly or transiently expressed.

DISCUSSION

JA is a key regulatory signaling molecule in plant defense. An important aspect of JA action is its synergistic interaction with ethylene in the induction of a subset of defense-related genes. In this study, we investigated the function of ORA59, a member of the ERF transcription factor family in Arabidopsis. We demonstrated that ORA59 integrates JA and ethylene signal inputs. By doing so, ORA59 controls the expression of a subset of JA- and ethylene-dependent genes, including *PDF1.2*. Expression of these genes in response to these signals, or after perception of certain pathogens, depends on ORA59, and no other transcription factor, including ERF1 or AtERF2, can bypass the requirement for ORA59.

The current commonly accepted model pointed to ERF1 as a key element in the integration of JA and ethylene signals for the regulation of defense genes in response to pathogens (Lorenzo et al., 2003; Gfeller et al., 2006). Several similarities exist between the transcription factors ORA59 and ERF1: (1) family-wide phylogenetic analysis using the conserved AP2/ERF domain showed that ORA59 and ERF1 group closely together in group IX of the ERF proteins (Nakano et al., 2006); (2) expression of the *ORA59* and *ERF1* genes is controlled by the JA and ethylene pathways (Fig. 1; Lorenzo et al., 2003); (3) ORA59 and ERF1 regulate a similar subset of JA- and/or ethylene-responsive defense-related genes when overexpressed (including *PDF1.2*, *ChiB*, and *HEL*; Supplemental Table S1; Lorenzo et al., 2003); (4) infection with the necrotrophic fungus *B. cinerea* induces the *ORA59* and *ERF1* genes (Fig. 5B; Berrocal-Lobo et al., 2002); and (5) overexpression of *ORA59* and *ERF1* leads to increased resistance to *B. cinerea* (Fig. 5A; Berrocal-Lobo et al., 2002). In activation assays using two different approaches, ORA59 and ERF1 were able to activate transcription from the *PDF1.2* promoter, whereas AtERF2, or its close homolog AtERF1, two other transcription factors within group IX of the ERF family, did not significantly activate *PDF1.2* promoter activity.

Taken together, these data suggest functional redundancy between ORA59 and ERF1 in JA- and ethylene-

dependent defense responses. However, the finding that expression of ORA59-regulated genes, including *PDF1.2*, in response to JA and/or ethylene treatments was severely compromised in *ORA59*-silenced plants revealed the essential role of ORA59. Moreover, plants silencing the *ORA59* gene showed increased susceptibility to *B. cinerea* infection, presumably by impaired expression of the ORA59-regulated defense genes, further supporting the crucial role of ORA59 in defense. The results also demonstrate that ERF1 alone is not sufficient to support wild-type levels of defense gene expression in response to JA and/or ethylene or to *B. cinerea* or *A. brassicicola* infection.

Our findings are summarized in the model in Figure 7. The specific function of ERF1 in JA and ethylene signaling is still unclear. ERF1 has been suggested to be part of a linear ethylene signal transduction cascade in which the transcription factor EIN3 was suggested to induce *ERF1* gene expression in response to ethylene (Solano et al., 1998). It is unlikely that ORA59 functions upstream or downstream from ERF1 in such a linear cascade, since *ORA59* and *ERF1* gene expression was unchanged in estradiol-induced XVE-*ERF1* and XVE-*ORA59* transformants, respectively (data not shown). One possibility is that ERF1 and ORA59 have separate specialized functions, for example, by differential expression of the corresponding genes in certain cell types or at certain developmental stages. Such a scenario is not supported by our data, since the effects of ERF1 (Lorenzo et al., 2003) and ORA59 on defense gene expression were measured at very similar developmental stages and in identical tissues (i.e. in young whole seedlings grown in tissue culture and in leaves of several-week-old soil-grown plants). It should be noted that defense gene expression in response to JA and ethylene is not completely abolished in RNAi-*ORA59* lines. The residual expression level might be mediated by ERF1 and/or AtERF15. Additional experiments using *ERF1* and *AtERF15* knockout plants and *ORA59/ERF1/AtERF15* double and triple knockout plants are needed to properly assess ERF1 and AtERF15 functions.

