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

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Abstract Plant defense against microbial pathogens depends on the action of several endogenously produced hormones, including jasmonic acid (JA) and ethylene (ET). In defense against necrotrophic pathogens, the JA and ET signaling pathways synergize to activate a specific set of defense genes including *PLANT DEFENSIN1.2* (*PDF1.2*). The APETALA2/Ethylene Response Factor (AP2/ERF)-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. The present study was aimed at the identification of elements in the *PDF1.2* promoter conferring the synergistic response to JA/ET and interacting with ORA59. We show that the *PDF1.2* promoter was activated synergistically by JA and the ET-releasing agent ethephon due to the activity of two GCC boxes. ORA59 bound in vitro to these GCC boxes and trans-activated the *PDF1.2* promoter in transient assays via these two boxes. Using the chromatin immunoprecipitation technique we were able to show that ORA59 bound the *PDF1.2* promoter in vivo. 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 type of 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.

Keywords Arabidopsis · ERF1 · Ethylene · Jasmonic acid · Transcription factor

Adel Zarei and Ana Paula Körbes have contributed equally to this study.

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Introduction

Plants undergo continuous exposure to various biotic and abiotic stresses in their natural environment. To respond optimally to environmental stresses including attack by microbial pathogens plants have evolved intricate mechanisms to perceive external signals and transduce them into the appropriate gene expression response (Pieterse et al. 2009). Perception of stress signals leads to the production of one or more of the secondary signaling molecules jasmonates (JAs), ethylene (ET), or salicylic acid (SA). JAs are a group of related lipid-derived signaling molecules including the namesake compound jasmonic acid (JA) (Wasternack 2007).

In general, it can be stated that SA-dependent gene expression responses are effective against pathogens with a biotrophic lifestyle, whereas defense genes activated by a combination of JA and ET are effective against pathogens

with a necrotrophic lifestyle (Glazebrook 2005). The JA/ET-dependent defense response is characterized by the production of antimicrobial PLANT DEFENSIN (PDF) proteins (Penninckx et al. 1998). In plants grown under sterile conditions *PDF1.2* mRNA accumulation is weakly induced by JA or ET, but is strongly induced by a combination of both hormones (Penninckx et al. 1998). In soil-grown plants, exogenous application of either JA or ethylene strongly induces PDF1.2 expression, presumably because the plants already produce low amounts of the signaling molecules. Mutant analysis shows that the response of soil-grown plants to either hormone is still dependent on functional JA and ET signaling pathways. Accumulation of *PDF1.2* mRNA is commonly used as a marker for activation of the JA/ET signaling pathway. To understand what this marker actually reports it is crucial to understand the regulation of *PDF1.2* mRNA accumulation.

A large number of transcription factors have been reported to affect *PDF1.2* mRNA accumulation when overexpressed or when inactivated by mutation or by RNA interference (RNAi). Positive effects were observed upon overexpression of the APETALA2/Ethylene Response Factor (AP2/ERF)-domain transcription factors ERF1 (Solano et al. 1998; Lorenzo et al. 2003), OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF-domain protein 59 (ORA59) (Pré et al. 2008), AtERF1 (Pré et al. 2008) or AtERF2 (Brown et al. 2003), or the basic leucine zipper (bZIP) transcription factor TGA5 (Zander et al. 2009). Knocking out the *AtMYC2* gene encoding a basic helix-loop-helix (bHLH) transcription factor (Boter et al. 2004; Lorenzo et al. 2004) or the *AtERF4* gene encoding an AP2/ERF-domain transcription factor with an ERF-associated repression (EAR) motif (McGrath et al. 2005) also stimulated the expression of the *PDF1.2* gene. Negative effects were observed upon overexpression of AtERF4 (McGrath et al. 2005), the WRKY transcription factor WRKY70 (Li et al. 2004), upon triple knockout of the *TGA2*, *5* and *6* transcription factor genes (Zander et al. 2009) or upon downregulation of *ORA59* expression via RNAi (Pré et al. 2008).

