Regulation of ORA59, a key modulator of disease resistance in Arabidopsis

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CHAPTER 2

Two GCC boxes and AP2/ERF-domain transcription factors ORA59 and ERF1 in jasmonate-ethylene mediated activation of the *PDF1.2* gene in Arabidopsis

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Abstract

Plant defense against pathogens and herbivores depends on the action of several endogenously produced hormones, including jasmonic acid (JA) and ET (ET). In certain defense responses, JA and ET signaling pathways synergize to activate a specific set of defense genes including *plant defensin 1.2* (*PDF1.2*). The APETALA2 (AP2)-domain transcription factor ORA59 acts as the integrator of the JA and ET signaling pathways and is the key regulator of JA- and ET-responsive *PDF1.2* expression. In this chapter we describe studies aimed at dissecting the interaction of ORA59 and the related transcription factor ERF1 with the *PDF1.2* promoter. We show that two GCC boxes in the *PDF1.2* promoter are important for trans-activation by ORA59 and ERF1 in transient assays and for *in vitro* binding. Using the chromatin immunoprecipitation technique we were able to show that ORA59 binds to the *PDF1.2* promoter *in vivo*. In stably transformed plants single mutation of either GCC box completely abolished the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ET-releasing agent ethephon. A tetramer of a single GCC box conferred JA/ethephon-responsive expression, demonstrating that the JA and ET signaling pathways converge to a single GCC box. Therefore ORA59 and two functionally equivalent GCC box binding sites form the module that enables the *PDF1.2* gene to respond synergistically to simultaneous activation of the JA and ET signaling pathways.

Introduction

Plants undergo continuous exposure to various biotic and abiotic stresses in their natural environment. To survive under such conditions, plants have evolved intricate mechanisms to perceive external signals, allowing optimal responses to environmental stresses including attack by herbivores or microbial pathogens (Fujita et al., 2006). Perception of stress signals leads to the production of one or more of the secondary signaling molecules jasmonic acid (JA), ethylene (ET), or salicylic acid (SA).

JA belongs to a family of signaling molecules, including certain precursors and derivatives, which are collectively known as jasmonates (JAs). Besides their role in some aspects of plant growth and development, such as production of viable pollen, JAs are major intermediate signaling molecules involved in defense against wounding, herbivore attack and pathogen infection (Creelman and Mullet, 1997; Turner et al., 2002). The SA and JA/ET dependent signaling pathways appear to modulate plant responses against different classes of pathogens (Thomma et al., 1999a). Arabidopsis plants impaired in JA or ET signaling pathways showed enhanced susceptibility to the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* (Penninckx et al., 1996; Thomma et al., 1998; Thomma et al., 1999a; Thomma et al., 1999b), demonstrating that JA and ET are important signal molecules for resistance against these pathogens.

A crucial step in the JA/ET-dependent defense response is the rapid transcription of genes coding for antimicrobial proteins (Penninckx et al., 1998) or enzymes involved in the biosynthesis of secondary metabolites (Memelink et al., 2001). Studying the mechanism 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.

Transcription factors belonging to a subgroup of the APETALA2 (AP2)-domain protein family known as the ET response factors (ERF) have emerged as important players in plant defense responses (Gutterson and Reuber, 2004). Proteins from the AP2/ERF-domain subfamily are characterized by a single AP2-type DNA-binding domain with a conserved amino acid sequence, and several members were shown to bind specifically to the sequence GCCGCC (Hao et al., 1998). This so-called GCC box is found in the promoters of several pathogen-responsive genes including *plant defensin 1.2* (*PDF1.2)*.

Constitutive overexpression of ERF1 and AtERF2 was shown to cause high levels of expression of the *PDF1.2* gene and other defense genes (Brown et al., 2003; Lorenzo et al., 2003; Solano and Ecker, 1998) and caused resistance to several fungi (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). It has been shown that the *ERF1* gene is synergistically induced by ET and JA and it was suggested that this transcription factor is a key element in integration of both signals for the regulation of defense genes (Lorenzo et al., 2003). Atallah, (2005) characterized the AP2/ERF-domain transcription factor ORA59, which was also transcriptionally induced by JA and ET in a synergistic manner. Overexpression of *ORA59* activated the expression of several JA- and ET-responsive defense-related genes including *PDF1.2,* and caused increased resistance against *B. cinerae* (Pré et al., 2008). Although several AP2/ERF-domain transcription factors

have been suggested to be positive regulators of *PDF1.2* gene expression (Brown et al., 2003; Lorenzo et al., 2003; Pré et al, 2008), a recent study showed 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 (Pré et al., 2008). Analysis of transgenic plants in which *ORA59* gene expression was silenced by RNAi, whereas the *ERF1* gene was normally expressed, showed that ORA59 is strictly required for *PDF1.2* gene expression in response to JA, JA/ET, and infection with necrotrophic fungi (Pré et al., 2008). Studies of the *PDF1.2* gene promoter (Brown et al., 2003; Manners et al., 1998) identified a GCC-box at positions -256 to -261 which is involved in the JA response. However interactions of the *PDF1.2* promoter with the relevant transcription factors ERF1 and especially ORA59 have not been reported, which prompted us to undertake the studies described in this chapter.

In the present study, we show that ORA59 and ERF1 trans-activate the *PDF1.2* promoter in transient assays via binding to two GCC boxes. Using the chromatin immunoprecipitation technique we were able to show that ORA59 binds the *PDF1.2* promoter *in vivo*. Interestingly, single mutation of either GCC box completely abolished the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ET-releasing agent ethephon.

Finally, we show that a tetramer of a single GCC box conferred JA/ethephon-responsive expression, demonstrating that the JA and ET signaling pathways converge to a single GCC box.

Results

ORA59 and ERF1 trans-activate the *PDF1.2* promoter in a dose-dependent manner

PDF1.2 promoter fragments containing 1186 bp (LF) or 277 bp (SF) upstream of the probable transcription start site (Manners et al., 1998) were fused to the β-glucuronidase (*GUS*) reporter gene (Figure 1a).

To study the dose-response relationship for trans-activation of the *PDF1.2* promoter by ORA59 (At1g06160) and ERF1 (At3g23240), Arabidopsis protoplasts were co-transformed with the SF promoter derivative fused to *GUS*, and variable amounts of effector plasmids carrying the *ORA59*, *ERF1* or *ORA47* (At1g74930) genes fused to the *CaMV 35S* promoter (Figure 1b). ORA59 and ERF1 activated the *SF-GUS* reporter gene 40 or 10 fold respectively, whereas the unrelated AP2/ERF-domain transcription factor ORA47 (Pré, 2006; Zarei, 2007) had no effect. As shown by Zarei (2007), ORA47 trans-activated the promoters of the *allene oxide cyclase* 1 and 2 genes in the protoplast assay, demonstrating that ORA47 is expressed and active. Previously we have shown that AtERF1 (At4g17500) and AtERF2 (At5g47220) did not significantly trans-activate the SF promoter derivative in a similar experimental setup (Pré et al., 2008). Together these observations indicate that ORA59 and ERF1 have a specific activating effect on the *PDF1.2* promoter. The trans-activation of the SF promoter was dose-dependent and increased up to 6 µg of effector plasmid, where after the response saturated.