The signal transduction cascade integrating ethylene and JA response pathways plays important biological functions in plants, in particular in microbial disease resistance responses. The whole purpose of signal transduction in cells is to activate a transcription factor, which in turn regulates the expression of response genes. The identification of a crucial transcription factor that integrates the JA and ethylene signals lies at the heart of a comprehensive understanding of the transduction of these signals. Our results add an important molecular component involved in JA signal transduction and in cross talk between JA and ethylene, as described among others in the Science Signal Transduction Knowledge Environment (Gfeller et al., 2006), and will have consequences for future studies on more upstream signal transduction components.

One question to be addressed in future studies is whether ORA59 is a crucial link in JA/ethylene-responsive COI1-dependent gene expression solely because it needs to be synthesized de novo upon induction of *ORA59* gene expression or whether there is an additional level of regulation acting on ORA59 protein activity. The JA-responsive transcription factor AtMYC2, which regulates a distinct subset of JA-responsive COI1-dependent genes, is also synthesized de novo in response to JA via gene induction, but it is additionally regulated at the protein level by interaction with putative repressors belonging to the jasmonate ZIM domain (JAZ) family (Chini et al., 2007). The JAZ proteins are degraded in a COI1-dependent manner in response to JA (Chini et al., 2007; Thines et al., 2007). Whether ORA59 interacts with members of the JAZ protein family or with members of a distinct class of regulatory proteins is an open question at the moment.

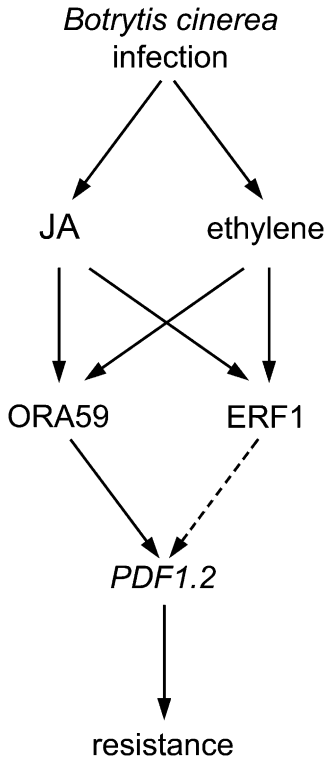

Figure 7. Model for the involvement of ORA59 and ERF1 in the defense of Arabidopsis plants against the necrotrophic fungus *B. cinerea*. In wild-type plants, infection with *B. cinerea* activates both JA and ethylene signaling pathways and leads to the expression of the *ORA59* and *ERF1* genes, encoding related ERF transcription factors. In infected *ORA59*-silenced lines, the absence of ORA59 results in impaired expression of the *PDF1.2* gene as well as increased susceptibility to the pathogen. The broken arrow indicates that the assessment of the role of ERF1 in regulating defense genes under the conditions indicated in the diagram requires analysis of an *erf1* knockout mutant.

MATERIALS AND METHODS

Biological Materials, Growth Conditions, and Treatments

Arabidopsis (*Arabidopsis thaliana*) wild-type plants, *coi1-1*, *ein2-1*, and *pad3-1* mutants, and all transgenic plants are in the genetic background of ecotype Columbia Col-0.