Direct interaction of these transcription factors with the *PDF1.2* promoter has not been reported except for TGA2 (Spoel et al. 2003), which was shown to bind in vitro without a documented in vivo relevance. In fact the TGA factors are thought to act indirectly (Zander et al. 2009). Among the positive regulators, ORA59 and ERF1 were shown to activate the *PDF1.2* promoter in transient assays in protoplasts (Pré et al. 2008), suggesting that they bind directly to the promoter. AtERF1 and AtERF2 were not active in this assay (Pré et al. 2008), indicating that they act indirectly. Analysis of transgenic plants in which *ORA59* gene expression was silenced by RNAi, whereas the *ERF1* gene was normally expressed, showed that *PDF1.2* expression in response to JA and to JA/ET was abolished (Pré et al. 2008). This establishes ORA59 as the main positive regulator of *PDF1.2* expression in response to JA/ET (Memelink 2009).

Studies of the *PDF1.2* promoter in transgenic plants showed that it can confer JA-responsive gene expression to a β-glucuronidase (*GUS*) reporter gene (Brown et al. 2003; Manners et al. 1998). The promoter was reported to be unresponsive to ET (Manners et al. 1998). It was not studied whether the *PDF1.2* promoter can confer synergistic gene expression to the combination of JA and ET. It is generally assumed that JA and ET affect *PDF1.2* mRNA accumulation at the transcriptional level, but it cannot be excluded that synergism is caused by stimulation of *PDF1.2* promoter activity by JA (Brown et al. 2003; Manners et al. 1998) and *PDF1.2* mRNA stabilization by ET. In this context it should be noted that the ET signal transduction component ETHYLENE INSENSITIVE 5 (EIN5) is an exoribonuclease which affects stability of certain mRNAs (Olmedo et al. 2006). Brown et al. (2003) identified one GCC-box in the *PDF1.2* promoter which is involved in the JA response. It was not studied whether this GCC box is also involved in the JA/ET response. GCC boxes are binding sites for AP2/ERF-domain transcription factors (Hao et al. 1998). However no binding studies were reported with the GCC box from the *PDF1.2* promoter and AP2/ERF-domain proteins.

The present study was aimed at the identification of elements in the *PDF1.2* promoter conferring the synergistic response to JA/ET and interacting with the key positive regulator ORA59. We show that the *PDF1.2* promoter was activated synergistically by JA and the ET-releasing agent ethephon due to the activity of two GCC boxes. ORA59 bound in vitro to these GCC boxes and trans-activated the *PDF1.2* promoter in transient assays via these two boxes. Using the chromatin immunoprecipitation technique we were able to show that ORA59 bound the *PDF1.2* promoter in vivo. 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 type of GCC box.

Materials and methods

Growth conditions and treatments

Arabidopsis thaliana ecotype Columbia (Col-0) was 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, 2,500 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 h with 4 μM estradiol. As control, seedlings were treated with dimethyl sulfoxide (DMSO; 0.1% v/v final concentration). 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 h with 50 μM JA (Sigma–Aldrich, St. Louis, MO) dissolved in DMSO (0.1% v/v final concentration), 1 mM of the ethylene-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 *PDF1.2* (At5g44420) promoter fragments LF and SF 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 BamHI and SalI and cloned in plasmid GusXX (Pasquali et al. 1994). Mutations m1, m2 and dm were generated according to the QuickChange Site-Directed Mutagenesis protocol (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 BamHI and BglII. Monomers were then tetramerized in a head-to-tail configuration using the BamHI and BglII sites. The tetramers were cloned as BamHI/BglII fragments in the plasmid GusSH-47 (Pasquali et al. 1994) digested with BamHI 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 BamHI and cloned in pRT101 (Töpfer et al. 1987). The *ERF1* (At3g23240) 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 BglII and cloned in BamHI-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 BglII cloned in pIC-20R (Marsh et al. 1984). The *ORA47* insert was excised with BglII and inserted into BamHI-digested pMOG183 (Mogen International, Leiden, The Netherlands), a pUC18 derivative carrying a double-enhanced CaMV 35 S promoter and the *nos* terminator separated by a BamHI site.

