ORA EST : functional analysis of jasmonate-responsive AP2/ERF-domain transcription factors in Arabidopsis thaliana

Pré, M.

Chapter 2

The Arabidopsis AP2/ERF-domain transcription factor ORA59, and not ERF1, integrates jasmonate and ethylene signal inputs in plant defense

Martial Pré, Mirna Atallah, Antony Champion, Martin de Vos, Corné M.J. Pieterse, and Johan Memelink

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Abstract

Plant defense against pathogen attack depends on the action of several endogenously produced secondary signaling molecules, including jasmonic acid (JA), ethylene and salicylic acid. In certain defense responses ethylene and jasmonate signaling pathways synergize to activate specific sets of defense genes. Here we describe the role of the Arabidopsis AP2/ERF-domain transcription factor ORA59 in ethylene and jasmonate signaling. *ORA59* (*At1g06160*) gene expression was induced by JA or ethylene, and synergistically induced by a combination of both hormones. Such induced expression required both JA and ethylene signaling pathways simultaneously. Overexpression of *ORA59* activated the expression of several JA- and ethylene-responsive defense-related genes, including the plant defensin gene *PDF1.2*, and caused increased resistance against the necrotrophic fungus *Botrytis cinerea.* In *ORA59*-silenced plants, expression of *PDF1.2* and other defense-related genes was blocked in response to JA and/or ethylene, or after infection with *B. cinerea* or *Alternaria brassicicola*. Moreover, these plants were also more susceptible to *B. cinerea* infection. Several AP2/ERF-domain transcription factors have been suggested to be positive regulators of *PDF1.2* gene expression. Here, we found that only ORA59 and ERF1 were able to function as transcriptional activators of *PDF1.2* gene expression, whereas AtERF2 and the related AtERF1 were not. Our results demonstrate that ORA59 is an essential integrator of the JA and ethylene signal transduction pathways, and thereby provide new insight in the nature of the molecular components involved in the crosstalk between these two hormones.

Introduction

Plant fitness and survival is dependent on the ability to mount fast and highly adapted responses to diverse environmental stress conditions including attack by herbivores or microbial pathogens. Perception of stress signals results in the production of one or more of the secondary signaling molecules jasmonic acid, ethylene, salicylic acid (SA) and abscisic acid.

Jasmonic acid belongs to a family of signaling molecules including certain precursors and derivatives, which are collectively indicated as jasmonates (JAs). Besides their roles in certain aspects of plant growth and development, such as anther dehiscence, fruit ripening and root growth, JAs are major intermediate signaling molecules involved in defense against wounding, attack by herbivores and infection by certain pathogens (Creelman and Mullet, 1997; Turner et al., 2002). Several studies revealed complex cross-talk relationships between

JA, ethylene and SA, which can act synergistically or antagonistically, in order to fine-tune the defense response (Kunkel and Brooks, 2002). Arabidopsis plants impaired in JA or ethylene signaling pathways showed enhanced susceptibility to the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* (Thomma et al., 1998 and 1999a; Penninckx et al., 1996), demonstrating that JA and ethylene are important signal molecules for resistance against these pathogens.

A crucial step in the JA-dependent defense response is the rapid transcription of genes coding for antimicrobial proteins or enzymes involved in the biosynthesis of secondary metabolites. Studying the mechanisms whereby the expression of these defense-related genes is regulated is therefore 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 of the plant-specific APETALA2 (AP2)-domain protein family known as the ethylene response factor (ERF) subfamily. Proteins from this AP2/ERF subfamily are characterized by a single AP2-type DNA-binding domain with a conserved amino acid sequence. Several *AP2/ERF* genes have been shown to be regulated by a variety of stress-related stimuli, such as wounding, JA, ethylene, SA, or infection by different types of pathogens (Liu et al., 1998; Chen et al*.,* 2002; Suzuki et al., 1998; Gu et al., 2000; Menke et al*.,* 1999; van der Fits and Memelink, 2001; Fujimoto et al., 2000; Solano et al., 1998). The transcription factor ERF1 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 (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). Constitutive overexpression of another AP2/ERF-domain transcription factor, AtERF2, was also shown to cause high levels of *PDF1.*2 and *ChiB* gene expression in transgenic Arabidopsis plants (Brown et al., 2003).

Atallah (2005) previously characterized 14 genes encoding AP2/ERF-domain proteins, which were rapidly induced by JA treatment in 10-days-old 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. In addition, expression of the *ORA59* gene was also induced by ethylene, and a combination of both JA and ethylene had a synergistic positive effect on *ORA59* mRNA accumulation (Atallah, 2005). Analysis of *ORA59* gene expression in Arabidopsis mutants revealed the necessity of intact JA and ethylene signaling pathways for JA-responsive expression of *ORA59* (Atallah, 2005). Similar

observations have been made for *ERF1* gene expression (Lorenzo et al., 2003). This suggests that multiple AP2/ERF-domain transcription factors are involved in JA-dependent transcriptional events, as well as in synergism between the JA and ethylene signaling pathways. These transcription factors might regulate distinct subsets of JA- and ethylene-responsive genes in certain cell types, or alternatively, they might be functionally redundant.

In this study, we show that ORA59 is a major component of the jasmonate- and ethylene-controlled regulatory network. Overexpression of *ORA59* induced the expression of a large number of defense-related genes including the *PDF1.2*, *ChiB* and *hevein-like* (*HEL*) genes. Expression of these genes in response to JA and/or ethylene, or after pathogen infection was dramatically reduced in plants showing post-transcriptional silencing of the *ORA59* gene. In addition, infection experiments with *B. cinerea* showed that plant resistance or susceptibility was directly linked to the presence or absence of ORA59, respectively. In transient transactivation assays as well as in transgenic plants inducibly overexpressing various AP2/ERF-domain transcription factors, ORA59 and ERF1, but not AtERF2 and AtERF1, were able to activate the *PDF1.2* promoter. Our findings show that ORA59, and not ERF1 or AtERF2, responds to JA and integrates JA and ethylene signals to regulate the expression of defense genes such as *PDF1.2* and *ChiB*, altering the current view of the molecular components involved in JA-responsive gene expression and in the crosstalk between JA and ethylene.

Results

ORA59 gene expression is controlled by the JA and ethylene signal transduction pathways

Previous analysis of *ORA59* gene expression in Arabidopsis mutants revealed the necessity of intact JA and ethylene signaling pathways for JA-responsive expression of *ORA59* (Atallah, 2005). In this respect, *ORA59* expression is similar to *ERF1* expression (Lorenzo et al., 2003). For *ERF1*, it was shown that ethylene-responsive expression also required intact JA and ethylene signaling pathways (Lorenzo et al., 2003). To establish whether *ORA59* expression was also 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; Feys et al., 1994; Guzman and Ecker, 1990). In accordance with previously reported 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, as described before (Atallah, 2005), a combined treatment with JA and ethephon led to a prolonged super-induction of *ORA59* gene expression. In response to ethephon, *ORA59* gene expression was strongly reduced in both mutants compared to wild-type (Figure 1).

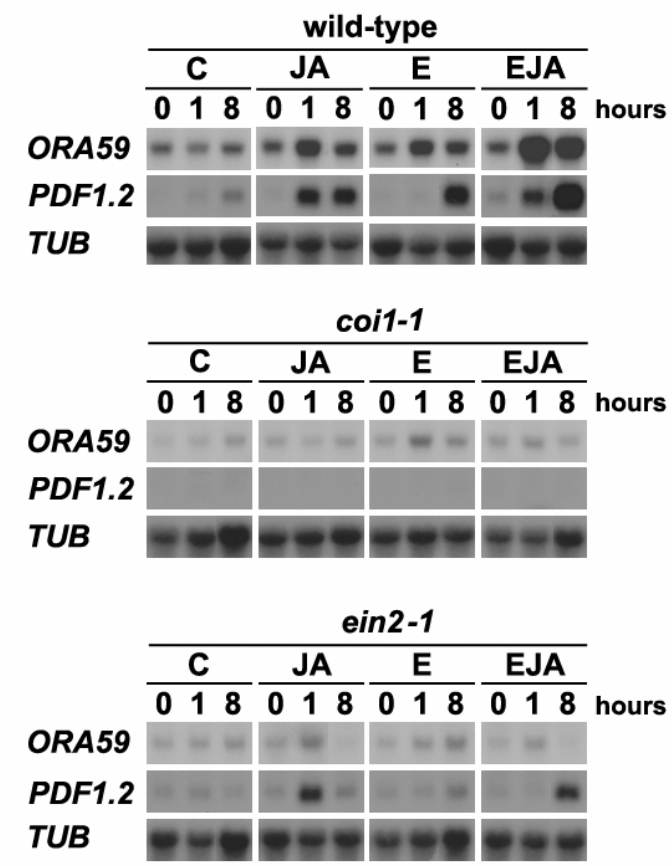

Figure 1. *ORA59* gene expression is controlled by the JA and ethylene signal transduction pathways. RNA was extracted from 14-days-old wild-type or mutant Arabidopsis seedlings treated with 50 µM jasmonic acid (JA), 1 mM ethephon (E; an ethylene releaser), a combination of both (EJA) or with the solvents (C), for the number of hours indicated. The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify the 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.

This indicates that the induction of *ORA59* gene expression by either JA, ethylene or a combination of both requires intact JA as well as ethylene signaling pathways for full responsiveness. Therefore, the responsiveness of the *ORA59* gene to JA and ethylene is

similar to *ERF1* expression in all aspects studied in this experiment. The *PDF1.2* defense-related gene is a well-characterized marker of the JA and ethylene signaling pathways. Expression of *PDF1.2* in response to JA and/or ethylene was similar to *ORA59* gene expression, except that it responded more slowly and less transiently in wild-type plants. Equal amounts of RNA were loaded on the gel as shown by the *β-tubulin* (*TUB*) mRNA level.

Genome-wide identification of putative ORA59 target genes

To characterize the genes regulated by ORA59, a genome-wide transcriptome analysis of *ORA59*-overexpressing plants using the Agilent Arabidopsis 3 Oligo microarray platform, which covers the full Arabidopsis genome, was performed. Two transgenic Arabidopsis lines expressing *ORA59* in an estradiol-inducible manner (XVE-*ORA59*) and two transgenic control lines expressing the *GUS* gene in an inducible manner (XVE-*GUS*) were grown for two weeks in liquid culture. Expression of the transgenes was induced by treating the samples with 2 µM estradiol and RNA was collected after 16 hours. Control samples were treated with the solvent DMSO for the same period of time. Microarray data analyses revealed that 405 genes had increased expression levels of at least 2-fold after induction of *ORA59* gene expression in both lines. From these 405 genes upregulated in XVE-*ORA59* lines, those genes that were also upregulated in one or both of the XVE-*GUS* lines were subtracted, resulting in 140 genes which were specifically upregulated in plants overexpressing the *ORA59* gene. As shown in Table 1, many of these genes are involved in defense against biotic or abiotic stress, signaling, primary and secondary metabolism or coding for other transcription factors. Several defense-related genes, such as *PDF1.2* (*a*, *b* and *c* genes), *HEL* and *ChiB* were highly expressed in plants overexpressing the *ORA59* gene. The expression of these genes is induced by JA or ethylene, and superinduced by a combination of both (Figure 1; Penninckx et al., 1998; Potter et al., 1993; Thomma et al., 1998). 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 hours (Figure 2). From the 140 genes upregulated in XVE-*ORA59* plants, 45% were upregulated in wild-type plants treated with JA for 8 hours (63 genes) while 69% were upregulated in wild-type plants treated with JA for 24 hours (96 genes). For plants treated simultaneously with JA and ethylene, proportions were even higher with 86% and 69% of common genes after 8 hours (121 genes) and 24 hours of treatment (96 genes), respectively (Figure 2).