Surface-sterilized seeds were grown for 10 d at 21°C in a growth chamber (16-h-light/8-h-dark photoperiod at 200 μE m^{-2} s^{-1} at 70% relative humidity) on a modified half-strength Murashige and Skoog medium (MA medium) solidified with 0.6% agar. Fifteen to 20 seedlings per sample were transferred to 50-mL polypropylene tubes (Sarstedt) containing 10 mL of MA medium and incubated on a shaker at 120 rpm for an additional 4 d before treatment. Seedlings were treated with 50 μM JA (Sigma-Aldrich), 1 mM ethephon (an ethylene-releasing compound; Sigma), or a combination of JA and ethephon. As controls, seedlings were treated with the respective solvents dimethyl sulfoxide (DMSO; 0.1%), sodium phosphate, pH 7 (0.5 mM), or a combination of both. Homozygous JA-insensitive *coi1-1* seedlings were selected on MA medium containing 50 μM JA. Transgenic plants carrying an XVE expression module were treated with 2 μM estradiol (Sigma) dissolved in DMSO. In order to reach similar expression levels of the transgene, transgenic XVE-*ORA59-TAP* and XVE-*ERF1-TAP* plants were treated with 4 μM estradiol. Induction treatment with methyl jasmonate was performed by dipping 5-week-old soil-grown plants in an aqueous solution containing 50 μM methyl jasmonate (Serva, Brunschwig Chemie) and 0.01% Silwet L-77. Control plants were dipped in 0.01% Silwet L-77.

Constructs and Plant Transformation

For the constitutive overexpressing plants, the *ORA59* (*At1g06160*) and hemagglutinin-tagged *AtERF1* (*At4g17500*) open reading frames were cloned into pRT101 in front of the CaMV 35S promoter. The 35S cassettes were cloned into the binary vector pCAMBIA1300 (http://www.cambia.org). The binary vector pCAMBIA1301 carrying the *GUS* gene under the control of the CaMV 35S promoter was used to generate control lines (1301 lines).

For the RNAi-*ORA59* lines, the *ORA59* open reading frame was cloned as an inverted repeat into the pHANNIBAL vector (GenBank accession no. AJ311872). For the RNAi control line *GUS* 5, the *GUS* open reading frame was cloned into pHANNIBAL. The pHANNIBAL expression cassettes were cloned into the binary vector pART27.

For the estradiol-inducible XVE lines, the *ORA59*, *AtERF1*, *AtERF2* (*At5g47220*), *GUS*, and C-terminal *TAP*-tagged *ORA59* and *ERF1* (*At3g23240*) open reading frames were cloned into the binary vector pER8. Details of plasmid construction are described in Supplemental Materials and Methods S1.

Binary vectors carrying the different constructs were introduced into *Agrobacterium tumefaciens*, and plants were transformed by floral dip. Transgenic plants were selected on MA medium containing 100 mg L^{-1} timentin and 20 mg L^{-1} hygromycin, except for pART27 transformants, which were selected on 100 mg L^{-1} timentin and 25 mg L^{-1} kanamycin.

The XVE-*ORA59*;*coi1-1* plants were obtained by fertilizing homozygous *coi1-1* ovules with pollen from transgenic XVE-*ORA59* plants. Heterozygous *coi1*/COI1 F1 siblings containing the transgene were selected on MA medium containing 20 mg L^{-1} hygromycin and were allowed to self-pollinate. F2 siblings homozygous for the *coi1* mutation and carrying the XVE-*ORA59* transgene were selected on MA medium containing 50 μM JA and 20 mg L^{-1} hygromycin.

Plant Infection

All genotypes were grown for 2 weeks on solid MA medium before transferring to sterile soil and cultivated for another 3 weeks at 24°C in a growth chamber (8-h-light/16-h-dark photoperiod at 200 μE m^{-2} s^{-1}) at 70% relative humidity. Because of the early-stage dwarf phenotype, seeds from transgenic line 35S:*ORA59 17* were sown 2 weeks earlier than the other genotypes. Therefore, soil-potted plants from this genetic background were allowed to grow for 5 weeks in order to reach a stage with rosette leaf size suitable for pathogen infection (Supplemental Fig. S3).