Binary constructs and plant transformation

The TAP insert was excised from pBS1479 (Puig et al. 2001) with BamHI and cloned into pC1300intB-35SnosBK (accession number AY560326) digested with BglII. 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 SalI/XhoI and cloned into pC1300intB-35SnosBK-TAP. The *ORA59-TAP* fusion was excised with SalI/SpeI from pC1300intB-35SnosBK-ORA59-TAP and introduced into the binary vector pER8 (Zuo et al. 2000) digested with XhoI/SpeI. 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 XbaI/XhoI and XbaI/HindIII, 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 EcoRI and SalI 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 EcoRI and XhoI and cloned in pASK-IBA45 digested with EcoRI and SalI. *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 EcoRI and SalI 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 harvested by centrifugation of cells lysed by freeze-thawing by denaturation in Ni–NTA binding buffer (5 mM imidazole, 0.5 M NaCl, 40 mM Tris–HCl pH 8.0) with 6 M urea and re-folded by a quick tenfold dilution in binding buffer without urea followed by 16 h dialysis against binding buffer. 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) antibodies 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, 1× Complete protease inhibitor Cocktail (Roche) and 0.5% v/v Triton ×100). After centrifugation at 15,000×*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 Xba and BglII. Wild-type and mutated versions of a GCC-like box from the *AOC2* promoter with the sequences 5′-GGA TCC TTT AGG GAC CGG CCA AAA GTA AGA TCT-3′ and 5′-GGA TCC TTT AGG GAT CGT CCA AAA GTA AGA TCT-3′ were cloned into pIC-20H digested with BamHI/BglII and fragments were excised with SalI and HindIII. 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)-0.5× 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 h, gels were dried on Whatman DE81 paper and exposed to Fuji X-ray films.

Transient expression assays

Protoplasts prepared from Arabidopsis 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 polyethylene 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 h 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 h 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) pre-absorbed 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 h 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 BamHI.

Results

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

PDF1.2 promoter fragments containing 1,187 bp (LF) or 278 bp (SF) upstream of the probable transcription start site (Manners et al. 1998) were fused to the β-glucuronidase (*GUS*) reporter gene (Fig. 1).

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 (Fig. S1). ORA59 and ERF1 activated the *SF-GUS* reporter gene 40- or tenfold respectively, whereas the unrelated AP2/ERF-domain transcription factor ORA47 had no effect. ORA47 trans-activated the promoter of the *ALLENE OXIDE CYCLASE 2* (*AOC2*) gene under these conditions in the protoplast assay (results not shown), 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

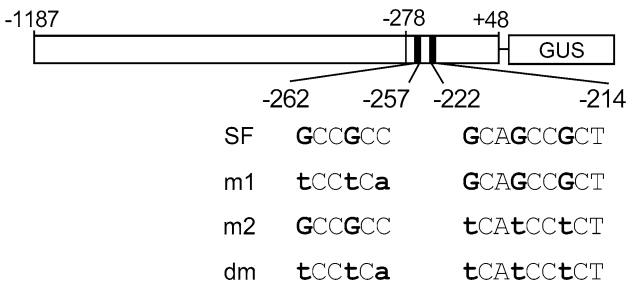

Fig. 1 PDF1.2 promoter constructs. Reporter constructs consisted of the *GUS* gene driven by wild-type or mutated LF (*long fragment* −1,187 to +48) or SF (*short fragment* −278 to +48) *PDF1.2* promoter derivatives. *Bold* and *lowercase* nucleotides indicate point mutations in GCC boxes. *Numbers* indicate positions relative to the start site of transcription