Table 1. Analysis of microarray data for genes up-regulated in XVE-*ORA59* lines

Gene annotation and putative function [1]	Fold-change: XVE-*ORA59*	wild-type: JA 8 hours	wild-type: JA 24 hours	wild-type: E+JA 8 hours	wild-type: E+JA 24 hours	AGI code
Defense						
chitinase B (ChiB)	34.0	3.3	4.8	7.0	18.7	At2g43580
chitinase	28.4	2.2	3.7	5.6	16.0	At2g43590
chitinase	25.6	2.4	3.7	5.9	13.8	At3g47540
legume lectin	21.2	2.1	5.5	11.8	10.2	At3g15356
legume lectin	16.6	— [2]	5.1	9.1	7.8	At3g16530
patatin	14.5	—	6.0	28.8	12.5	At2g26560
plant defensin PDF1.3	11.6	—	—	16.9	22.8	At2g26010
plant defensin PDF1.2b	11.1	2.5	—	34.2	25.7	At2g26020
avrRpt2-induced AIG2 protein (AIG2)	11.1	3.3	6.4	9.7	9.2	At3g28930
plant defensin PDF1.1	10.8	—	4.4	—	-2.2	At1g75830
plant defensin PDF1.2c	10.6	2.1	6.6	21.1	21.7	At5g44430
pathogenesis-related protein	9.0	—	3.1	2.7	5.4	At4g25780
osmotin-like protein (OSM34)	8.5	—	2.8	3.4	8.2	At4g11650
plant defensin PDF1.2a	7.9	2.2	7.4	31.7	16.9	At5g44420
pathogenesis-related protein	7.7	—	—	—	—	At4g33710
GCN5-related N-acetyltransferase (GNAT)	6.4	—	—	3.3	—	At4g37670
stress-responsive protein	6.0	—	—	2.6	2.8	At5g01410
jacalin lectin	5.8	—	3.4	3.3	—	At1g52130
basic endochitinase	5.5	—	—	—	6.8	At3g12500
jacalin lectin	5.3	—	2.5	3.2	—	At5g49860
hevein-like protein (HEL)	5.0	—	2.8	3.5	5.1	At3g04720
curculin-like (mannose-binding) lectin	4.0	—	2.7	4.0	3.1	At5g18470
disease resistance protein (TIR-NBS class)	3.9	2.5	3.3	4.3	4.5	At1g72910
disease resistance protein (TIR-NBS class)	3.9	4.0	6.2	4.8	4.9	At1g72900
disease resistance protein (TIR-NBS-LRR class)	3.7	—	—	4.3	—	At1g56540
jacalin lectin	3.3	—	—	—	—	At5g38550
avirulence-responsive protein	3.2	2.1	2.0	2.5	2.1	At1g33930
glycine-rich protein	3.1	2.3	2.8	2.7	2.7	At1g02710
legume lectin	3.1	—	—	—	—	At5g03350
Transcription						
ORA59	15.6	—	2.2	5.5	4.4	At1g06160
AP2 domain-containing transcription factor	11.5	3.0	5.0	10.8	7.7	At4g06746
AP2 domain-containing transcription factor	9.9	—	—	4.8	3.7	At2g31230
IAA20	6.7	—	—	—	—	At2g46990
floral homeotic protein APETALA1 (AP1)	5.1	—	4.8	3.0	—	At1g69120
Dof-type zinc finger domain-containing protein	4.8	—	—	—	—	At3g50410
WRKY family transcription factor	4.2	2.7	3.1	3.1	—	At3g56400
no apical meristem (NAM) family protein	3.9	9.8	10.2	14.1	7.8	At2g43000
zinc finger (C3HC4-type RING finger)	3.8	—	—	—	—	At2g26130
zinc finger (C3HC4-type RING finger) family protein	3.4	—	—	—	—	At5g27420
zinc finger (HIT type) family protein	3.0	3.0	3.1	3.1	2.8	At5g63830
Shikimate/Tryptophan metabolism						
tryptophan synthase, beta subunit 2 (TSB2)	4.7	3.0	4.3	5.0	6.0	At4g27070
tryptophan synthase, beta subunit 1 (TSB1)	4.2	2.2	3.5	4.0	4.8	At5g54810
anthranilate synthase beta subunit (ASB1)	3.9	4.3	5.1	8.2	7.5	At1g25220
3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 1 (DHS1)	3.7	6.1	5.5	4.8	5.9	At4g39980
anthranilate synthase beta subunit	3.6	—	5.9	8.6	7.5	At1g24807
indole-3-glycerol phosphate synthase (IGPS)	3.0	2.4	2.6	3.7	4.3	At2g04400

List of genes upregulated in XVE-*ORA59* transgenic lines overexpressing the *ORA59* gene and their fold-change ratios in wild-type plants after hormone treatment.

All genes listed had a fold-change ≥ 2 (P-values ≤ 0.001) in both XVE-*ORA59* transgenic lines overexpressing *ORA59* compared to non-induced XVE-*ORA59* lines. For each gene, the fold-change in wild-type plants treated with jasmonic acid (JA) or with a combination of ethephon and JA (E+JA) compared to control-treated plants, is also indicated. In bold are the genes whose expression was confirmed by RNA blot analyses.

[1]Annotations are as given by the MIPS Arabidopsis thaliana Genome Database (MAtDB; http://mips.gsf.de/proj/thal/db/index.html) except for *ORA59*.

[2]Fold-change values with P-values ≥ 0.001 were considered statistically non-reliable and therefore are represented by a bar (-).

Table 1 (continued). Analysis of microarray data for genes up-regulated in XVE-*ORA59* lines

Gene annotation and putative function [1]	Fold-change XVE-*ORA59*	wild-type JA 8 hours	wild-type JA 24 hours	wild-type E+JA 8 hours	wild-type E+JA 24 hours	AGI code
Signaling						
transducin family protein / WD-40 repeat family protein	3.9	3.2	3.5	3.4	3.2	At5g58760
mitogen-activated protein kinase kinase (MAPKK7)	3.9	4.1	5.9	16.3	9.5	At1g18350
mitogen-activated protein kinase kinase (MAPKK9)	3.4	4.4	5.5	13.7	9.4	At1g73500
Protein modification / synthesis / degradation / transport						
phosphorylase	17.6	13.3	23.0	20.6	17.9	At4g24350
phosphorylase	12.9	12.9	24.0	17.0	14.3	At4g24340
protein kinase	10.7	—	2.7	5.7	6.1	At5g10520
expressed protein similar to phosphatase	9.5	—	—	—	—	At1g73010
protein kinase	5.0	—	—	—	—	At2g23770
proteasel (pfpI)-like protein (YLS5)	4.9	—	2.5	3.5	5.0	At2g38860
cysteine proteinase	4.4	—	—	—	—	At4g36880
protein disulfide isomerase	3.3	2.4	2.9	2.7	3.2	At1g21750
mitochondrial import inner membrane translocase subunit	3.2	2.2	2.5	3.1	—	At1g17530
serine/threonine protein kinase	3.1	3.0	3.0	3.4	3.2	At1g66880
polyubiquitin (UBQ4)	3.1	2.1	2.6	3.0	2.5	At5g20620
Ran-binding protein 1a (RanBP1a)	3.0	2.4	2.4	2.6	2.6	At1g07140
Primary Metabolism/ Secondary metabolism						
anthocyanin 5-aromatic acyltransferase (AN5-AT)	26.8	2.2	7.7	17.7	14.8	At5g61160
UDP-glucoronosyl/UDP-glucosyl transferase family protein	23.6	2.8	3.4	15.3	12.9	At1g07260
2-oxoacid-dependent oxidase, putative (DIN11)	12.5	14.9	56.1	36.5	35.3	At3g49620
aldo/keto reductase	11.2	—	—	8.2	—	At1g59950
glycosyl hydrolase	10.4	—	2.9	5.2	7.0	At4g16260
pyruvate decarboxylase	7.9	—	—	4.4	4.4	At5g54960
S-adenosyl-L-methionine:carboxyl methyltransferase family protein	7.6	—	—	4.6	—	At1g15125
S-adenosyl-L-methionine:carboxyl methyltransferase family protein	6.5	—	4.0	15.5	7.6	At1g66700
lipase	5.8	—	—	—	—	At1g30370
O-methyltransferase	5.8	2.4	4.6	6.7	3.0	At1g21100
O-methyltransferase	5.7	—	3.5	4.4	2.2	At1g21130
alcohol dehydrogenase	5.3	—	2.0	6.4	3.8	At1g64710
terpene synthase/cyclase	5.1	—	—	2.4	—	At3g29110
malate oxidoreductase	4.9	—	—	3.0	2.8	At5g25880
UDP-glucoronosyl and UDP-glucosyl transferase	4.9	11.2	8.7	12.6	19.9	At2g15490
bifunctional dihydrofolate reductase-thymidylate synthase	4.7	—	—	—	—	At2g21550
5'-adenylylsulfate reductase (APR3)	4.5	4.0	3.6	5.5	4.7	At4g21990
phosphofructokinase	4.3	2.7	3.5	2.8	—	At5g47810
sulfotransferase	4.1	11.8	19.3	38.2	30.2	At5g07010
Fe-S metabolism associated domain-containing protein	4.1	—	2.7	9.2	5.5	At1g67810
UDP-glucoronosyl/UDP-glucosyl transferase family protein	4.0	5.7	8.5	14.8	9.7	At4g27570
sulfotransferase	4.0	8.5	12.5	27.9	16.1	At5g07000
glycerophosphoryl diester phosphodiesterase family protein	3.7	—	3.4	3.8	—	At5g43300
D-3-phosphoglycerate dehydrogenase	3.6	4.6	4.3	9.3	7.3	At4g34200
D-3-phosphoglycerate dehydrogenase	3.5	4.5	4.6	7.6	7.4	At1g17745
2-oxoacid-dependent oxidase	3.5	—	4.6	23.0	22.4	At3g49630
guanylate kinase 1 (GK-1)	3.5	—	—	—	—	At2g41880
glutamate-cysteine ligase	3.5	5.9	7.6	9.2	8.6	At4g23100
(S)-2-hydroxy-acid oxidase	3.4	2.7	3.1	3.4	—	At3g14130
sulfate adenylyltransferase 1	3.3	5.2	3.9	6.0	5.6	At3g22890
tropinone reductase	3.2	—	6.8	9.4	8.5	At2g29350
Cytochrome P450s						
cytochrome P450 (CYP1)	9.3	2.1	4.1	8.0	6.0	At4g22710
cytochrome P450 71B22	5.6	—	—	3.2	3.2	At3g26200
cytochrome P450	5.0	—	9.7	5.6	—	At1g64930
cytochrome P450	4.6	—	—	2.2	—	At4g00360
Cell wall						
pectinesterase	5.7	—	2.4	3.1	2.6	At3g14310
invertase/pectin methylesterase inhibitor	4.4	4.3	3.3	3.8	—	At3g47380

Table 1 (continued). Analysis of microarray data for genes up-regulated in XVE-*ORA59* lines