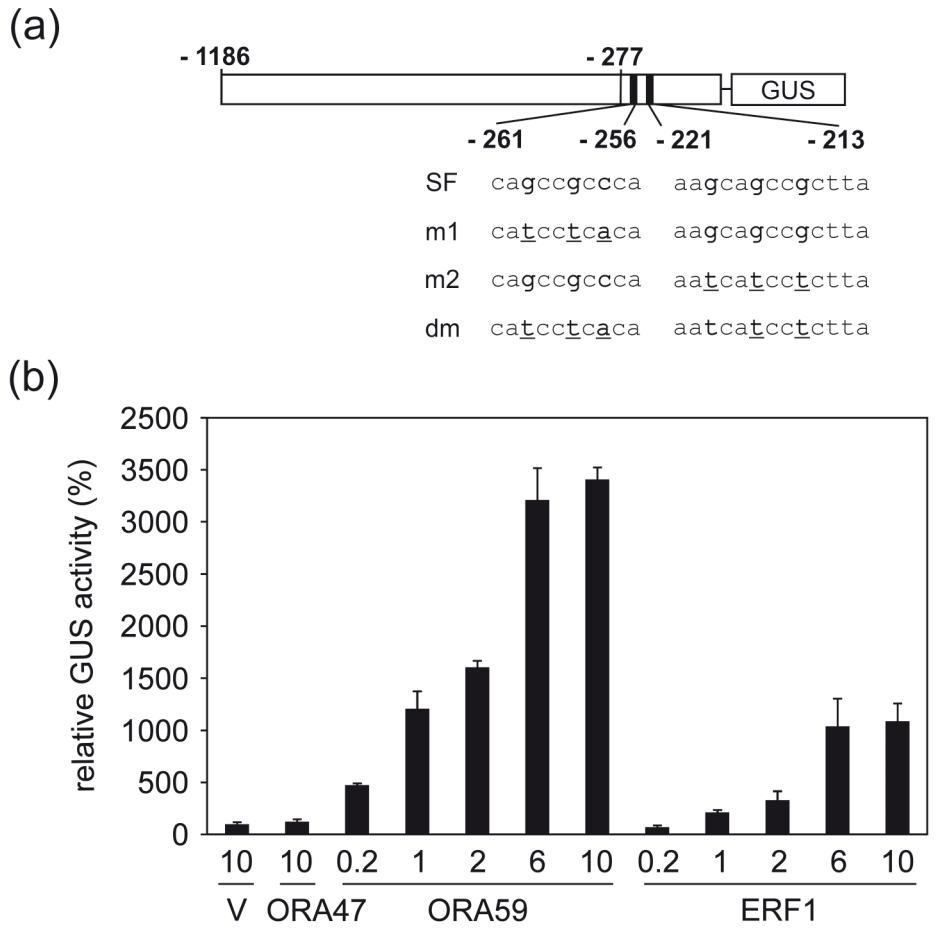

Figure 1. ORA59 and ERF1 trans-activate the *PDF1.2* promoter in a dose-dependent manner. **(a)** Constructs used in trans-activation assays. Reporter constructs consisted of the *GUS* gene driven by wild-type or mutated LF (long fragment) or SF (short fragment) *PDF1.2* promoter derivatives. Bold and underlined nucleotides indicate point mutations in GCC boxes. Numbers indicate positions relative to the start site of transcription. **(b)** Arabidopsis protoplasts were co-transformed with 2 µg of wild-type *SF-GUS* and variable amounts in µg of effector plasmids. The effector constructs consisted of an expression vector carrying the *CaMV 35S* promoter without or with the *ORA59*, *ERF1* or *ORA47* genes. The *Renilla* luciferase (LUC) gene fused to the *CaMV 35S* promoter served as a reference gene to correct for differences in transformation and protein extraction efficiencies. Values represent means ± SE of triplicate experiments and are expressed relative to the vector (v) control set as 100%.

ORA59 and ERF1 trans-activate the *PDF1.2* promoter independently via two GCC boxes

Transient expression assays revealed that the short SF derivative conferred GUS expression to a level similar as found with the long LF derivative both with ORA59 as well as ERF1 (Figure

2a), indicating that all cis-acting elements interacting with these two transcription factors are contained within the SF derivative. In the SF derivative a GCC box at positions -261 to -256 was pointed out as being important for the JA-responsive activity of this promoter derivative (Brown et al., 2003). To study whether ORA59 and ERF1 act via this GCC box, we mutated it generating the m1SF promoter derivative (Figure 1a). This mutation reduced GUS activity conferred by ORA59 and ERF1 1.5-2 fold, indicating that it is important but that there are other sequences interacting with these transcription factors. Indeed, there is another GCC-like box at positions -221 to -213. Therefore we generated m2SF and dmSF promoter derivatives carrying mutations in the second GCC box and in both GCC boxes, respectively. The m2 mutation reduced *PDF1.2* promoter activity about 2-fold, similar to the m1 mutation. The double mutant version was activated 5-6 fold less efficiently by ORA59 and ERF1 than the wild-type derivative. These results indicate that the two GCC boxes are functionally equivalent and are the main sites interacting with ORA59 and ERF1 (Figure 2a).

To find out whether there is a synergistic effect of ORA59 and ERF1 on activation of the *PDF1.2* promoter, we co-transformed identical amounts of effector plasmids carrying ORA59 or ERF1 alone or in combination with the SF-*GUS* reporter construct. The results show that ORA59 and ERF1 act additively instead of synergistically (Figure 2b), indicating that they act independently via interaction with the same target sites.

ORA59 and ERF1 bind to the two GCC-boxes in the *PDF1.2* promoter *in vitro*

To establish whether ORA59 and ERF1 indeed bind the GCC boxes in the *PDF1.2* promoter as suggested by the trans-activation experiments, we produced recombinant proteins expressed in *Escherichia coli*. Analysis of the proteins by staining of an SDS-PAA gel with coomassie brilliant blue (results not shown) or immunoblot analysis with Penta-His horse-radish-peroxidase antibody conjugate (Figure 3a) showed a single main reactive band in each protein preparation. Although ORA59 and ERF1 have similar predicted sizes of around 30 kDa, ORA59 migrated in the denaturing gel system at a position corresponding to 42 kDa, which might be due to a specific structure of the protein. ORA59 expressed in Arabidopsis protoplasts also migrated at the same position (results not shown), which makes it unlikely that the aberrant migration is due to a post-translational modification.