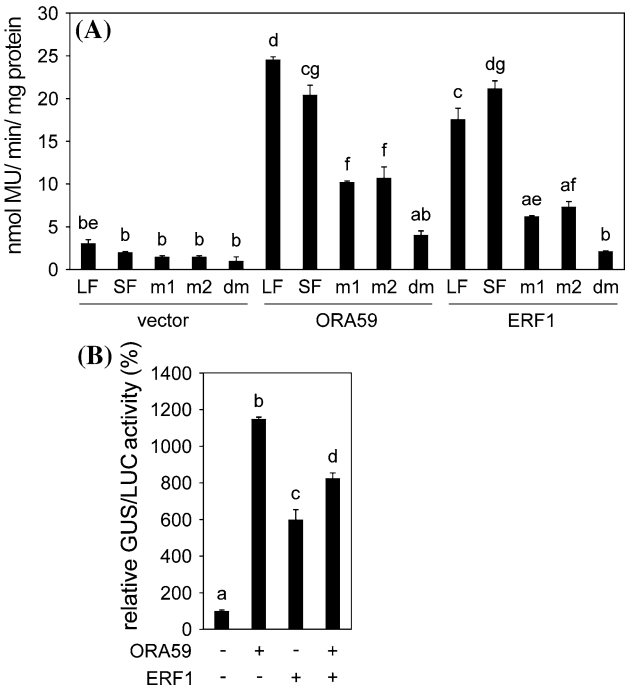

Fig. 2 ORA59 and ERF1 trans-activate the *PDF1.2* promoter independently via two GCC boxes. **a** Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying different versions of the *PDF1.2* promoter shown in Fig. 1 fused to *GUS* and overexpression vectors without or with the *ORA59* or *ERF1* genes driven by the CaMV 35S promoter. 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. Letters above the *bars* indicate different groups as determined by one-way ANOVA ($P < 0.05$)

plasmid, where after the response saturated. Based on these observations, in the following trans-activation experiments 6 μg of effector plasmids were used for maximum responses (Figs. 2a, 6a) and 0.5–1 μg for studies on synergism between ORA59 and ERF1 (Fig. 2b).

ORA59 and ERF1 trans-activate the *PDF1.2* -278 promoter independently via two GCC boxes

Transient expression assays revealed that the short SF derivative conferred GUS expression to a similar level as the long LF derivative both with ORA59 as well as ERF1 (Fig. 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 (GCCGCC) at positions −262 to −257 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 SFm1 promoter derivative (Fig. 1). This mutation reduced GUS activity conferred by ORA59 and ERF1 1.5–twofold, indicating that it is important but that there are other sequences interacting with these transcription factors. Indeed, there is another GCC-like box (GCAGCCGCT) at positions −222 to −214 (Fig. 1). Therefore we generated SFm2 and SFdm promoter derivatives carrying mutations in the second GCC box and in both GCC boxes, respectively. The m2 mutation reduced *PDF1.2* promoter activity about twofold, similar to the m1 mutation. The double mutant version was activated five to sixfold 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 (Fig. 2a).

To find out whether is 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 as a mixture with the SF-*GUS* reporter construct. The results show that the mixture conferred an activity which was intermediate between the activity of each separate effector, indicating that ORA59 and ERF1 act additively instead of synergistically (Fig. 2b).

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* with N- and C-terminal Strep and His tags, respectively. Levels of intact ORA59 protein in the soluble fraction of *E. coli* extracts were almost undetectable. However high amounts of ORA59 and ERF1 proteins were present in the insoluble fraction, presumably in inclusion bodies. Therefore proteins in the insoluble fraction were dissolved in buffer with 6 M urea, proteins were renatured by dilution and dialysis, and full-length ORA59 and ERF1 were isolated by sequential Ni–NTA and Strep-Tactin affinity chromatography. Analysis of the proteins by staining of an SDS-PAA gel with coomassie brilliant blue (not shown) or immunoblot analysis with anti-His antibodies conjugated to horseradish peroxidase (Fig. 3a) showed a single main reactive band in each protein preparation. Although the tagged ORA59 and ERF1 proteins have similar predicted sizes of 31 and 29 kDa, respectively, 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 an artifact due to the *E. coli* expression system.