Gene annotation and putative function [1]	Fold-change XVE-*ORA59*	wild-type JA 8 hours	wild-type JA 24 hours	wild-type E+JA 8 hours	wild-type E+JA 24 hours	AGI code
Cell wall						
pectinesterase	5.7	—	2.4	3.1	2.6	At3g14310
invertase/pectin methylesterase inhibitor	4.4	4.3	3.3	3.8	—	At3g47380
Redox						
peroxidase	24.8	—	3.5	6.5	14.1	At4g08780
peroxidase	14.1	—	3.4	5.2	10.0	At4g08770
peroxidase	11.2	—	—	2.3	13.3	At3g03670
glutathione S-transferase	6.6	2.1	2.3	4.9	3.7	At1g27130
glutathione S-transferase	4.8	—	2.1	3.1	3.1	At4g02520
glutathione S-transferase	4.8	3.1	3.4	2.6	3.2	At2g30870
peroxidase	4.6	—	—	2.2	2.8	At4g11290
glutathione S-transferase	4.6	—	—	3.6	—	At1g69920
glutathione S-transferase	4.5	—	4.8	4.0	2.9	At1g02930
glutathione S-transferase	4.5	—	—	2.9	3.3	At2g02930
glutathione S-transferase	4.5	—	—	—	3.1	At1g10370
peroxidase	4.1	—	2.9	5.8	6.8	At4g37520
thioredoxin family protein	3.6	2.9	3.7	3.5	—	At4g32580
glutathione S-transferase	3.5	—	4.2	3.8	2.4	At1g02920
respiratory burst oxidase protein D (RbohD)	3.3	—	—	3.1	3.9	At5g47910
RNA processing						
DEAD box RNA helicase (DRH1)	3.7	2.7	3.0	3.3	3.1	At3g01540
U2 snRNP auxiliary factor small subunit	3.3	2.1	3.2	3.8	—	At1g27650
U2 snRNP auxiliary factor small subunit	3.1	3.2	3.3	3.2	3.0	At5g42820
Hormone metabolism						
ACC synthase 6 (ACCS6)	6.8	—	—	2.8	—	At4g11280
nitrilase 4 (NIT4)	4.8	—	5.5	18.7	14.5	At5g22300
Transport						
MATE efflux family protein	14.2	4.0	12.9	17.9	14.5	At3g23550
uclacyanin II	7.9	—	—	4.2	3.9	At2g44790
sigA-binding protein identical to SigA binding protein	5.6	—	—	2.7	—	At3g56710
proton-dependent oligopeptide transport (POT) family protein	5.2	4.8	8.4	14.4	10.3	At4g21680
ubiquinol-cytochrome C reductase iron-sulfur subunit	3.3	2.9	2.9	3.3	3.2	At5g13430
glycerol-3-phosphate transporter	3.1	—	—	4.7	6.0	At3g47420
Unclassified proteins						
heavy-metal-associated domain-containing protein	17.9	—	—	2.4	—	At5g52720
hypothetical protein	16.9	—	6.5	5.8	10.0	At1g13490
expressed protein	15.3	—	2.1	6.0	3.2	At5g25250
expressed protein	5.5	—	—	2.5	—	At1g52270
FAD-binding domain-containing protein	5.2	—	4.0	14.2	—	At1g26380
sigA-binding protein-related low similarity to SigA binding protein	5.1	—	—	3.1	—	At2g41180
hypothetical protein	5.0	—	—	—	—	At5g44565
expressed protein	3.6	2.3	—	—	—	At2g16485
expressed protein	3.5	-2.6	-2.8	—	2.1	At5g42530
expressed protein	3.5	—	—	3.4	—	At3g57400
expressed protein	3.3	4.6	3.9	6.9	7.2	At2g27260
unknown protein	3.2	3.0	2.7	3.0	3.7	At1g05130
expressed protein	3.2	2.1	3.3	4.2	3.2	At5g35525
extracellular dermal glycoprotein	3.2	—	3.1	2.9	—	At1g03230
expressed protein	3.2	2.9	2.4	2.7	2.2	At1g68120
expressed protein	3.1	2.7	3.1	3.1	—	At4g31080

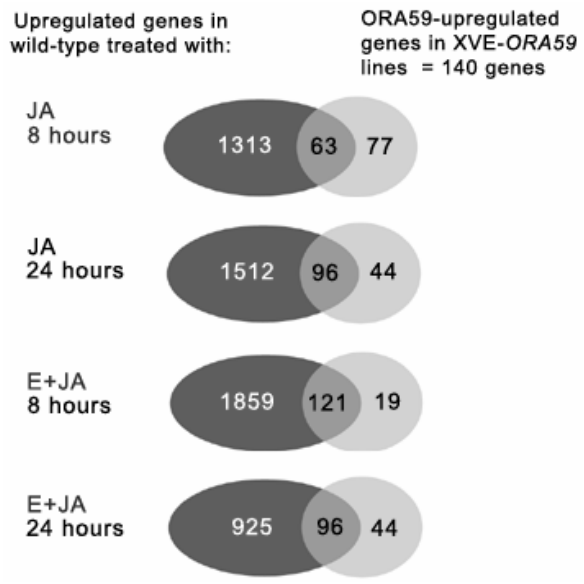

Figure 2. Venn diagrams presenting the results of the genome-wide transcriptome analyses of the putative ORA59 target genes. Genes that were significantly upregulated in two-weeks-old XVE-*ORA59* lines overexpressing *ORA59* (140 genes) were compared with genes induced in two-weeks-old wild-type (Col-0) plants treated with 50 µM jasmonic acid (JA) or 50 µM JA and 1 mM of the ethylene-releaser ethephon (E+JA) for 8 and 24 hours. In each ellips is the number of genes upregulated in response to the particular treatment indicated. All genes had a fold-change ≥ 2 (*P*-values ≤ 0.001). Indicated in the overlapping areas are the number of genes commonly upregulated in XVE-*ORA59* lines and in wild-type plants after the specified treatment. Numbers in the nonoverlapping areas of the ellipses represent genes specifically upregulated under the given conditions.

These results show that a large number of ORA59-upregulated genes are responsive to JA alone or in combination with ethephon. The microarray data were confirmed by RNA blot analyses for a selected set of genes (Figure 3). Estradiol-induced activation of *ORA59* gene expression in XVE-*ORA59* line #10 resulted in high expression levels of all genes tested, including *PDF1.2, ChiB*, *HEL, AN5-AT* and *AIG2.* Expression of these genes was not induced in the estradiol-treated XVE-*GUS* control line #7. Consistent with the microarray data (Table 1), the expression of several genes encoding enzymes of the shikimate/tryptophan biosynthesis pathway, such as *IGPS*, *ASB1* and *TSB1*, was increased in the XVE-*ORA59* line overexpressing *ORA59* (Figure 3). Similar results were obtained with RNA samples originating from another independent XVE-*ORA59* line treated with estradiol (line #6; data not shown).

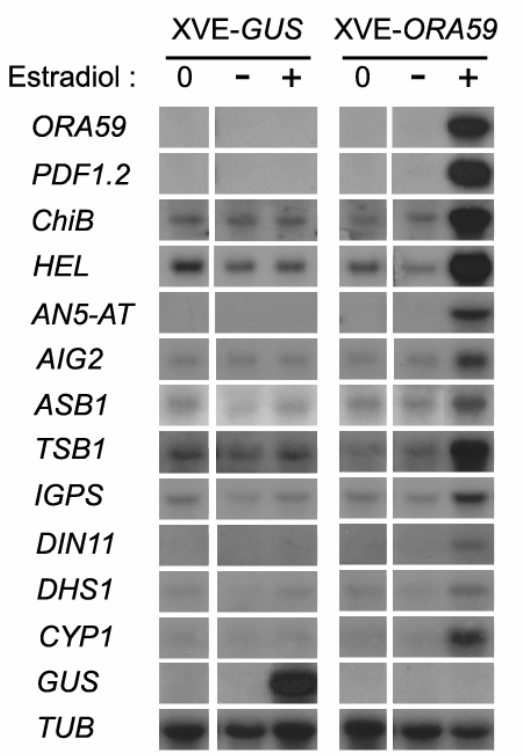

Figure 3. Verification of putative ORA59 target genes obtained from the microarray data by RNA blot analyses. Expression levels of a selected set of genes upregulated in plants overexpressing *ORA59* identified from the microarray data were analyzed in 2-weeks-old plantlets from XVE-*ORA59* transgenic line #10 grown in liquid medium. XVE-*GUS* transgenic line #7 was used as a control. RNA samples were collected from plants which were untreated (0), or treated for 16 hours with 2 µM estradiol (+) or with the solvent DMSO at 0.1% final concentration (-).The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

ORA59 functions downstream from COI1

The expression studies indicate that ORA59 regulates several JA- and ethylene-responsive genes (Table 1). Figure 2 shows that only a small proportion of genes induced by JA or JA and ethylene (10 % at most) are regulated by ORA59. 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 defense gene set. 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 4, estradiol-induced expression of *ORA59* resulted in high expression levels of the target genes *PDF1.2* and *HEL* in a COI1-independent manner. This result demonstrates that 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*. In contrast, estradiol-induced expression of *ORA59* in the *coi1-1* mutant background did not lead to high expression of the *ChiB* gene (Figure 4), indicating that regulation of the *ChiB* gene by ORA59 is controlled by COI1-dependent molecular mechanisms.

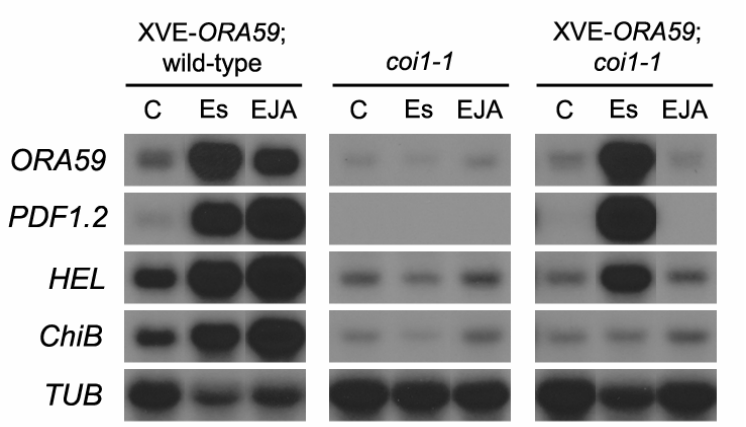

Figure 4. *ORA59* overexpression activates target gene expression without requiring COI1. Two-weeks-old *coi1-1* mutant plants, and plants containing the XVE-*ORA59* expression module in the wild-type and in the *coi1-1* backgrounds were treated for 8 hours with 50 µM jasmonic acid and 1mM ethephon (EJA), 5 µM estradiol (Es) or the solvents DMSO and Na-phosphate (C). The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

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 the pHANNIBAL vector (Wesley et al., 2001). In plants this construct directs the expression of an RNA consisting from 5' to 3' of the anti-sense *ORA59* ORF, the *Pdk* intron and the sense *ORA59* ORF, which can fold in a hairpin structure, generating after splicing a double-stranded *ORA59* RNA. Transgenic *ORA59*-silenced plants growing under normal conditions displayed no visible aberrant phenotype compared to wild-type plants. RNA blot analyses using a probe specific

for *ORA59* revealed a smeary signal in five out of seven independent lines (Figure 5A). A similar autoradiographic pattern was obtained using the *Pdk* intron as a probe (*PDKi*; Figure 5A), suggesting that the smear corresponds to the complete inverted repeat-intron RNA and its degradation products. In these transgenic lines, *ORA59* mRNA was undetectable after treatment with JA. In contrast, transgenic line #5 showed wild-type *ORA59* gene expression in response to JA, whereas line #13 showed reduced JA-induced expression. Induction of the *PDF1.2* and *HEL* genes in response to JA treatment was severely compromised in those *ORA59*-silenced lines with undetectable *ORA59* mRNA (Figure 5B). Furthermore, the reduced *PDF1.2* and *HEL* transcript abundance correlated with the *ORA59* expression level in individual lines, indicating that the JA-induced expression of these defense genes is dependent on ORA59.