Next, the binding of ORA59 and ERF1 proteins to radiolabeled SF, mSF, and dmSF fragments was studied in electrophoretic mobility shift assays. The unrelated AP2/ERF-domain transcription factor ORA47 was used as a control at an amount that gave clear complex formation with a binding site from the promoter of the target gene *allene oxide cyclase 2* (*AOC2*; Figure 3b). As shown in Figure 3b, ORA59 and ERF1 were able to bind to the SF fragment, in contrast to ORA47. Binding of ORA59 and ERF1 was partially decreased when the GCC box at positions -261 to -256 was mutated, and completely abolished when both GCC boxes were mutated. Although the GCC box at positions -221 to -213 clearly contributed to binding to the SF fragment, mutation of this

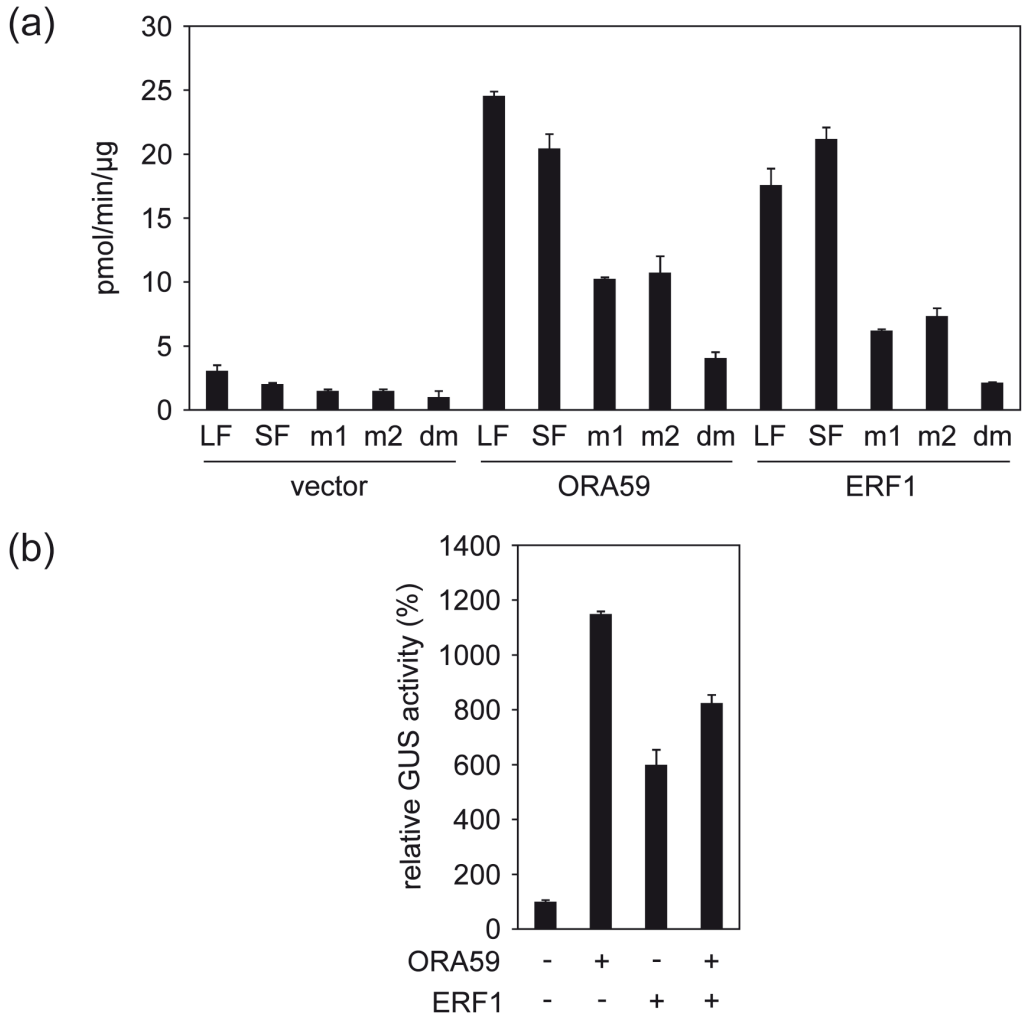

Figure 2. ORA59 and ERF1 trans-activate the *PDF1.2* promoter independently via two GCC boxes. **(a)** Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying different versions of the *PDF1.2* promoter shown in Figure 1A fused to *GUS* and overexpression vectors without or containing the *ORA59* or *ERF1* genes driven by the *CaMV 35S* promoter. Protein concentrations were used to correct for differences in protein extraction efficiencies. Values represent means ± SE of triplicate experiments. **(b)** The *SF-GUS* reporter plasmid was co-transformed with 1 µg of overexpression vectors carrying ORA59 or ERF1, or with a combination of 0.5 µg of each overexpression plasmid. Values represent means ± SE of triplicate experiments and are expressed relative to the vector control.

GCC box alone had relatively little effect on binding. These EMSA experiments confirm that these two GCC boxes are the main binding sites for ORA59 and ERF1 in the SF derivative of the *PDF1.2* promoter.

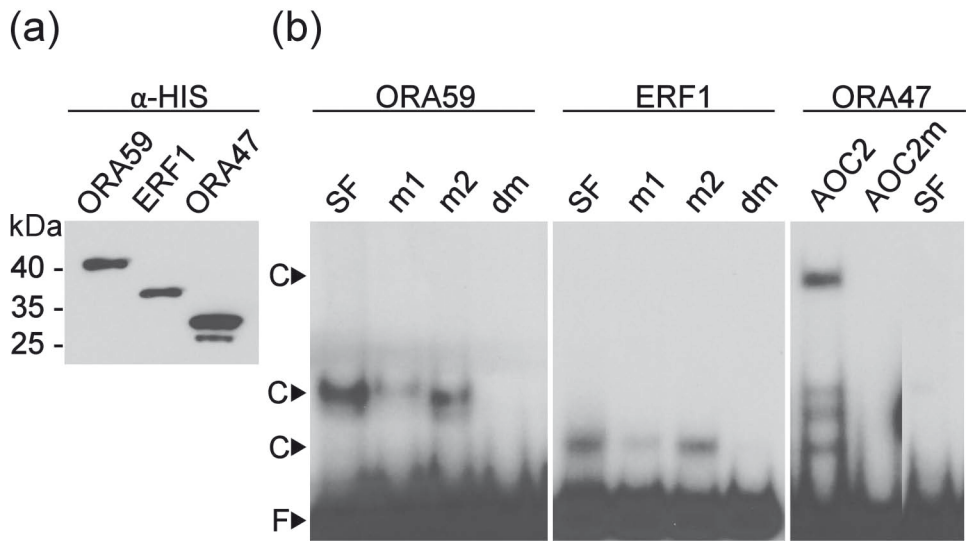

Figure 3. ORA59 and ERF1 bind to two GCC boxes in the *PDF1.2* promoter *in vitro*. **(a)** After SDS-PAGE recombinant proteins were detected with Penta-His HRP antibody conjugate following Western blotting. Protein size markers are indicated in k Dalton. **(b)** EMSAs were performed with recombinant ORA59 and ERF1 proteins and radio-labeled SF, m1, m2 or dm fragments. ORA47 protein and wild-type or mutated AOC2 fragment were used as a control. The arrow heads mark the positions of protein-DNA complexes (C) and free probes (F).