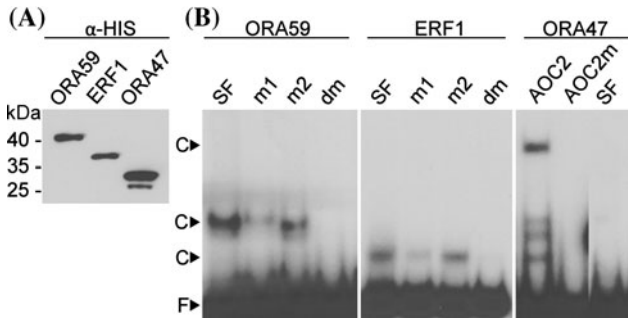

Fig. 3 ORA59 and ERF1 bind to two GCC boxes in the *PDF1.2* promoter in vitro. **a** After SDS–PAGE and Western blotting recombinant proteins were detected with anti-His HRP-conjugated antibodies. Positions of protein size markers are indicated in k Dalton. **b** EMSAs were performed with recombinant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

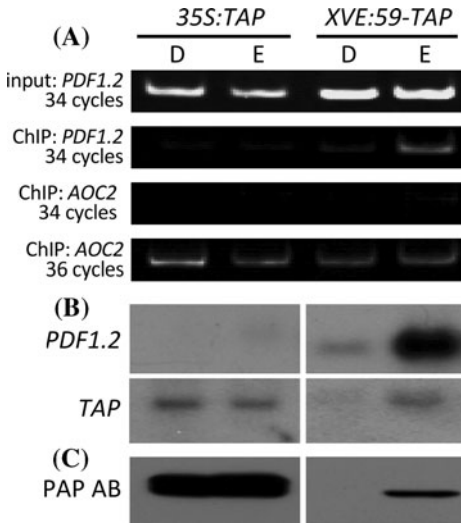

Fig. 4 ORA59 binds to the *PDF1.2* promoter in vivo. Arabidopsis seedlings from XVE-ORA59-TAP line #4 and 35S-TAP line #7 were treated with 4 μM estradiol (*E*) or 0.1% DMSO (*D*). RNA and protein was extracted for Northern and Western blot analysis of transgene expression. Sonicated chromatin prepared 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 preparations were used as templates 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) antibodies

Next, the binding of ORA59 and ERF1 proteins to radiolabeled wild type and mutated SF 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 *AOC2* promoter (Fig. 3b). As shown in Fig. 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 −262 to −257 was mutated, and completely abolished when both GCC boxes were mutated. Although the GCC box at positions −222 to −214 clearly contributed to binding to the SF fragment, mutation of this 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.

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

The trans-activation experiments as well as the in 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 (Fig. S2). Maximum levels of *ORA59-TAP* mRNA and protein were observed after 16–24 h. 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 (Fig. 4b).

Based on the results from the expression analysis, seedlings treated with 4 μM estradiol or 0.1% DMSO for 16 h 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 (Fig. 4b, 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 (Fig. 4a, 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 *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 (Fig. 4a). 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 *PDF1.2* promoter derivative SF, we generated stably transformed plants containing the *GUS* fusion constructs shown in Fig. 1 via *Agrobacterium*-mediated transformation. T2 seedlings from eight independent transgenic lines for each construct were treated with JA, the ET-releasing agent ethephon or both for 24 h. 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 (Fig. 5). 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 −262 to −257 reduced activity to the level observed with the wild-type promoter after control treatment, whereas mutation of the GCC box at positions −222 to −214 left a very low residual response to JA/ethephon. Mutation of both GCC boxes strongly reduced *PDF1.2* promoter activity to levels below the level of the wild-type promoter after control treatment. Therefore in stably transformed plants the two GCC boxes were functionally equivalent and were both necessary for JA- and JA/ethephon-responsive activity of *PDF1.2* promoter derivative SF.