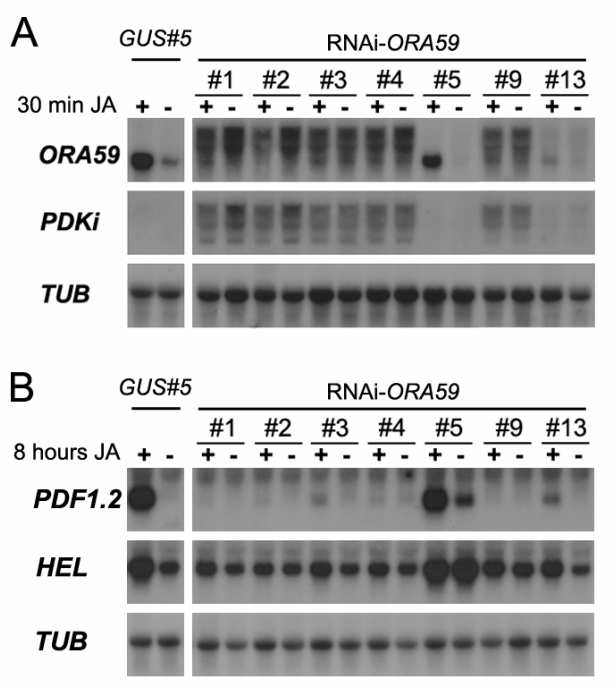

Figure 5. The *ORA59* gene is silenced in the majority of independent transgenic RNAi-*ORA59* lines. Ten-days-old seedlings from control line (*GUS* #5) or seedlings from several independent RNAi-*ORA59* transgenic lines (indicated by numbers) were treated for 30 minutes (min) **(A)** or for 8 hours **(B)** with 50 µM jasmonic acid (+) or with 0.1% of the solvent DMSO (-).The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

Transgenic line RNAi-*ORA59* #9 was used for further analyses. As shown in Figure 6A, the JA- and ethephon-induced expression of several genes identified by the microarray analysis, including *PDF1.2*, *AN5-AT* and *HEL* genes, was dramatically reduced in *ORA59*-silenced

plants, compared to the control line. 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, two possible functionally equivalent transcription factors, were determined (Figure 6A). The JA- and ethephon-induced gene expression levels of the two putative functional homologues *ERF1* and *AtERF2* in the *ORA59*-silenced line were similar to those observed in the control line, indicating that ERF1 and AtERF2 protein levels are likely to remain unchanged in the *ORA59*-silenced line. Equal loading of RNA on gel was checked by hybridization with the *TUB* gene (Figure 6A and B).

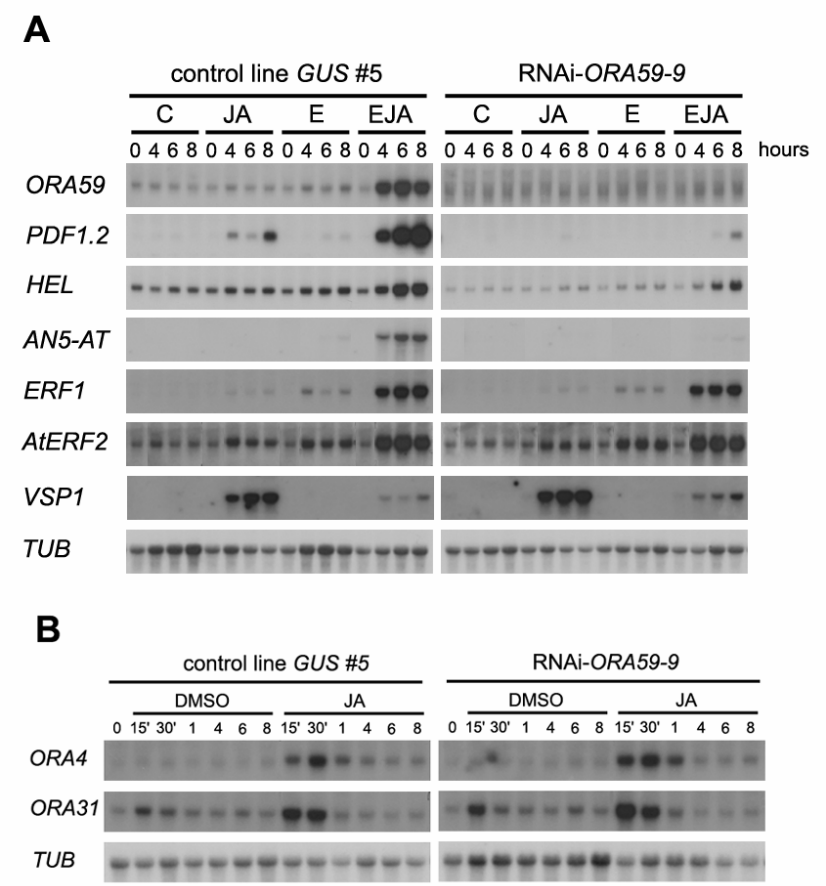

Figure 6. Specific silencing of the *ORA59* gene severely compromises the JA- and/or ethylene-responsive expression of several defense genes. **(A).** Two-weeks-old seedlings from the representative RNAi-*ORA59* transgenic line #9 or from the control line *GUS* #5 were treated for the number of hours indicated with 50 µM jasmonic acid (JA), 1 mM ethephon (E), a combination of both (EJA), or the solvents DMSO and Na-phosphate (C). **(B).** Two-weeks-old seedlings from the same lines as in **(A)** were treated for 15 and 30 minutes or for the number of hours indicated with 50 µM jasmonic acid (JA) or 0.1 % of the solvent DMSO. The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

RNAi-mediated silencing is based on targeted degradation of the endogenous mRNA (Hamilton and Baulcombe, 1999). mRNA degradation is triggered by sequence-specific short 21 to 25 nucleotide (nt) oligomers derived from the double-stranded RNA molecule (Hamilton and Baulcombe, 1999). Two JA-responsive AP2/ERF genes, *ORA4* and *ORA31* (Atallah, 2005), have 21 to 23 nt-long perfect sequence homologies to the *ORA59* gene in the highly conserved region encoding the AP2 domain, and are therefore putative targets for mRNA degradation. Expression levels of the *ORA4* and *ORA31* genes in response to JA treatment were similar to those observed in control plants, indicating that in *ORA59*-silenced plants, silencing was specifically directed at *ORA59* mRNA (Figure 6B). These results demonstrate that ORA59 is responsible for the activation of a subset of JA- and ethylene-responsive genes including *PDF1.2*, *AN5-AT,* 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 expression of these genes.

ORA59 controls resistance against the necrotrophic fungus *Botrytis cinerea*

In Arabidopsis, the JA and ethylene signal transduction pathways are involved in resistance against the necrotrophic fungi *B. cinerea* and *A. brassicicola* (Thomma et al., 1998 and 1999a; Ferrari et al., 2003; Penninckx et al., 1996). 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 these pathogens.

Several independent transgenic lines constitutively overexpressing the *ORA59* gene were constructed. As shown by RNA gel blot analyses, the ORA59-regulated genes *PDF1.*2, *HEL* and *ChiB* were highly expressed in the 35S:*ORA59* lines compared to control lines (Figure 7).

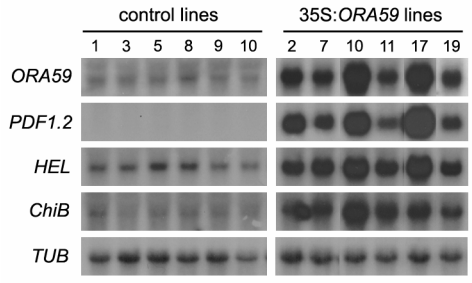

Figure 7. ORA59 target gene expression in constitutive *ORA59*-overexpressing plants. RNA was extracted from ten-days-old seedlings from several (indicated by numbers) independent 35S:*ORA59* lines and control lines (1301). The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

The level of defense gene expression correlated with the level of *ORA59* gene expression in each independent line. In general, independent 35S:*ORA59* lines showed a severe dwarf phenotype under normal growth conditions, similar to that observed with transgenic plants

overexpressing *ERF1* (Solano et al., 1998). As shown in Figure 8, 35S:ORA59 plants from line #10 and #17 were smaller than wild-type plants when grown on normal medium.

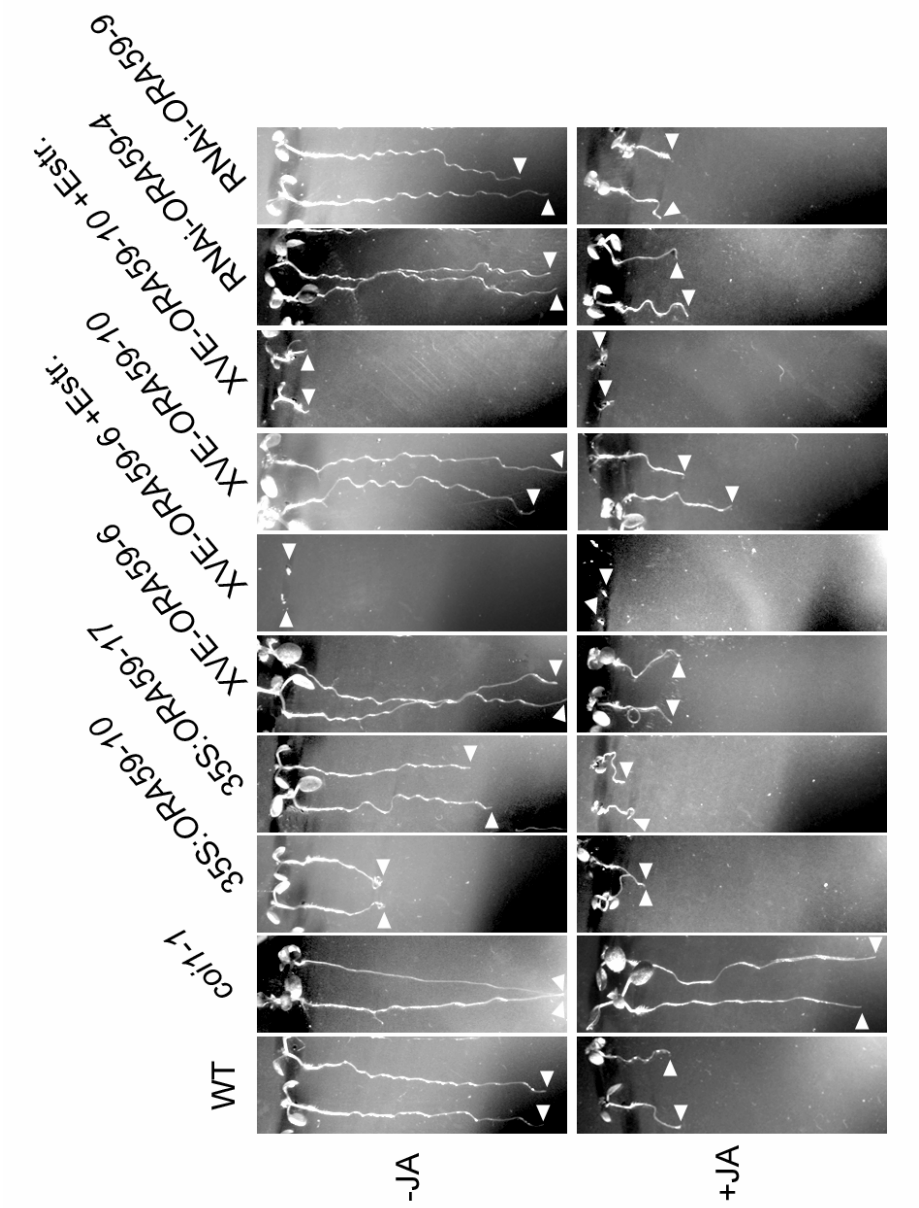

Figure 8. *ORA59* overexpression results in dwarf plants but ORA59 does not participate in root growth inhibition by JA. Two-weeks-old Arabidopsis wild-type, *coi1* mutant, and transgenic plants constitutively (35S:*ORA59*) and inducibly (XVE-*ORA59*) overexpressing or silencing (RNAi-*ORA59*) the *ORA59* gene were grown on plates containing (+JA) or lacking (-JA) 50 µM jasmonic acid. In addition, XVE-*ORA59* plants were grown with or without 4 µM estradiol (Estr.). The position of the root tip is indicated with an arrowhead.

In the presence of JA, wild-type and overexpressing plants were equally sensitive to JA with respect to root growth inhibition. In contrast, the JA-insensitive *coi1-1* mutant did not show reduced root growth in response to JA. The relationship between reduction of plant size and *ORA59* overexpression was further analyzed with the inducible XVE-*ORA59* plants. In the absence of estradiol, XVE-*ORA59* plants from independent lines #6 and #10 showed wild-type phenotypes when cultured either on normal medium or on medium containing JA. However, plant growth was dramatically impaired in the XVE-*ORA59 #10* line on medium inducing the *ORA59* transgene, and JA treatment had additional negative effect on growth. Seeds from the line XVE-*ORA59 #6* did not even germinate on medium containing estradiol or JA/estradiol. This indicates that overexpression of *ORA59* has a negative effect on plant growth. Transgenic *ORA59*-silenced plants grown on medium containing JA showed reduced root growth similar to wild-type plants, demonstrating that ORA59 is not involved in root growth inhibition by JA. These results suggest that a high and constant expression of the ORA59-regulated genes, including the defense related genes *PDF1.2, HEL* and *ChiB*, generates a stress condition that compromises plant growth. However, ORA59 does not control the branch of the JA response responsible for root growth inhibition.