Botrytis cinerea and *Alternaria brassicicola* were grown on potato dextrose agar plates for 2 weeks at 22°C. Spores were harvested as described by Broekaert et al. (1990). Spores from *B. cinerea* were incubated in half-strength potato dextrose broth for 2 h prior to inoculation. For inoculation with *B. cinerea* and *A. brassicicola*, 3-μL droplets of spore suspension (5×10^5 and 1×10^6 spores mL^{-1}, respectively) were deposited on four to six leaves of each plant (15–20 plants per genotype). *B. cinerea*-infected leaves were gently wounded with a needle where the droplet was deposited. After inoculation, plants were maintained under high relative humidity with the same temperature and photoperiod conditions.

Disease ratings were assigned to the inoculated leaves of each plant, as indicated in the legend to Figure 5. A Pearson χ^2 test was used to evaluate whether distributions of disease ratings were statistically different between the wild type and other genotypes. For gene expression analysis, infected and noninfected leaves from several inoculated plants of each genotype were collected at days 2 and 4 after inoculation with *B. cinerea* and at days 2 and 7 after inoculation with *A. brassicicola*.

Gene Expression Analyses

Total RNA was extracted from frozen tissue by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% ethanol and resuspended in water. Ten-microgram RNA samples were subjected to electrophoresis on 1.5% agarose/1% formaldehyde gels and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Sciences). DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. Primer sequences are listed in Supplemental Materials and Methods S1. For reverse transcription-PCR, aliquots of 20 μg of total RNA were treated with DNaseI, phenol extracted, and precipitated with ethanol. RNA integrity was verified on gel. Aliquots of 4 μg of DNaseI-treated RNA were reverse-transcribed with oligo$(dT)_{12-18}$ and Moloney murine leukemia virus reverse transcriptase (Promega). Aliquots corresponding to 0.16 μg of reverse-transcribed RNA were amplified for 20 cycles of 1 min at 92°C, 45 s at 50°C, and 45 s at 72°C using SuperTaq (SphaeroQ) and primers specific for the *ORA59*, *AtERF15*, and *Actin7* (At5g09810) genes. In pilot experiments with increasing cycle numbers, it was determined that 20 cycles were well within the linear range of amplification for all three genes. Control PCR containing equivalent amounts of DNaseI-treated RNA that was not reverse transcribed did not yield bands. Aliquots corresponding to 0.06 μg of reverse-transcribed and PCR-amplified RNA were run on 5% polyacrylamide gels. Ethidium bromide-stained bands were recorded on a Bio-Rad GelDoc XR system and were quantified using Bio-Rad Quantity One software. Details of microarray experiments are given in Supplemental Materials and Methods S1.

Transient Expression Assays

Protoplasts prepared from an Arabidopsis Columbia cell suspension were cotransformed with a reporter plasmid carrying *PDF1.2* promoter-*GUS*, effector plasmids carrying *AtERF1*, *AtERF2*, *ERF1*, or *ORA59* genes fused to the CaMV 35S promoter, and a reference plasmid carrying the *Renilla LUCIFERASE* (*LUC*) gene under the control of the CaMV 35S promoter. As controls, *PDF1.2* promoter-*GUS* was cotransformed with the corresponding empty effector vectors. Protoplasts were transformed with the three constructs in a ratio of 1:1:3 (*GUS*:*LUC*:effector plasmid). Protoplasts were harvested at 18 h after transformation and frozen in liquid nitrogen. *GUS* reporter gene expression was related to *LUC* expression to correct for transformation and protein extraction efficiency. Average GUS-LUC ratios from triplicate experiments were expressed relative to the respective vector controls.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Venn diagrams comparing putative ORA59 target genes with JA- and JA/ethylene-responsive genes detected in genome-wide transcriptome analyses.

Supplemental Figure S2. Verification with RNA-blot analyses of putative ORA59 target genes.

Supplemental Figure S3. *ORA59*-overexpressing plants show a dwarf phenotype.

Supplemental Table S1. Microarray data of genes up-regulated by ORA59.

Supplemental Materials and Methods S1. Details of microarray analyses, DNA cloning procedures, plant transformation, transient expression assays, and PCR primer sequences.

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