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 −262 to −257 were generated and fused to the TATA box of the CaMV 35S promoter and the *GUS* reporter gene. We tested whether the GCC box tetramer could support transcriptional activation by ORA59 or ERF1 in a transient assay. ORA59 and ERF1 strongly trans-activated the artificial promoter construct with ORA59 as the strongest activator as with the native *PDF1.2* promoter (Fig. 6a). The mutant GCC box tetramer was not activated, 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 (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 with a strong synergistic effect of the JA/ethephon combination (Fig. 6b). The results show that the JA and ET signaling pathways converge to a single type of GCC box sequence.

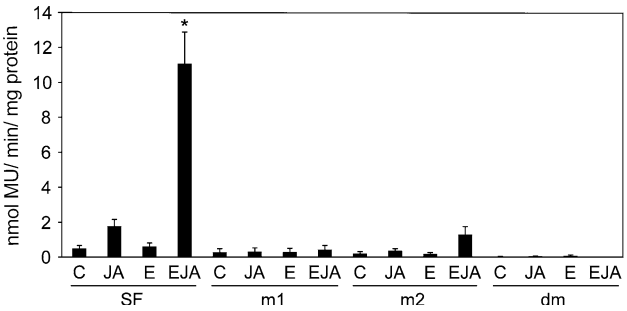

Fig. 5 Both GCC boxes are essential for JA- and ethephon-responsive expression of *PDF1.2* promoter derivative SF in stably transformed Arabidopsis plants. Each *bar* represents average GUS activity values determined in pools of 10 T2 seedlings from 8 independent transformed lines for each construct corrected for protein concentration ± SE. Seedlings were control-treated (*C*) or treated with 50 μM JA, 1 mM of the ET-releasing agent ethephon (*E*) or both (*EJA*) for 24 h. The asterisk marks the only value that was different from any of the others in a one-way ANOVA ($P < 0.05$)

Discussion

In defense against necrotrophic pathogens, the JA and ET signaling pathways synergize to activate a specific set of defense genes including *PDF1.2* (Penninckx et al. 1998). The AP2/ERF-domain transcription factor ORA59 acts as the integrator of the JA and ET signaling pathways and is