ORA59 binds to the *PDF1.2* promoter *in vivo*

The transactivation experiments as well as the i*n vitro* binding studies suggest that ORA59 binds directly to the *PDF1.2* promoter *in vivo* to regulate gene expression. We wanted to confirm this directly using chromatin immunoprecipitation analysis (ChIP). Therefore we constructed plants expressing ORA59 with the tandem affinity purification (TAP; Puig et al., 2001) tag attached to its C-terminal end under control of the estradiol-inducible XVE system (Zuo et al., 2000). Following screening of the transformants for the *ORA59-TAP* mRNA level, line #4 was selected for further analysis.

We first verified that the ORA59-TAP fusion protein was expressed and functional. In addition we wanted to determine the optimal induction conditions prior to harvesting plant samples for ChIP analysis. Following addition of 4 µM estradiol or the solvent DMSO the kinetics of mRNA and protein accumulation were followed (Figure 4). Maximum levels of ORA59-TAP mRNA and protein were observed after 16 to 24 hours. Estradiol treatment also induced the ORA59 target gene *PDF1.2*, but with slower kinetics, showing that the ORA59-TAP fusion protein is functionally active. DMSO-treated transgenic plants did not express ORA59-TAP or *PDF1.2*. Estradiol treatment had no effect on *PDF1.2* expression in control plants (Figure 5b).

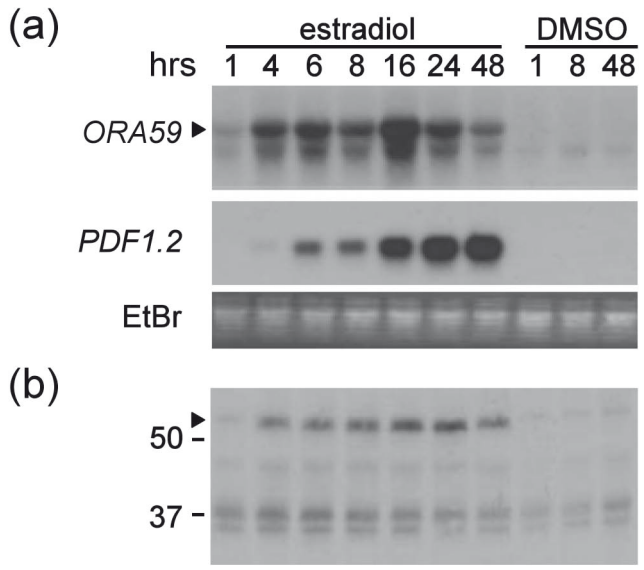

Figure 4. The ORA59-TAP protein is inducibly expressed and functional. Fifteen days-old T2 seedlings from XVE-ORA59-TAP line #4 cultured in liquid medium were treated for varying times in hours with 4 µM estradiol or the solvent DMSO at a final concentration of 0.1%. **(a)** Northern blot analysis. Gel blots were hybridized with the indicated probes. The arrowhead indicates the position of the *ORA59-TAP* mRNA. The ethidium bromide (EtBr) stained gel is shown as a control for RNA loading. **(b)** Western blot analysis. The protein samples were separated by SDS-PAGE followed by Western blotting and immuno-probing with the Peroxidase anti-Peroxidase (PAP) antibody, which has affinity for the protein A part of the TAP tag. The arrowhead indicates the position of the ORA59-TAP fusion protein. Positions of protein size markers are indicated in k Dalton.

Based on the results from the expression analysis, seedlings treated with 4 µM estradiol or 0.1% DMSO for 16 hours were used for ChIP analysis. Transgenic seedlings expressing only the TAP tag under control of the *CaMV 35S* promoter were similarly treated as controls. Protein and mRNA analysis of the harvested samples prior to formaldehyde cross linking showed that the ORA59-TAP fusion protein was induced by estradiol treatment and was functional as judged by the induction of *PDF1.2* expression (Figures 5b and c). The *35S*-TAP seedlings expressed the TAP mRNA and protein, but as expected did not express the *PDF1.2* gene. PCR analysis using *PDF1.2* primers of the chromatin prepared following formaldehyde cross linking of the samples showed that equivalent amounts of DNA were present (Figure 5a, input). ChIP was performed using IgG Sepharose beads, which have strong affinity for the protein A part of the TAP tag. PCR analysis of the recovered DNA with primers flanking the GCC boxes in the *PDF1.2* promoter revealed that this genomic region was overrepresented in the preparation from XVE-ORA59-TAP seedlings treated with estradiol. Primers specific for the promoter of the unrelated *AOC2* gene did not show amplification of a fragment after the same number of PCR cycles. After 36 PCR cycles an