Because of its mild dwarf phenotype (Figure 8) and its constitutive overexpression of *ORA59* and of the ORA59 target genes (Figure 7), the 35S:*ORA59* line #17 was chosen for subsequent disease resistance tests. In order to reach a plant size similar to the other genotypes, the 35S:*ORA59* plants were allowed to grow for seven weeks before inoculation, as compared to five weeks for all other genotypes. Disease resistance tests were performed at the same time for all genotypes. Mature leaves of five- to seven-weeks-old wild-type plants, JA-insensitive *coi1-1* mutant plants, and transgenic 35S:*ORA59* line #17 and RNAi-*ORA59* line #9 were inoculated with *B. cinerea* ($5x10^5$ spores/mL). Figure 9A shows the disease progression in three representative leaves of each genotype scored 5 days post inoculation. Infected wild-type leaves were relatively tolerant to this inoculum density of *B. cinerea*, while *coi1-1* plants showed increased susceptibility with a large percentage of leaves with spreading necrotic lesions (Figure 9B), confirming previous findings (Thomma et al., 1998). Interestingly, 35S:*ORA59 #17* 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. 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 that were collected 2 and 4 days after inoculation. Inoculation of wild-type plants resulted in an increase in the mRNA level of *ORA59* both locally and systemically (Figure 9C). In the severely diseased JA-insensitive

coi1-1 mutant, *B. cinerea* infection did not result in the induction of *ORA59*. 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.

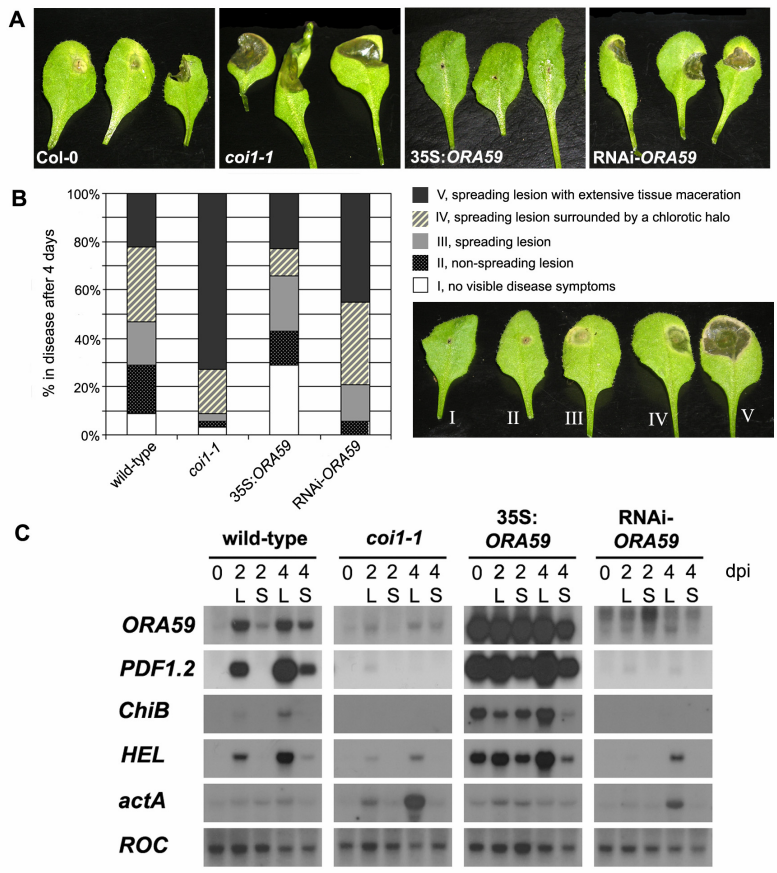

Figure 9. ORA59 is involved in resistance against *Botrytis cinerea*. Disease severity was scored in Arabidopsis wild-type plants, *coi1-1* mutants and transgenic plants overexpressing (35S:*ORA59-17*) or silencing (RNAi-*ORA59-9*) the *ORA59* gene. Disease ratings were performed 4 days after inoculation. **(A).** Representative disease symptoms at 4 days after inoculation. (**B).** Distribution of disease severity classes. Disease severity is expressed as the percentage of leaves falling in disease severity classes: I, no visible disease symptoms; II, non-spreading 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 represent 60 to 100 leaves of 15 to 20 plants per genotype. The experiment was repeated three times with similar results. (**C).** Infected local (L) and non-infected systemic (S) leaves from several inoculated plants of each genotype were collected at day 0, day 2 and day 4 after inoculation (dpi) with *B. cinerea* and RNA was extracted. The RNA gel blot was hybridized with the indicated probes. The *ROC* probe was used to verify RNA loading.

The *actin* (*actA*) gene from *B. cinerea* (Benito et al., 1998) was used as a molecular marker of disease progression. As shown in Figure 9C, the *actA* gene was expressed in local leaves of the susceptible *coi1-1* and RNAi-*ORA59* plants four days after infection, indicating growth of the pathogen *B. cinerea* in these leaves. Hybridization with the *ROC* gene, encoding a cytosolic cyclophilin, showed equal loading of RNA. These results show a strong correlation between the effects of *ORA59* expression levels on defense gene expression and on resistance against *B. cinerea.*

To test whether ORA59 also controls resistance against *A. brassicicola*, five-weeks-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*. Disease ratings were assessed 7 days after inoculation. In comparison to wild-type Col-0 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 towards this pathogen (Thomma et al., 1998 and 1999b). In contrast to *coi1-1*, the level of resistance against *A. brassicicola* in *ORA59*-silenced plants did not differ from that of wild-type plants (data not shown). RNA blot analyses of infected and systemic leaves revealed that *A. brassicicola* induced *ORA59* gene expression in wild-type plants, both locally and systemically (Figure 10). Similar to *coi1-1* mutant plants, *ORA59*-silenced plants were strongly impaired in *ORA59* and ORA59 target gene expression in response to *A. brassicicola* (Figure 10), consistent with the results from *B. cinerea* infection.

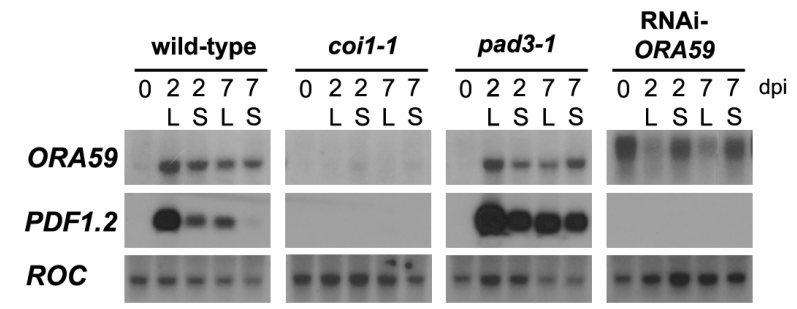

Figure 10. *PDF1.2* gene expression induced by infection with *Alternaria brassicicola* is impaired in *ORA59*-silenced plants. Four-weeks-old Arabidopsis wild-type plants, *coi1-1* and *pad3-1* mutant plants and transgenic plants silencing the *ORA59* gene (RNAi-*ORA59*) were inoculated with *A. brassicicola*. Three-µL droplets containing 10^6 spores/mL were applied on mature leaves. Infected (L) and non-infected (S) leaves of several inoculated plants of each genotype were collected at day 0, day 2 and day 7 after inoculation and RNA was extracted. The RNA gel blot was hybridized with the indicated probes. The *ROC* probe was used to verify RNA loading.

Hybridization with the *ROC* gene showed equal loading of RNA. Thus, although ORA59 is responsible for defense gene expression and the *ORA59* and *PDF1.2* mRNA levels are increased after *A. brassicicola* infection, ORA59 and its target genes do not play an important role in the resistance against this pathogen under the conditions of this experiment.

Resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was found to be affected both in plants impaired in salicylic acid (SA) signaling and in JA and ethylene response mutants (Pieterse et al., 1998), suggesting that resistance against this pathogen is controlled by a combined action of SA, JA and ethylene. To characterize the involvement of ORA59 in resistance against *P. syringae*, above-ground parts of wild-type and transgenic plants overexpressing *ORA59* were inoculated with this pathogen and infection rate was determined by scoring disease symptoms on the leaves. Transgenic plants did not show elevated resistance against *P. syringae* compared to wild-type plants, indicating that ORA59 does not control the genes essential for the JA-dependent defense response against *P. syringae* (data not shown). These results indicate that ORA59 regulates genes that are specific for defense against particular pathogens, such as *PDF1.2*, *ChiB* and *HEL* in the case of *B. cinerea*.

Which AP2/ERF-domain transcription factors regulate *PDF1.2*?

Our results show that ORA59 is the crucial regulator of several defense-related genes including *PDF1.2.* Loss-of-function studies revealed that only ORA59, and no other AP2/ERF-domain transcription factor or member of another class of transcriptional regulators, was sufficient to activate the expression of *PDF1.2* in response to JA and ethephon treatments (Figure 6A) or to infection with *B. cinerea* (Figure 9C) or *A. brassicicola* (Figure 10). These results appear to contradict a previous report showing that constitutive overexpression of the AP2/ERF-domain transcription factor *ERF1* gave rise to elevated *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). Similarly, constitutive overexpression of *AtERF1*, a close homologue of *AtERF2*, led to high *PDF1.2* expression levels (Figure 11A). One possible explanation for the apparent discrepancy with our results is that overexpression of *ERF1*, *AtERF*1 or *AtERF2* causes a stress condition, manifested also by the dwarf phenotype, which indirectly leads to *PDF1.2* expression. To address the question 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 *PDF1.2* gene in stably transformed Arabidopsis plants expressing *ORA59, ERF1, AtERF1* and *AtERF2* in an estradiol-inducible manner. Under non-induced conditions, the *AP2/ERF* transgene is silent.

We speculated 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 11B, 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 estradiol-treated XVE-*ORA59* line, indicating that ORA59 activity was not significantly affected by the fusion with a TAP tag. Similarly, ERF1 activity appears to be preserved in the TAP fusion protein. Two to three independent lines per construct were tested with essentially similar results (data not shown).