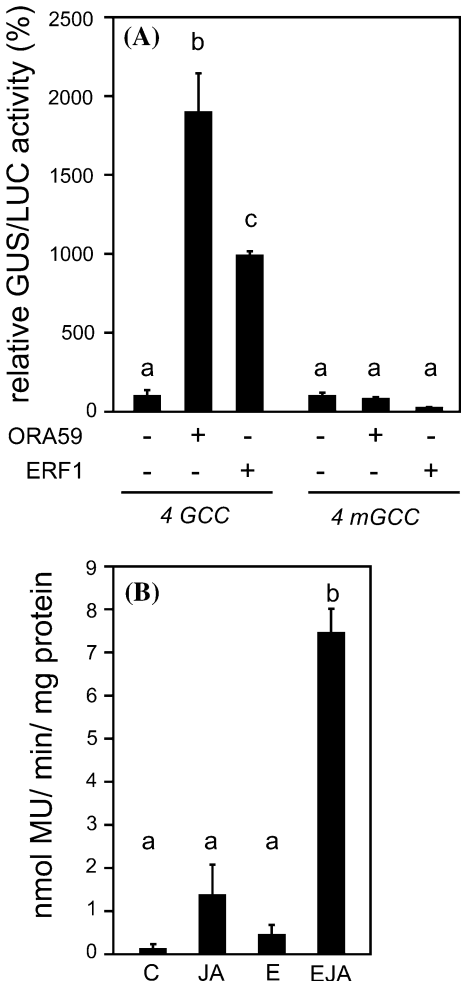

Fig. 6 A GCC box tetramer is sufficient to confer JA- and ethephon-responsive expression in stably transformed Arabidopsis plants. **a** Arabidopsis protoplasts were co-transformed with 2 μg of wild-type or mutated *4GCC-GUS* and 6 μg of overexpression vectors containing *ORA59* or *ERF1* driven by the 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 corresponding vector control set at 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 ± SE. Seedlings were control-treated (*C*) or treated with 50 μM JA, 1 mM of the ET-releasing agent ethephon (*E*) or both (*EJA*) for 24 h. Letters above the *bars* indicate different groups as determined by one-way ANOVA ($P < 0.05$)

the key regulator of JA- and ET-responsive *PDF1.2* expression (Pré et al. 2008). Here we aimed at characterizing the *PDF1.2* promoter sequences responsible for synergistic expression in response to JA/ET and for interaction with ORA59.

We show that two GCC boxes in the *PDF1.2* promoter were essential for in vitro binding to ORA59 and for transactivation by ORA59 in Arabidopsis protoplasts. Using the chromatin immunoprecipitation technique we were able to show that ORA59 bound the *PDF1.2* promoter in vivo. In transgenic plants both GCC boxes were also essential and mutation of either GCC box resulted in a dramatic reduction of the expression of the *PDF1.2* promoter in response to JA/ethephon or to JA alone. This latter finding is consistent with the report of Brown et al. (2003) on the effect of the single mutation of the GCC box at positions −262 to −257 on JA-responsive gene expression. Combined with the data reported by Pré et al. (2008), our findings strengthen the notion that ORA59 is the key regulator of JA/ET-responsive *PDF1.2* expression. Previously we have shown that ORA59 can activate the *PDF1.2* promoter in a transient assay in protoplasts and upon constitutive or inducible overexpression in stably transformed plants (Pré et al. 2008). In addition downregulation of ORA59 expression via RNAi abolished JA/ET-responsive *PDF1.2* expression (Pré et al. 2008).

Both in transient assays as well as in stably transformed plants the GCC boxes were functionally equivalent. However, a difference was that in the transient assay mutation of a single GCC box had a moderate effect, whereas in stably transformed plants mutation of a single GCC box abolished *PDF1.2* promoter activity. One possible explanation is that the *PDF1.2* promoter in the context of a stably established chromatin structure in transgenic plants requires two GCC boxes to be activated in response to JA/ET, whereas a single GCC box is not sufficient for opening up the chromatin structure for transcription. In transiently transformed protoplasts the introduced plasmids probably have not assembled into a proper chromatin structure. Another possibility is that in protoplasts the JA/ET signaling pathways are partially active due to wounding associated with cell wall removal, resulting in opening of the chromatin structure of the *PDF1.2* promoter. In support of either possibility is the observation that the *PDF1.2* promoter has a clear basal expression level in protoplasts in the absence of effectors, whereas in stably transformed plants there is no detectable basal expression.

The function of ERF1 is somewhat mysterious at the current level of understanding. We show here that ERF1 also bound to the GCC boxes in vitro and activated the *PDF1.2* promoter through interaction with the GCC boxes in the protoplast assay. ERF1 can activate the expression of the *PDF1.2* gene when constitutively (Solano et al. 1998; Lorenzo et al. 2003) or inducibly (Pré et al. 2008) overexpressed in stably transformed plants, but when *ORA59* expression is knocked out by RNAi ERF1 is not able to support expression of *PDF1.2* in response to JA, JA/ethephon or infection with *Botrytis cinerea* (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. In addition the EMSA experiments did not indicate differential binding to the two GCC boxes, since the effects of GCC box mutations were similar for ORA59 and ERF1.

The *PDF1.2* promoter conferred a synergistic gene expression response, demonstrating that JA and ET act synergistically at the transcriptional level. The synergistic effect of JA and ethylene 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 ethylene signaling pathways converge to a single GCC box sequence. If the two GCC boxes would have been functionally different, a tetramer of a single GCC box would not have been able to confer a similar synergistic response to JA/ethephon as the native *PDF1.2* promoter. 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 ethylene signaling pathways.