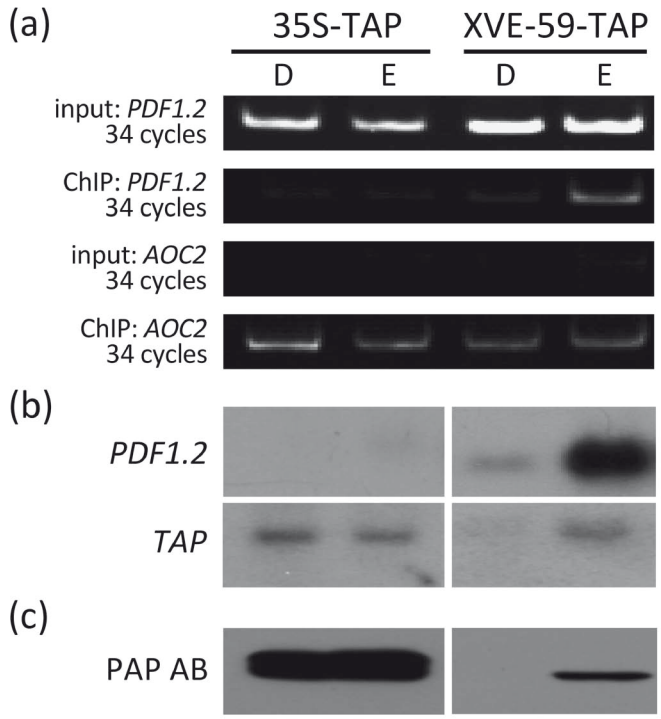

Figure 5. ORA59 binds to the *PDF1.2* promoter *in vivo*. Seedlings from XVE-ORA59-TAP line #4 and *35S*-TAP line #7 were treated with 4 µM estradiol (E) or 0.1% DMSO (D). RNA and protein was extracted for Northern and Western blot analysis of transgene expression. Sonicated chromatin prepared from the remainder of the tissue samples was used in ChIP with IgG Sepharose which has affinity for the TAP tag. **(a)** ChIP analysis. Input chromatin or recovered chromatin was used as template in PCR with cycle number and gene-specific primers as indicated. **(b)** Northern blot analysis with probes as indicated. **(c)** Western blot analysis with Peroxidase anti-Peroxidase (PAP) antibody.

AOC2 fragment was amplified to similar levels in all samples, which indicates that based on this background contamination equivalent amounts of immuno-precipitated DNA were used for the PCR reactions (Figure 5a). The results therefore show that the ORA59-TAP fusion protein binds directly to the *PDF1.2* promoter *in vivo*.

Effects of GCC box mutations on JA- and ethephon-responsive expression of *PDF1.2* promoter derivative SF in stably transformed Arabidopsis plants

The expression of the *PDF1.2* gene is synergistically induced by a combination of JA and ET (Penninckx et al., 1998). To study the contribution of the two GCC boxes to JA- and ET-responsive activity of the *PDF1.2* promoter derivative SF, we generated stably transformed

plants containing the *GUS* fusion constructs shown in Figure 1a via *Agrobacterium*-mediated transformation. T2 seedlings from eight independent transgenic lines for each construct were treated with JA, ethephon or both for 24 hrs. Consistent with the accumulation of endogenous *PDF1.2* mRNA (Penninckx et al., 1998), *PDF1.2* promoter activity was relatively weakly induced by JA or ethephon alone, but strongly induced by the combination (Figure 6). Mutation of either GCC box dramatically decreased *PDF1.2* promoter activity in response to JA or JA/ethephon. Mutation of the GCC box at positions -261 to -256 reduced activity to the level observed with the wild-type promoter after control treatment, whereas mutation of the GCC box at positions -221 to -213 left a very low residual response to JA/ethephon. Mutation of both GCC boxes reduced *PDF1.2* promoter activity to very low levels below the level of the wild-type promoter after control treatment. Whereas both GCC boxes were important for transient trans-activation and *in vitro* binding by ORA59 and ERF1, it turns out that mutation of a single GCC box reduced JA- and JA/ethephon-responsive expression of *PDF1.2* promoter derivative SF close to background levels.

A GCC box tetramer is sufficient to confer JA- and ethephon-responsive expression in stably transformed Arabidopsis plants

The synergistic effect of JA and ET on *PDF1.2* promoter activity could be due either to convergence of the signaling pathways on each of the two GCC boxes, or it could be due to the separate action of each of the signaling pathways on a distinct single GCC box. To distinguish between these possibilities, tetramers of the wild-type and mutant (m1) GCC box between positions -261 to -256 were generated and fused to the TATA box of the *CaMV 35S* promoter and the *GUS* reporter gene. We tested whether a tetramer of the short GCC box could support transcriptional activation by ORA59 and ERF1 in a transient assay. ORA59 and ERF1 strongly transactivated the artificial promoter construct with ORA59 as the strongest activator (Figure 7a). The mutant GCC box tetramer was completely inactive, demonstrating that the transcription factors activated the artificial promoter via binding to the GCC boxes. Analysis of transgenic seedlings containing the tetramer constructs revealed that none of the lines transformed with the mutant GCC box tetramer showed GUS activity after hormone treatment (results not shown). The wild-type GCC box tetramer conferred JA- and JA/ethephon-responsive gene expression which was qualitatively and quantitatively similar to the native *PDF1.2* promoter. The results show that the JA and ET signaling pathways converge to a single GCC box sequence.

Discussion

In certain defense responses, JA and ET signaling pathways synergize to activate a specific set of defense genes including *PDF1.2* (Penninckx et al., 1998). The AP2-domain transcription

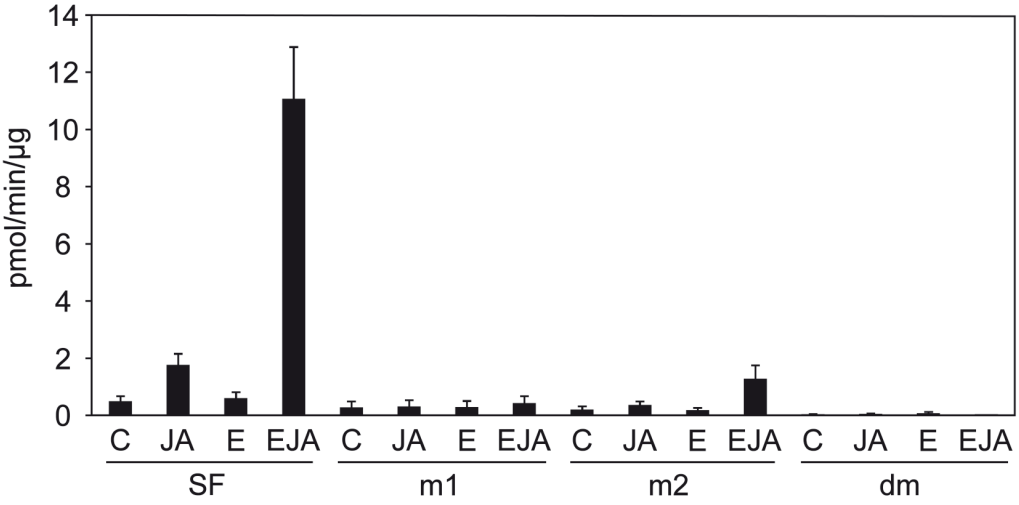

Figure 6. Both GCC boxes are essential for JA- and ethephon-responsive expression of *PDF1.2* promoter derivative SF in stably transformed Arabidopsis plants. Each bar represents average GUS activity values determined in pools of 10 T2 seedlings from 8 independent transformed lines for each construct corrected for protein concentration ± SE. Seedlings were control-treated (C) or treated with 50 µM JA, 1 mM of the ethylene-releasing agent ethephon (E) or both (EJA) for 24 hrs.

factor ORA59 acts as the integrator of the JA and ET signaling pathways and is the key regulator of JA- and ET-responsive *PDF1.2* expression (Pré et al., 2008). Here we aimed at dissecting the interaction of ORA59 and the related transcription factor ERF1 with the *PDF1.2* promoter. We show that two GCC boxes in the *PDF1.2* promoter were important for *in vitro* binding to ORA59 and ERF1 and were functionally equivalent in transactivation assays using these transcription factors. Using the chromatin immunoprecipitation technique we were able to show that ORA59 bound the *PDF1.2* promoter *in vivo*. Interestingly, single mutation of either GCC box resulted in a dramatic reduction of the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ET-releasing agent ethephon.