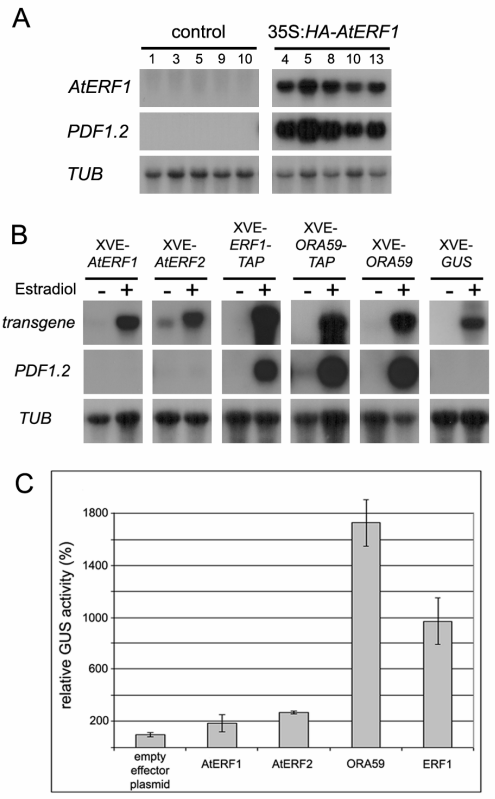

Figure 11. ORA59 and ERF1 are activators of *PDF1.2* gene expression *in planta.* **(A)** *PDF1.2* gene expression in *AtERF1*-overexpressing plants. RNA was extracted from ten-days-old seedlings from several independent 35S:*HA-AtERF1* and control lines (indicated by numbers). **(B).** Estradiol-induced *PDF1.2* gene expression in stably transformed XVE lines. Two-weeks-old Arabidopsis seedlings from transgenic lines carrying the *AtERF1*, *AtERF2*, *ORA59* or *GUS* gene under the control of the inducible XVE system (XVE-*AtERF1* #1, XVE-*AtERF2* #1, XVE-*ORA59* #10 and XVE-*GUS* #7, respectively) or carrying the *ORA59* or *ERF1* gene fused to the *TAP* tag under the control of the inducible XVE system (XVE-*ORA59-TAP* #17 and XVE-*ERF1-TAP* #16, respectively) were treated for 24 hours with estradiol (+) or the solvent DMSO (-). The top panels were hybridized with separate AP2/ERF or GUS probes and exposed for 8 - 24 hours. Expression levels cannot be directly compared but are similar within a 3-fold change. 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 co-transformed with a reporter plasmid carrying *PDF1.2*-promoter-*GUS* and effector plasmids carrying the *AtERF1*, *AtERF2*, *ERF1* or *ORA59* gene fused to the CaMV 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 co-transformed to correct for transformation and protein extraction efficiencies. Values represent means ± SE of triplicate measurements.

In general, we observed that *PDF1.2* gene expression level was slightly higher in XVE-*ORA59* lines compared to XVE-*ERF1* lines. In a second approach, we analyzed the ability of ORA59, ERF1, AtERF1 and AtERF2 to transactivate the *PDF1.2* promoter in transient expression assays. Arabidopsis protoplasts were co-transformed 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 co-transformation with *ORA59* and *ERF1* effector plasmids, respectively, compared to the empty effector plasmid (Figure 11C). No significant activation of the *GUS* reporter gene was observed with effector plasmids carrying *AtERF1* or *AtERF2* genes. Both experimental approaches demonstrate that ORA59 and ERF1 are able to function as transcriptional activators of *PDF1.2* gene expression, whereas AtERF1 and AtERF2 do not activate *PDF1.2* gene expression when inducibly or transiently expressed.

***ORA59* overexpression does not repress *VSP1* expression in response to JA**

In transgenic plants overexpressing the *ERF1* gene, JA treatment failed to express efficiently the JA-responsive *VSP2* gene, encoding the vegetative storage protein 2 (Lorenzo et al., 2004), suggesting that ERF1 represses a branch of the JA signaling pathway responsible for the expression of a subset of JA-responsive genes including *VSP2*. This prompted us to test whether ORA59 could also act as a negative regulator of that part of the JA response. Two-weeks-old transgenic XVE-*ORA59* line #6 was treated with estradiol for 16 hours prior to treatment with JA for 8 hours. Transgenic XVE-*ERF1-TAP* line 16 was used as control for the negative effect of *ERF1* overexpression on *VSP* expression in response to JA. Expression of the *VSP1* gene was analyzed by RNA gel blot hybridization and *VSP1* transcript levels in the XVE-*ORA59* lines were compared to those observed in the XVE-*ERF1-TAP* line and in a XVE-*GUS* control line.

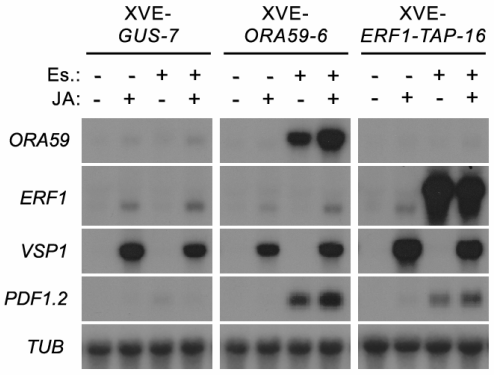

Figure 12. *ORA59* overexpression does not repress *VSP1* expression in response to JA. Two-weeks-old inducible XVE-*ORA59* (line #6), XVE-*ERF1-TAP* (line #16) and XVE-*GUS* (line #7) transgenic Arabidopsis plants were treated for 16 hours with 4 µM estradiol (Es.; +) or with DMSO (-) prior to treatment for 8 hours with 50 µM jasmonic acid (JA; +) or with DMSO (-). The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

The *VSP1* and *VSP2* nucleotide sequences share 93% identity and therefore it is likely that hybridization with the complete coding sequence of the *VSP1* gene would also detect the *VSP2* mRNA. As shown in Figure 12, JA treatment induced the expression of the *VSP1* gene in all lines. Estradiol-induced overexpression of *ORA59* prior to JA treatment did not result in reduced *VSP1* expression in the JA-treated XVE-*ORA59* line, demonstrating that *ORA59* overexpression does not lead to reduced *VSP1* gene expression in response to JA. This shows that under our experimental conditions, ORA59 does not act antagonistically on the JA signaling pathway involved in the activation of a subset of genes, including the *VSP* genes. This result is consistent with the observation that silencing of the *ORA59* gene in plants did not lead to a significant increase in *VSP1* expression in response to JA compared to control plants (Figure 6). Surprisingly, overexpression of *ERF1* prior to JA treatment failed to repress *VSP1* expression in the JA-treated XVE-*ERF1-TAP* line under our experimental conditions. This finding is in contradiction with the previous results showing that constitutive overexpression of *ERF1* inhibits *VSP* induction in response to JA (Lorenzo et al., 2004). After 16 hours of treatment with 4 µM estradiol, the transgene in XVE lines is expressed at a level that is at least, similar to that obtained in transgenic lines overexpressing a transgene under the control of the strong constitutive promoter CaMV 35S (Chapter 5). Estradiol-induced expression of *PDF1.2* indicates that both ORA59 protein and ERF1-TAP fusion protein are functional in their respective line (as demonstrated in Figure 11). Identical results were obtained with XVE-*ORA59* line 10, XVE-*ORA59-TAP* lines 4 and 17, and XVE-*ERF1-TAP* line 2 (data not shown).

Discussion

Jasmonates are key regulatory signaling molecules in plant defense. JAs interact with ethylene in the regulation of plant wound and defense responses against pests or pathogen attack. An important aspect of JA- and ethylene-dependent defense responses is their synergistic interaction in the induction of many defense-related genes.

In this study, we investigated the function of ORA59, a member of the AP2/ERF-domain transcription factor family in Arabidopsis. Here, we demonstrate 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, strictly depends on ORA59, and no other transcription factor, including ERF1 or AtERF2, can bypass the requirement for ORA59.

Transcriptome analysis is a powerful tool to unravel transcription factor function and to identify genes positively or negatively regulated by a given transcription factor. Most microarray analyses are set up as comparisons of transcript profiles in plants constitutively overexpressing the transcription factor of interest against a certain control genotype, such as the wild-type background. Despite the many advantages of the constitutive overexpression strategy, one needs to be cautious when interpreting phenotypes that are caused by the constitutive overexpression of transcription factors. Due to its abundance or inappropriate expression in time and space, the protein could exert new activities that it does not in the wild-type (Zhang, 2003). Constitutive overexpression of *ORA59* caused dwarfism. We speculated that this severe phenotype is due to a general stress condition caused by the constitutive activation of ORA59-regulated genes, and that this neomorphic phenotype is likely to cause the activation of a variety of genes which are not directly controlled by ORA59. Therefore we used transgenic Arabidopsis plants in which the *ORA59* gene was under the control of an inducible promoter to increase the likelihood that upregulated genes are directly controlled by ORA59. One advantage is that, in the non-induced condition, plants are similar to wild-type, as the *ORA59* transgene is silent, thereby avoiding neomorphic phenotypes. A second advantage is that the microarray experiments were performed by comparing RNA samples from induced and non-induced plants of the same genetic background, limiting changes in expression of unspecific genes due to the transgene insertion site in the genome or other differences between independent transgenic lines. Therefore, we speculate that ORA59 is likely to be a direct regulator of the majority, if not all, of the genes identified by the microarray experiment.

Genome-wide transcriptome analyses revealed that overexpression of *ORA59* positively modulated the expression of a large number of genes involved in defense (including *PDF1.2, ChiB* and *HEL*), cell signaling and protein modification (including genes encoding kinases and phosphorylases) and shikimate/ tryptophan biosynthesis (such as *ASB1*, *TSB1* and *IGPS*). The involvement of ORA59 in integration of ethylene and JA signal inputs was supported by the observation that the majority of the ORA59-upregulated genes were also induced in wild-type plants treated with JA alone or in combination with ethylene, as revealed by microarray experiments. In fact, it is entirely possible that all of the ORA59-upregulated genes are regulated by JA and/or ethylene at earlier or later time points than those selected for the microarray experiments (8 and 24 hours).

Supporting evidence for the regulatory role of ORA59 was provided by the findings that the JA- and/or ethylene-induced expression of the putative ORA59-regulated genes, including *PDF1.2, AN-5AT* and *HEL*, was severely compromised in *ORA59*-silenced plants. In contrast, expression of two other reported putative regulators of the *PDF1.2* gene, *ERF1* and *AtERF2*

(Solano et al., 1998: Brown et al., 2003) was still induced by JA and/or ethylene treatments in *ORA59*-silenced plants, indicating that ERF1 or AtERF2 are unable to induce *PDF1.2* expression in response to JA or ethylene in these plants. The transcription factor ERF1 was suggested to be a key element in the integration of JA and ethylene signals for the regulation of defense genes (Lorenzo et al., 2003). However, our results demonstrate that it is ORA59, and not ERF1 or any other transcription factor, that integrates both signals in order to activate JA- and ethylene-responsive genes, such as *PDF1.2*, leading to adapted defense responses. By means of two different approaches, only ORA59 and ERF1 were able to activate *PDF1.2* expression in Arabidopsis, whereas AtERF2, or its close homologue AtERF1, did not activate *PDF1.2* gene expression. This suggests that the observations that constitutive overexpression of *AtERF1* (this study) or *AtERF2* (Brown et al., 2003) induced the expression of *PDF1.2* in stably transformed plants, are artefacts that are likely caused by non-specific stress effects. All of our data on ERF1 are consistent with those reported by Lorenzo et al. (2003). Although these data therefore appear to be correct, additional data obtained from plants impaired in *ERF1* expression is needed to assign a proper function for ERF1. From our findings, we assume that it is not ERF1, but ORA59, that integrates JA and ethylene signal inputs to regulate defense gene expression under conditions where JA and ethylene were exogenously applied to young seedlings, or in response to infection with *B. cinerea* or *A. brassicicola* (conditions similar to those used by Lorenzo et al.). This further supports the necessity of interpreting constitutive overexpression results in conjunction with data from other approaches, such as inducible overexpression, or analysis of RNAi or knock-out mutants.

Defense against infection by the necrotrophic fungi *B. cinerea* and *A. brassicicola* is dependent on both JA and ethylene signaling, and mutations in either of these signal transduction pathways increase susceptibility (Thomma et al., 1998 and 1999a; Penninckx et al., 1996). Overexpression of *ORA59* increases resistance to *B. cinerea*, whereas plants silencing the *ORA59* gene show increased susceptibility to this pathogen, presumably by impaired expression of the ORA59-regulated defense genes. Several *B. cinerea* susceptibility loci have been identified in Arabidopsis (Mengiste et al., 2003; Veronese et al., 2004). The *bos1* (*Botrytis susceptible1*) mutant was found to be affected in resistance against *B. cinerea*. The *BOS1* gene encodes a R2R3MYB transcription factor. However, the *BOS1* gene seems to be involved in resistance against a broad range of biotic and abiotic stresses. Furthermore, *PDF1.2* transcript levels were similar in *bos1* mutants compared to wild-type in response to *B. cinerea* (Mengiste et al., 2003), indicating that the R2R3MYB transcription factor BOS1 regulates a different set of genes than ORA59 does. The *bos3* mutant shows a dramatic decrease in *PDF1.2* expression in response to *B. cinerea* infection, similar to the decrease

observed in *ORA59*-silenced plants. The *BOS3* locus maps to a region on chromosome 1, which is close to the *ORA59* gene location (Veronese et al., 2004). This suggests that the *bos3* mutation affects the *ORA59* gene. However, the different phenotypic responses to *A. brassicicola* infection observed in *bos3* mutants (susceptible; Veronese et al., 2004) and in *ORA59*-silenced plants (resistant) indicate that the *bos3* mutation does not affect the *ORA59* gene.