In a previous report a single GCC box at positions -261 to -256 was pointed out as being responsible for the JA-responsive activity of a *PDF1.2* promoter derivative which is very similar to our SF derivative (Brown et al., 2003). Mutation of this GCC box had only a moderate effect on trans-activation of the *PDF1.2* promoter by ORA59 or ERF1 in transient assays or on *in vitro* binding of these transcription factors. Simultaneous mutation of a second GCC box at positions -221 to -213 knocked out *in vitro* and transient *in vivo* interactions of ORA59 and ERF1 with *PDF1.2* promoter derivative SF.

The function of ERF1 is somewhat mysterious at the current level of understanding. ERF1 can induce the expression of defense genes when overexpressed, but is not able to support JA- or JA/ethephon-responsive expression of defense genes when *ORA59* expression is knocked out by

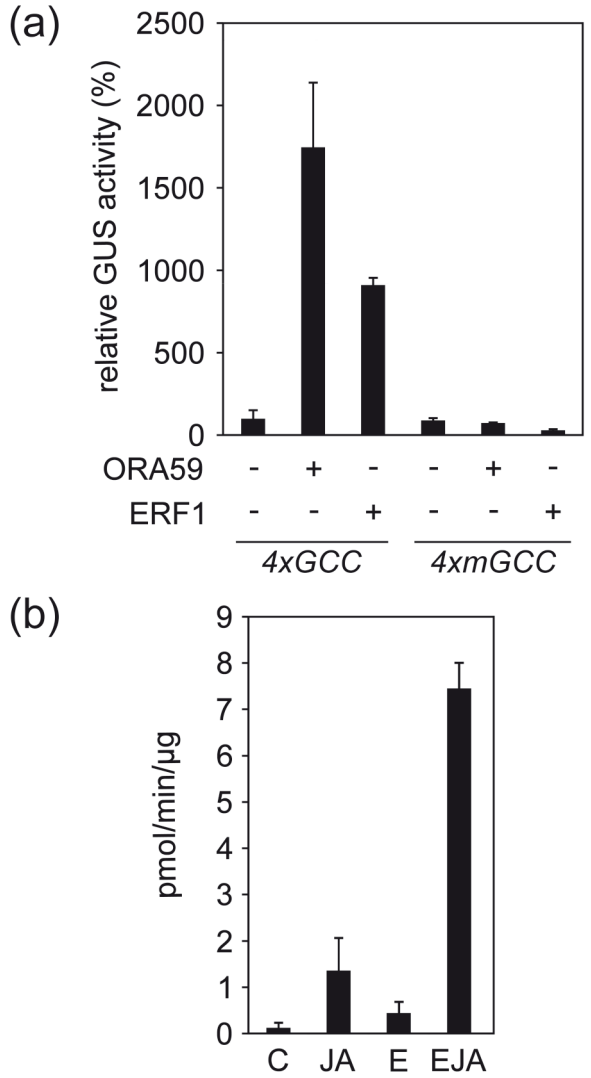

Figure 7. A GCC box tetramer is sufficient to confer JA- and ethephon-responsive expression in stably transformed Arabidopsis plants. **(a)** Arabidopsis protoplasts were co-transformed with 2 µg of wild-type or mutated 4xGCC-GUS and 6 µg of overexpression vectors containing *ORA59* or *ERF1* driven by the *CaMV 35S* promoter. The *Renilla* luciferase (LUC) gene fused to the *CaMV 35S* promoter served as a reference gene to correct for differences in transformation and protein extraction efficiencies. Values represent means ± SE of triplicate experiments and are expressed relative to the vector (v) control set as 100%. **(b)** Each bar represents average GUS activity values determined in pools of 10 T2 seedlings from 4 independent transformed 4GCC lines corrected for protein concentration in the same samples ± SE. Seedlings were control-treated (C) or treated with 50 µM JA, 1 mM of the ethylene-releasing agent ethephon (E) or both (EJA) for 24 hrs.

RNAi (Pré et al., 2008). One option could be that ERF1 acts synergistically with ORA59 on *PDF1.2* expression, for example by differential binding of these two proteins to the two GCC boxes. We tested this idea by comparing *PDF1.2* promoter activity levels in response to ORA59 and ERF1 separately or combined, but we did not find evidence for synergism.

Quite surprising in view of the other results presented here was the observation that single mutation either GCC box completely abolished the response of the *PDF1.2* promoter derivative SF in stably transformed plants in response to JA or JA/ethephon. Although this finding is consistent with the previous report of Brown et al. (2003) on the effect of the single mutation of the GCC box at positions -261 to -256, it was unexpected since the other assays highlighted the importance of both GCC boxes. There are several explanations for this apparent inconsistency. One likely option is that the *PDF1.2* promoter in the context of a chromatin structure in stably transformed plants requires two GCC boxes to be activated in response to JA whereas a single GCC box is not sufficient for opening up the chromatin structure for transcription.

The synergistic effect of JA and ET on *PDF1.2* promoter activity could be due either to convergence of the signaling pathways on each of the GCC boxes, or it could be due to the separate action of each of the signaling pathways on a single distinct GCC box. The wild-type GCC box tetramer conferred JA- and JA/ethephon-responsive gene expression, showing that the JA and ET signaling pathways converge to a GCC box sequence. Therefore ORA59 and two functionally equivalent GCC box binding sites form the module that enables the *PDF1.2* gene to respond synergistically to simultaneous activation of the JA and ET signaling pathways. The GCC box tetramer construct may also have applications as an artificial minimal JA/ET responsive promoter to dissect mechanisms of the synergistic effect of JA and ET and of the antagonistic effect of JA and SA, since it is likely to show less complex regulation than the native *PDF1.2* promoter, which contains also binding sites for other transcription factors such as the TGA proteins (Spoel et al., 2003).

Materials and Methods

Growth conditions and treatments

Arabidopsis thaliana ecotype Columbia (Col-0) is the genetic background for all wild type and transgenic plants. Following stratification for 3 days at 4°C, surface-sterilized seeds were germinated for 10 days at 21°C in a growth chamber (16 h light/8 h dark, 2500 lux at 70% humidity) on plates containing MA medium (Masson and Paszkowski, 1992) with 0.6% agar supplemented with 20 mg/L hygromycin for selection of transgenic plants. Batches of 15-20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt) containing 10 ml liquid MA medium without antibiotic and the tubes were incubated on a shaker at 120 rpm for 4 additional days before treatments. Transgenic plants carrying an XVE expression module containing the *ORA59* gene fused to the TAP tag were treated for 16 hrs with 4 µM estradiol. As control, seedlings were treated with 0.1% DMSO. Transgenic seedlings carrying the *PDF1.2* promoter derivatives LF, SF,

m1, m2 or dm or the wild-type and mutant GCC box tetramers fused to *GUS* were treated for 24 hrs with 50 µM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO; 0.1% v/v final concentration), 1 mM of the ET-releasing compound ethephon (Sigma-Aldrich) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) or a combination of JA and ethephon. As control, seedlings were treated with 0.1% DMSO and 0.5 mM sodium phosphate pH 7.

***PDF1.2* promoter and constitutive overexpression constructs**

Arabidopsis genomic DNA was used as template for the amplification of LF and SF fragments with forward primers 5'-CGG GAT CCA TGC AGC ATG CAT CGC CGC ATC-3' or 5' CGG GAT CCC CAT TCA GAT TAA CCA GCC GCC C-3', respectively, and the reverse primer 5'-GCG TCG ACG ATG ATT ATT ACT ATT TTG TTT TCA ATG-3'. Amplified products were digested with *Bam*HI and *Sal*I and cloned in plasmid GusXX (Pasquali et al., 1994). Mutations m1, m2 and dm were generated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and primers 5'-CCA TTC AGA TTA ACC ATC CTC ACC TGT GAA CGA TG-3' or 5'-CAT TAG CTA AAA GCC GAA TCA TCC TCT TAG GTT ACT TTA GAT ATC G-3', and their respective reverse complementary primers. For the construction of the *GCC box tetramers*, wild-type and mutant GCC-box monomers of the *PDF1.2* promoter were cloned by annealing the oligonucleotides 5'-GATC CTT AAC CAG CCG CCC ATG TGA-3' and 5'-GAT CTC ACA TGG GCG GCT GGT TAA G-3', and 5'-GATC CTT AAC CAT CCT CAC ATG TGA-3' and 5'-GAT CTC ACA TGT GAG GAT GGT TAA G-3', respectively, and ligating them into the plasmid pIC-20H (Marsh et al., 1984) digested with *Bam*HI and *Bgl*II. Monomers were then tetramerized in a head-to-tail configuration using the *Bam*HI and *Bgl*II sites. The tetramers were cloned as *Bam*HI/*Bgl*II fragments in the plasmid GusSH-47 (Pasquali et al., 1994) digested with *Bam*HI such that the orientation of the GCC-boxes relative to the downstream ORF was the same as in the *PDF1.2* promoter. 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', digested with *Bam*HI and cloned in pRT101 (Töpfer et al., 1987). The *ERF1* ORF was PCR-amplified using the primer set 5'-GAA GAT CTT CAT CAC CAA GTC CCA CTA TTT TC-3' and 5'-GAA GAT CTC ATA TGG ACC CAT TTT TAA TTC AGT CC-3', digested with *Bgl*II and cloned in *Bam*HI-digested pRT101. The *ORA47* (At1g74930) ORF was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-GAA GAT CTC ATA TGG TGA AGC AAG CGA TGA AG-3' and 5'-GAA GAT CTT CAA AAA TCC CAA AGA ATC AAA G-3' and following digestion with *Bgl*II cloned in pIC-20R (Marsh et al., 1984). The *ORA47* insert was excised with *Bgl*II and inserted into pMOG183 (Mogen International, Leiden, The Netherlands) digested with *Bam*HI.

Binary constructs and plant transformation

The TAP insert was excised from pBS1479 (Puig et al., 2001) with *Bam*HI and cloned into pC1300intB-35SnosBK (accession number AY560326) digested with *Bgl*II. pC1300intB-35SnosBK is a derivative of the binary vector pCAMBIA1300 carrying a *CaMV 35S* expression cassette. The

ORA59 ORF lacking the stop codon (*ORA59*-Δstop) was amplified by PCR with the primer set 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' and cloned in pGEM-T Easy (Promega). The *ORA59* ORF was excised from pGEM-T Easy with *Sal*I/*Xho*I and cloned into pC1300intB-35SnosBK-TAP. The *ORA59-TAP* fusion was excised with *Sal*I/*Spe*I from pC1300intB-35SnosBK-ORA59-TAP and introduced into the binary vector pER8 (Zuo et al., 2000) digested with *Xho*I/*Spe*I. The *PDF1.2* promoter derivatives SF, m1, m2 and dm fused to *GUS* and the tetrameric constructs *4xGCC:GUS* and *4xmGCC:GUS* were cloned into binary vector pMOG22λCAT (Pasquali et al., 1994; Menke et al., 1999) with *Xba*I/*Xho*I and *Xba*I/*Hin*dIII, respectively. The pMOG22λCAT binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993). 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.

Protein production and immunoblot analysis

ORA59, ERF1 and ORA47 proteins were produced with N and C terminal Strep and His tags, respectively. *ORA59* was amplified with the primer set 5'-CGG AAT TCA ATG GAA TAT CAA ACT AAC TTC-3' and 5'-CGG TCG ACC CTT GAG AAC ATG ATC TCA TAA G-3', digested with *Eco*RI and *Sal*I and cloned in pASK-IBA45plus (IBA Biotagnology, Göttingen, Germany). *ERF1* was amplified with the primer set 5'-CGG AAT TCA ATG GAC CCA TTT TTA ATT CAG-3' and 5'-CGG TCG ACC CTT GCC AAG TCC CAC TAT TTT C-3', digested with *Eco*RI and *Xho*I and cloned in pASK-IBA45 digested with *Eco*RI and *Sal*I. *ORA47* was amplified with the primer set 5'-CGG AAT TCA ATG GTG AAG CAA GCG ATG AAG-3' and 5'-CGG TCG ACC CTT GAA AAT CCC AAA GAA TC-3', digested with *Eco*RI and *Sal*I and cloned in pASK-IBA45plus. The proteins were expressed in *Escherichia coli* strain BL21(DE3)pLysS (Novagen). Since the large majority of ORA59 and ERF1 proteins was insoluble and the remaining soluble part was mostly degraded, proteins were purified from inclusion bodies by denaturation in binding buffer (5 mM Imidazole, 0.5 mM NaCl, 40 mM Tris-HCl pH 8.0) with 6 M urea and re-folded by a quick 10-fold dilution in binding buffer without urea followed by 16 h dialysis. All proteins were purified by sequential Ni-NTA agarose (Qiagen) and Strep-Tactin sepharose (IBA) chromatography according to the Novagen His tag and the IBA Strep tag purification protocols. Proteins were separated by 10% (w/v) SDS-PAGE and transferred to Protan nitrocellulose (Schleicher & Schuell) by semi-dry electroblotting. Recombinant proteins isolated from *E.coli* were detected with Penta-His HRP antibody conjugate (Qiagen 1:20000), following blocking with Penta-His HRP blocking agent. TAP-tagged proteins expressed in plants were detected with Peroxidase anti-peroxidase (PAP; Sigma-Aldrich 1:10000) antibody and 5% nonfat dry milk as blocking agent.