Surprisingly, the *ORA59*-silenced plants showed resistance to *A. brassicicola* infection, similar to wild-type Col-0 plants. ORA59 and its target genes are unlikely to play a role in defense against this pathogen, under the conditions of this disease resistance test, as their gene expression patterns are not correlated with *A. brassicicola* susceptibility. In addition to camalexin production, presumably other JA- and ethylene-dependent defense responses, regulated by another transcription factor than ORA59, are required for effective resistance against this necrotrophic pathogen.

The AP2/ERF-domain transcription factors ORA59 and ERF1 appear to regulate the same set of JA- and ethylene-responsive genes (Table 1; Lorenzo et al., 2003), suggesting functional redundancy of these two proteins. However, our loss-of-function results with *ORA59*-silenced lines clearly demonstrate that ORA59 is absolutely necessary for expression of these defense genes in response to hormone treatments and *B. cinerea* or *A. brassicicola* infection, and that ERF1 is not involved in defense gene expression in response to these signals. Our findings are summarized in the model in Figure 13.

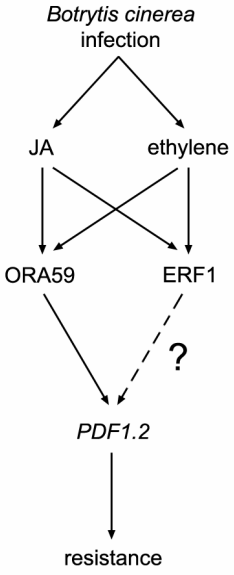

Figure 13. Model for the involvement of ORA59 and ERF1 in response to *Botrytis cinerea* in Arabidopsis. In wild-type plants, infection with *B. cinerea* activates both JA and ethylene signaling pathways and leads to the expression of *ORA59* and *ERF1* genes. In infected *ORA59*-silenced lines, the absence of ORA59 results in impaired expression of the *PDF1.2* gene, as well as increased susceptibility against the pathogen. In these lines, the JA- and ethylene-induced expression of *ERF1* does not lead to *PDF1.2* expression, demonstrating that ORA59 is the key regulator that is strictly required for the integration of the JA and ethylene signal inputs, resulting in gene activation. The exact role of ERF1 in defense awaits analyses of gene expression and pathogen susceptibility in plants impaired in *ERF1* expression.

The exact function of ERF1 in defense gene expression awaits analysis of ERF1 loss-of function plants generated via knockout or RNAi. 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 induced XVE-*ERF1* and XVE-*ORA59* transformants, respectively (unpublished results). One possibility is that ERF1 has no essential function. Alternatively, ERF1 and ORA59 might have separate specialized functions, for example by differential expression of the corresponding genes in certain cell types or at certain developmental stages. Our results show that some discrepancy exists between ORA59 and ERF1 functions. Overexpression of *ORA59* and *ERF1* activated the expression of the basic endochitinase *ChiB* gene (*b-CHI*; Table 1; Solano et al., 1998). In the *coi1* mutant background, *ORA59* overexpression resulted in high expression levels of the target genes *PDF1.2* and *HEL* (Figure 4), demonstrating that ORA59 functions downstream from COI1 as the terminal integrator of the JA and ethylene signal inputs leading to *PDF1.2* and *HEL* gene expression. In contrast, *ORA59* overexpression failed to induce *ChiB* expression in the *coi1* mutant (Figure 4), indicating that the ORA59-mediated regulation of the *ChiB* gene is controlled by COI1-dependent signaling events. A similar experiment performed with *ERF1* showed that *ERF1* overexpression induced *ChiB* expression without the requirement for COI1 (Lorenzo et al., 2003), suggesting a distinct role between ORA59 and ERF1 in the regulation of a number of JA- and ethylene-responsive genes including *ChiB*.

Lorenzo et al. (2004) showed that the JA-induced expression of the *VSP2* gene, encoding a vegetative storage protein, was largely prevented in constitutive *ERF1*-overexpressing plants, suggesting a role for ERF1 in repressing the JA-induced expression of a subset of JA-responsive genes. In our study, a similar experimental set-up showed that *ORA59* overexpression had no effect on the JA-induced expression of the *VSP1* gene, a close homologue of *VSP2*. Similarly, JA-induced *VSP1* expression level in ORA59-silenced plants was comparable to that in control plants. These results suggest that ORA59 and ERF1 have distinct roles in JA and ethylene signaling. However, *ERF1* overexpression could not suppress the JA-induced expression of *VSP1* within a period of 8 hours of JA treatment, contradicting the findings of Lorenzo et al. (2004). Our results indicate that the negative role of ERF1 on a subset of JA-responsive genes can not be attributed to a direct effect of *ERF1* overexpression on the expression of these genes, but might be due to a late response

resulting from *ERF1* overexpression. The discrepancy of the findings from constitutive and inducible *ERF1*-overexpressing plants may also suggest that the results observed in 35S:*ERF1* transgenic plants are artifacts due to non-specific stress effects (discussed above).

Materials and Methods

Biological materials, growth conditions and treatments

Arabidopsis thaliana wild-type plants, *coi1-1*, *ein2-1* and *pad3-1* mutants, and all transgenic plants are in the genetic background of ecotype Col-0. Seeds were surface-sterilized by incubation for 1 minute in 70 % ethanol, 15 minutes in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for three hours (http://plantpath.wisc.edu/~afb/vapster.html).

Surface-sterilized seeds were transferred to 250 ml Erlenmeyer flasks containing 50 ml MA medium (Masson and Paszkowski, 1992) or grown on plates containing MA medium supplemented with 0.6% agar. Transgenic plants from T2 or T3 generations were selected on solid MA medium containing 100 mg/L timentin and either 25 mg/L kanamycin for ORA59-silenced plants or 20 mg/L hygromycin for ORA59-overexpressing plants. Following stratification for 3 days at 4ºC, seeds were incubated at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days or otherwise as indicated. Seeds in liquid medium were placed on a shaker at 120 rpm. Alternatively, seedlings were first selected on solid MA medium supplemented with the above mentioned appropriate antibiotics or 50 µM JA (for selection of *coi1-1* seedlings) for 10 days, after which 15 to 20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml MA medium and incubated on a shaker for 4 additional days before treatment. Seedlings were treated for different time periods with 50 µM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.1% final concentration), 1 mM of the ethylene-releasing compound ethephon (Sigma) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) or a combination of JA and ethephon. As controls, seedlings were treated with 0.1% DMSO, 0.5 mM sodium phosphate pH 7 or a combination of both. Transgenic plants carrying an XVE expression module containing the *ORA59*, *GUS*, *AtERF1* or *AtERF2* gene were treated with 2 µM estradiol (Sigma) dissolved in DMSO (0.2% final concentration). In order to reach similar expression levels of the transgene, transgenic plants carrying an XVE expression module containing the ORA59 or ERF1 gene fused to the TAP tag were treated with 4 µM estradiol. As control, seedlings were treated with 0.2% DMSO.

Botrytis cinerea and *Alternaria brassicicola* strain MUCL 20297 were grown on potato dextrose agar plates for 2 weeks at 22°C. Spores were harvested as described by Broekaert et al. (1990).

Binary constructs and plant transformation

The *ORA59* (*At1g06160*) open reading frame (ORF) was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-CGG GAT CCA TAT GGA ATA TCA AAC TAA CTT C-3' and 5'-CGG GAT CCT

CAA GAA CAT GAT CTC ATA AG-3'. The *ORA59* ORF digested with BamHI was inserted in pBluescript SK+ (Stratagene, La Jolla, CA) such that the 5'-end of the *ORA59* ORF was flanking the HindIII restriction site to generate pSK-ORA59.

For the construction of transgenic lines constitutively overexpressing *ORA59*, the *ORA59* ORF digested with BamHI was cloned into pRT101 (Töpfer et al., 1987) to generate pRT101-ORA59. The Cauliflower Mosaic Virus (CaMV) 35S cassette containing the *ORA59* ORF in sense orientation was excised with PstI and cloned into the binary vector pCAMBIA1300 (accession number AF234296). The binary vector pCAMBIA1301 (accession number AF234297) carrying the *GUS* gene under the control of the CaMV 35S promoter was used to generate control lines (1301 lines).

For the construction of transgenic lines showing post-transcriptional silencing of the *ORA59* gene, the *ORA59* ORF digested with BamHI was cloned into pIC-20H (Marsh et al., 1984) digested with BglII such that the 5'-end of the *ORA59* ORF flanked the EcoRI restriction site. The *ORA59* insert was excised from pIC-20H with EcoRI/XhoI and cloned into the pHANNIBAL vector (accession number AJ311872) to generate pHAN-ORA59as. To create an inverted repeat, the *ORA59* ORF was excised from pSK-ORA59 with XbaI/HindIII and cloned into pHAN-ORA59as to generate pHAN-ORA59sas. For the construction of the RNAi control line *GUS* #5, the *GUS* ORF was excised from GusSH (Pasquali et al., 1994) with SalI/HindIII and cloned into pHANNIBAL digested with XhoI/HindIII. The pHANNIBAL expression cassettes were cloned into the binary vector pART27 (Gleave, 1992) using NotI.

For the construction of plants with estradiol-responsive transgene expression (XVE), the *GUS* ORF was excised from pGUSN358→S (Clontech, Palo Alto, CA) with SalI/EcoRI and cloned into pBluescript SK+ to generate pSK-GUS. The *AtERF1* and *AtERF2* open reading frames were amplified by PCR from Arabidopsis genomic DNA using the following primer sets respectively: 5'-CGG GAT CCA TAT GAC GGC GGA TTC TCA ATC-3' and 5'-CGG GAT CCT TAT AAA ACC AAT AAA CGA TC-3'; 5'-CGG GAT CCA TAT GTA CGG ACA GTG CAA TAT AG-3' and 5'-CGG GAT CCT TAT GAA ACC AAT AAC TCA TC -3'. *AtERF1* and *AtERF2* were cloned as BamHI fragments into pBluescript SK+. The *ORA59*, *AtERF2* and *GUS* ORFs were excised from pRT101-ORA59, pSK-AtERF2 and pSK-GUS respectively, with XhoI/XbaI and cloned into the binary vector pER8 (Zuo et al, 2000) digested with XhoI/SpeI. The *AtERF1* ORF was excised from pSK-AtERF1 with ApaI/SpeI and cloned into pER8 digested with the same enzymes.

For the construction of the XVE-*ERF1-TAP* and XVE-*ORA59-TAP* lines, the *ERF1-TAP* and *ORA59-TAP* cassettes were created by mutating the stop codon at the 3' of the *ERF1* and *ORA59* genes and then fused in frame with a *TAP* tag at the 3'end, generating a protein-tag fusion with the tag at the C-terminus. *ERF1* and *ORA59* were amplified by PCR with the primer set 5'-ACG CGT CGA CAA AAT GGA CCC ATT TTT AAT TCA GTC C- 3' and 5'-CCG CTC GAG CCT TGC CAA GTC CCA CTA TTT TC- 3' and 5'-ACG CGT CGA CAA AAT GGA ATA TCA AAC TAA CTT C- 3' and 5'- CCG CTC GAG CCT TGA GAA CAT GAT CTC ATA AG-3', respectively, and cloned in pGEM-T Easy (Promega, Madison, WI). The *TAP* insert was excised from pBS1479 (Puig et al., 2001) with BglII and cloned into pCAMBIA1300 digested with BamHI. The *ERF1-ΔSTOP* and *ORA59-ΔSTOP* inserts were excised from pGEM-T Easy with SalI/XhoI and cloned into pCAMBIA-*TAP*. The *ERF1-TAP* and *ORA59-TAP* cassettes were excised with SalI/SpeI from pCAMBIA1300-TAP and introduced into the binary vector pER8 digested with XhoI/SpeI.