Plant proteins were extracted by grinding frozen tissue samples (0.2 g) in liquid nitrogen and thawing the powder in 0.25 ml protein extraction buffer (PBS buffer; 137 mM NaCl, 27 mM KCl, 100 mM $NaHPO_4$, 2 mM K_2HPO_4, pH 7.4, 1x Complete protease inhibitor Cocktail (Roche) and 0.5% Triton X100). After centrifugation at 15000 x g for 10 min at 4°C, supernatants were transferred to clean tubes, frozen in liquid nitrogen, and stored at -80°C. Protein concentrations

were determined using the Bio-Rad protein assay reagent with bovine serum albumin as the standard. Detection was carried out by incubating the blots in 10 ml luminol solution (250 µM sodium luminol (Sigma-Aldrich), 0.1 M Tris-HCl pH 8.6, 0.01% H_2O_2) mixed with 60 µl enhancer solution (67 µM p-hydroxy coumaric acid (Sigma-Aldrich) in DMSO and exposure to X-ray films (Fuji, Tokyo, Japan).

Electrophoretic mobility shift assays

PDF1.2 promoter derivatives SF, m1, m2 and dm were isolated from the GusXX plasmid with *XbaI* and *Bgl*II. Wild-type and mutated versions of a GCC-like box from the *AOC2* promoter with the sequences 5'-GGAT CCT TTA GGG ACC GGC CAA AAG TAAGATCT-3' and 5'-GGAT CCT TTA GGG ATC GTC CAA AAG TAAGATCT-3' were cloned into pIC-20H digested with *Bam*HI/*Bgl*II (Marsh et al., 1984) and fragments were excised with *Sal*I and *Hind*III. Promoter fragments were labeled by filling in the overhangs with the Klenow fragment of DNA polymerase I and α-^{32}P-dCTP. DNA binding reactions containing 0.1 ng of end-labeled DNA probe, 500 ng of poly(dAdT)-poly (dAdT), binding buffer (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% glycerol), and protein extract in a 10 µl volume, were incubated for 30 min at room temperature before loading on 5% w/v acrylamide/bisacrylamide (37:1)-0.5x Tris-Borate-EDTA gels under tension. Binding buffer conditions were optimized for ORA59 and ORA47 protein by addition of 25 ng of sonicated herring sperm DNA and 1 mM or 0.25 mM DTT to the binding buffer, respectively. After electrophoresis at 125 V for 1 hour, gels were dried on Whatman DE81 paper and exposure to Fuji X-ray films.

Transient expression assays

Protoplasts prepared from *Arabidopsis thaliana* cell suspension ecotype Col-0 were co-transformed with plasmids carrying one of the *PDF1.2*-promoter-*GUS* versions, effector plasmids carrying *ORA59*, *ERF1* or *ORA47* fused to the *CaMV 35S* promoter and the p2rL7 plasmid (De Sutter et al., 2005) carrying the *Renilla reniformis* luciferase (LUC) gene under the control of the *CaMV 35S* promoter. As controls, co-transformations of *PDF1.2*-promoter-*GUS* with the empty pRT101 expression vector and the p2rL7 plasmid were carried out. Protoplasts were transformed using polyET glycol as described previously (Schirawski et al., 2000) with the three constructs in a ratio of 2:2:6 (µg GUS:LUC:effector plasmid). To study a possible synergistic effect of ORA59 and ERF1 a ratio of 2:2:1 (µg GUS:LUC:effector plasmid) was chosen. The protoplasts were harvested 18 hrs after transformation and were frozen in liquid nitrogen. GUS and LUC activity assays were performed as described (van der Fits and Memelink, 1997; Dyer et al., 2000). GUS activities were related to LUC activities in the same samples to correct for differences in transformation and protein extraction efficiencies. Alternatively, differences in protein extraction efficiencies were corrected for protein concentration.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed according to Bowler et al. (2004) with some modifications. Two grams of 2 weeks-old seedlings constitutively overexpressing TAP (line #7) or seedlings from XVE-ORA59-TAP transgenic line #4 treated with 0.1% DMSO or 4 µM estradiol for 16 hours in liquid MA medium were harvested. A small part of the samples was used for mRNA and protein detection. The rest was infiltrated with 1% formaldehyde to crosslink protein and DNA and chromatin sonicated to an average size of 400 bp was prepared. IgG Sepharose 6 fast flow (GE Healthcare) preabsorbed with salmon sperm DNA (0.1 mg/ml) and BSA (1 mg/ml) in ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl) was added to chromatin preparations and the mixtures were rotated at 4°C for 6 hrs to bind TAP or TAP-fusion protein. After 5 times washing the beads (Bowler et al., 2004), DNA recovered from the beads and sonicated chromatin input were reverse cross linked and analyzed by semi-quantitative PCR. The *PDF1.2* promoter was amplified for 34 cycles using the primer set 5'-TAT ACT TGT GTA ACT ATG GCT TGG-3' and 5'-TGT TGA TGG CTG GTT TCT CC-3' located up and down stream of the two GCC-boxes. For amplification of the *AOC2* promoter the primer set 5'-CAT GTA TTT TCA TTC CAA GAG CAG C-3' and 5'-GAT GCT TTG GGA GGA ATT TGG-3' was used at 34 or 36 cycles.

RNA extraction and Northern blot analysis

Total RNA was isolated from tissue ground in liquid nitrogen by extraction with two volumes of phenol buffer (1:1 mixture of phenol containing 0.1% w/v 8-hydroxyquinoline and buffer containing 100 mM LiCl, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 100 mM Tris) and one volume of chloroform. After centrifugation, the aqueous phase was re-extracted with one volume of chloroform. RNA was precipitated overnight with LiCl at a final concentration of 2 M, washed twice with 70% ethanol, and resuspended in water. Northern blot analyses were performed as described (Memelink et al., 1994). Briefly, 10 µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose/1% v/v 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 washing were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed to Fuji X-ray films. The *PDF1.2* probe was PCR amplified from Arabidopsis genomic DNA using the primer set 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'. The TAP probe was excised from pBS1479 (Puig et al., 2001) with *Bam*HI.

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