For the construction of the constitutive *AtERF1*-overexpressing plants, *AtERF1* (*At4g17500*) was amplified by PCR on genomic DNA with the primer set 5'- GGG GTA CCA AAA TGT ACC CAT ACG ATG TTC CAG ATT ACG CTG GTT ACC CAT ACG ATG TTC CAG ATT ACG CTG AGC TCA TGA CGG CGG ATT CTC AAT C–3' and 5'-CGG GAT CCT TAT AAA ACC AAT AAA CGA TC-3' and cloned in pGEM-T Easy. The resulting sequence encodes the AtERF1 protein with a double haemagglutinin (HA) epitope tag at its N-terminal end. The *HA-AtERF1* insert was excised with KpnI/BamHI and cloned into pRT101. The CaMV 35S-cassette containing *HA-AtERF1* was excised with HindIII and introduced into pCAMBIA1300.

The binary vectors pCAMBIA1300-ORA59, pCAMBIA1300-HA-AtERF1 and pCAMBIA1301 were introduced into *Agrobacterium tumefaciens* strain LBA1115 (containing the Vir plasmid pSDM3010). The binary vectors pART27-ORA59, pART27-GUS, pER8-ORA59 and pER8-AtERF2 were introduced into *A. tumefaciens* strain LBA4404 while pER8-GUS, pER8-AtERF1, pER8-ERF1-TAP and pER8-ORA59-TAP were introduced into *A. tumefaciens* strain EHA105. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MA medium containing 100 mg/L timentin and 20 mg/L hygromycin, except for pART27 transformants which were selected on 25 mg/L 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 hygromycin and were allowed to selfpollinate. 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 hygromycin.

Plant infection with pathogens

All genotypes were grown for 2 weeks on solid MA medium containing suitable antibiotics at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux). Seedlings were transferred to sterile soil and cultivated for another 3 weeks in a growth chamber with a 8 h day (1400 lux at 24ºC) and 16 h night (20ºC) cycle at 65% relative humidity. Due to their early stage dwarf phenotype, seeds from the 35S:*ORA59 #17* transgenic plants were sown 2 weeks earlier than the other genotypes. Soil-potted plants from this genetic background were therefore allowed to grow 5 weeks in order to reach a stage with rosette leaf size suitable for pathogen infection.

For inoculation with fungal pathogens, 3-µL droplets of spore suspension were deposited on 4-6 mature leaves of each plant. Inocula used were 5.10^5 and 1.10^6 spores/mL for *Botrytis cinerea* and *Alternaria brassicicola*, respectively. *B. cinerea* spores were incubated in half-strength potato dextrose broth for 2 hours prior to inoculation. Moreover, *Botrytis*-infected leaves were gently wounded with a needle where the spore-containing droplet was deposited. After inoculation, plants were maintained under high relative humidity with the same temperature and photoperiod conditions. In each experiment, 15-20 plants per genotype were inoculated.

Disease ratings were assessed at day 4 and day 7 after inoculation with *B. cinerea* or *A. brassicicola*, respectively. Disease ratings were assigned to the inoculated leaves of each plant, as described by Ton et al. (2002) with minor modifications. Briefly, intensity of disease symptoms and lesion size were classified: I, no visible disease symptoms; II, non-spreading 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. For gene expression analysis, infected and non-infected leaves from several inoculated plants of each genotype were collected at day 2 and day 4 for *B. cinerea*-infected plants, and at day 2 and day 7 for *A. brassicicola*-infected plants. Leaf tissue was frozen in liquid nitrogen and stored at –80ºC.

Northern blot 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. As described by Memelink et al. (1994), 10 µg RNA samples were subjected to electrophoresis on 1.5% agarose/1% formaldehyde gels, and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). All probes were ^{32}P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications (Chapter 3). Blots were exposed on X-ray films (Fuji, Tokyo, Japan).

DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5'- GGG GTA CCG GAT CCT CTT AAG TGG AGA GTT TTC C-3' and 5'- GCT CTA GAC TCG AGG CCA CGG TGG CTT CTT TTC C-3' for *ORA59* (*At1g06160*); 5'- GGG GTA CCG GAT CCA CGG CGG ATT CTC AAT CTG ATT ATG-3' and 5'- GCT CTA GAC TCG AGC TAA CAT ATC CTC AGA ATC G-3' for *AtERF1* (*At4g17500*); 5'- GGG GTA CCG GAT CCA TGT ACG GAC AGT GCA ATA TAG-3' and 5'- GCT CTA GAC TCG AGC GAT GAT GAC GTG TCA AAA TGG-3' for *AtERF2* (*At5g47220*); 5'- GGG GTA CCG GAT CCT TAA TAA CGG CGT TAA CTC ACG-3' and 5'- GCT CTA GAC TCG AGT CGC CGG AGG CTT ATT TTC CTC-3' for *ORA4* (*At2g44840*); 5'- GGG GTA CCG GAT CCA GAT CCA TGG ATG AAG CAC GAA TC-3' and 5'- GCT CTA GAC TCG AGA ATG GCG GTT TAC GAA TTT GAG-3' for *ORA31* (*At5g47230*); 5'-AAT GAG CTC TCA TGG CTA AGT TTG CTT CC-3' and 5'-AAT CCA TGG AAT ACA CAC GAT TTA GCA CC-3' for *PDF1.2* (*At5g44420*); 5'-GCT TCA GAC TAC TGT GAA CC-3' and 5'-TCC ACC GTT AAT GAT GTT CG-3' for *ChiB* (*At3g12500*); 5'-CGG GAT CCA TAT GAA GAT CAG ACT TAG CAT AAC-3' and 5'-CGG GAT CCT CAA ACG CGA TCA ATG GCC GAA AC-3' for *HEL* (*At3g04720*); 5'-TGT CCC ACT CTC GTT CTT TG-3' and 5'-TCA AGT CCG GCT GGA ACA TTG-3' for *AN5-AT* (*At5g61160*); 5'-ATG ACA AGC TCC GAT CAA TC-3' and 5'-TCA AGC CGA AGC CGG AGA AG-3' for *AIG2* (*At3g28930*); 5'-ATG GCG GCT TCT ACA TTG TAC-3' and 5'-CTA TGT CAG CTT CTC GGA CTC-3' for *ASB1* (*At1g25220*); 5'-CAG GCA CCT CTG CTA CTT TC-3' and 5'-ATA GCT CCA TGG AGG ACA CC-3' for *TSB1* (*At5g54810*); 5'-GCA ATT CTC GAT CCG AGC TC-3' and 5'-CTC TAC TTG GAG AAG CCT TC-3' for *IGPS* (*At2g04400*); 5'-CAG TCC TTT ACT GGC CAA ATG- 3' and 5'- GGG TCT CTA AGC ATC TTT CC- 3' for *DIN11* (*At3g49620*); 5'-TCC ACC AGA TCT ATC TAC GG- 3' and 5'- GCA GCG TAA CCT CCA GTG GC- 3' for *DHS1* (*At4g39980*); 5'-ATG GGA ACA GCG TCG TCT AAG- 3' and 5'- GAG GTT GAC TGG TGA TCC TTC- 3' for *CYP1* (*At4g22710*); 5'-CGG GAT CCA TGA AAA TCC TCT CAC TTT-3' and 5'-CCC TCG AGT TAA GAA GGT ACG TAG TAG AG-3' for *Vegetative Storage Protein 1* (*VSP1, At5g24780*); 5'-CGG AAT TCA TGA GAG AGA TCC TTC ATA TC-3' and 5'-CCC TCG AGT TAA GTC TCG TAC TCC TCT TC-3' for *TUB* (*At5g44340*); 5'-CGG GAA GGA TCG TGA TGG A-3' and 5'-CCA ACC TTC TCG ATG GCC T-3' for *ROC* (*At4g38740*). The BamHI/NcoI

and EcoRI/HindIII fragments from the *ERF1* (*At3g23240*) and *actA* (Benito et al., 1998) open reading frames, respectively, were used as probes.

Microarray analyses

Wild-type plants (Columbia ecotype), two transgenic Arabidopsis lines expressing *ORA59* in an inducible manner (XVE-*ORA59-6* and *-10*) and two transgenic control lines expressing the *GUS* gene in an inducible manner (XVE-*GUS-7* and *-15*) were used for microarray experiments. Biological samples containing 15-20 seedlings were grown for 2 weeks in polypropylene tubes containing liquid MA medium as described above. Three replicates were used for each treatment or time point. After treatment with estradiol, JA or JA/ethephon (or with appropriate control solutions), total RNA was extracted as described above and subsequent purification was performed using NucleoSpin RNA II columns (Macherey-Nagel, Düren, Germany). RNA integrity of each biological sample was checked using Agilent Lab-on-a-chip 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA). RNAs of the three replicates were then pooled and checked again for RNA integrity. From each pooled sample, 20 µg were labeled using the fluorescent direct label method as described by the supplier (Agilent Technologies). Control-treated samples were Cy5-labeled whereas estradiol-, JA- or JA/ethephon-treated samples were Cy3-labeled. Hybridizations were performed using Agilent Arabidopsis 3 Oligo (44K) Arrays according to manufacturer's recommendations. Scan images were converted to raw data with the Agilent Feature Extraction software (version 7.5) using default parameters. Data were analyzed using the Rosetta Resolver software (Agilent Technologies). For the microarray experiments using wild-type plants, duplicate arrays were performed for each condition using RNA samples from independent plants. To increase confidence in measurements, log ratio values of the two replicates were combined using default parameters of the Rosetta Resolver software.

Data obtained from the two XVE-*GUS* hybridizations were used to remove genes with differential expression presumably due to estradiol or due to the estradiol-activated chimeric XVE transcription factor (Zuo et al., 2000). Genes were considered "induced" when they were upregulated in both XVE-*ORA59* hybridization experiments and not induced in either of the XVE-*GUS* hybridization experiments. Log ratio values of the "induced" genes from the two XVE-*ORA59* lines were combined. Expression differences with fold-change ≥ 2 and with *P*-values ≤ 0.001 were considered significant. The raw data files plus the details of the labelling and hybridization experiments have been deposited in the public microarray database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-460.

Transient expression assays

A 1183 base pair fragment of the *PDF1.2* (*At5g44420*) promoter was amplified by PCR on genomic DNA with the primer set 5'-CGG GAT CCA TGC AGC ATG CAT CGC CGC ATC -3' and 5'-GCG TCG ACG ATG ATT ATT ACT ATT TTG TTT TCA ATG -3' and cloned in pGEM-T Easy (Promega). The *PDF1.2* insert was excised with BamHI/SalI and cloned in the vector GusXX (Pasquali et al., 1994). Protoplasts prepared from *Arabidopsis thaliana* cell suspension ecotype Col-0 were co-transformed with plasmids carrying *PDF1.2*-promoter-*GUS*, effector plasmids carrying *AtERF1*, *AtERF2*, *ERF1* or *ORA59* genes fused to the CaMV 35S promoter and the p2rL7 plasmid (De Sutter et al., 2005) carrying the *Renilla*

luciferase (*LUC*) gene under the control of the CaMV 35S promoter. As a control, co-transformation of *PDF1.2*-promoter-*GUS* with an empty vector (pRT101 or pMOG184 (Mogen International, Leiden, The Netherlands)) and the p2rL7 plasmid was carried out. Protoplasts were transformed using polyethylene glycol as described previously (Schirawski et al., 2000) with the three constructs in a ratio of 1:1:3 (*GUS*:*LUC*:effector plasmid). Protoplasts were harvested 18 hours after transformation and frozen in liquid nitrogen. GUS and LUC activity assays were performed as described by van der Fits and Memelink (1997) and Dyer et al. (2000) respectively, for each individual transformation. *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.

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