It is likely that the JA and ET signaling pathways converge on the transcription factor ORA59. The question remains then how the JA and ET signaling pathways act on ORA59. One mechanism is by synergistically stimulating the expression of the *ORA59* gene (Pré et al. 2008), which implies that unidentified upstream transcription factors are activated. One of those transcription factors could be EIN3. There is evidence suggesting that EIN3 regulates the expression of the *ERF1* gene (Solano et al. 1998), and the *ERF1* and *ORA59* genes have very similar patterns of expression (Lorenzo et al. 2003; Pré et al. 2008). In addition the JA and ET signaling pathways could also activate ORA59 at the protein level. The JA signaling pathway is thought to activate the transcription factor AtMYC2 by promoting the degradation of the Jasmonate ZIM domain (JAZ) repressors (Chini et al. 2007; Thines et al. 2007). It is possible that members of the JAZ protein family repress ORA59 activity, although there is no evidence reported for JAZ-ORA59 interaction. Alternatively distinct repressor proteins could regulate ORA59 activity in response to JA/ET. The GCC box tetramer construct may have applications as an artificial minimal JA/ET-responsive promoter to dissect mechanisms of the synergistic effect of JA and ET or 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).

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References

Boter M, Ruíz-Rivero O, Abdeen A, Prat S (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev 18:1577–1591

Bowler C, Benvenuto G, Laflamme P, Molino D, Probst AV, Tariq M, Paszkowski J (2004) Chromatin techniques for plant cells. Plant J 39:776–789

Brown RL, Kazan K, McGrath KC, Maclean DJ, Manners JM (2003) A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of Arabidopsis. Plant Physiol 132:1020–1032

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448:666–671

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

De Sutter V, Vanderhaeghen R, Tilleman S, Lammertyn F, Vanhoutte I, Karimi M, Inze D, Goossens A, Hilson P (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. Plant J 44:1065–1076

Dyer BW, Ferrer FA, Klinedinst DK, Rodriguez R (2000) A noncommercial dual luciferase enzyme assay system for reporter gene analysis. Anal Biochem 282:158–161

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43:205–227

Hao D, Ohme-Takagi M, Sarai A (1998) Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. J Biol Chem 273:26857–26861

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218

Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 16:319–331

Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 15:165–178

Lorenzo O, Chico JM, Sánchez-Serrano JJ, Solano R (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell 16:1938–1950

Manners JM, Penninckx IAMA, Vermaere K, Kazan K, Brown RL, Morgan A, Maclean DJ, Curtis MD, Cammue BPA, Broekaert WF (1998) The promoter of the plant defensin gene *PDF1.2* from Arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. Plant Mol Biol 38:1071–1080

Marsh JC, Erfle M, Wykes EJ (1984) The pIC plasmids and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32:481–485

Masson J, Paszkowski J (1992) The culture response of *Arabidopsis thaliana* protoplasts is determined by the growth conditions of donor plants. Plant J 2:829–833

McGrath KC, Dombrecht B, Manners JM, Schenk PM, Edgar CI, Maclean DJ, Scheible WR, Udvardi MK, Kazan K (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. Plant Physiol 139:949–959

Memelink J (2009) Regulation of gene expression by jasmonate hormones. Phytochemistry 70:1560–1570

Memelink J, Swords KMM, Staehelin LA, Hoge JHC (1994) Southern, Northern and Western blot analysis. In: Gelvin SB, Schilperoort RA, Verma DPS (eds) Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, pp F1–F23

Menke FLH, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18:4455–4463

Olmedo G, Guo H, Gregory BD, Nourizadeh SD, Aguilar-Henonin L, Li H, An F, Guzman P, Ecker JR (2006) ETHYLENE-INSENSITIVE5 encodes a 5′–>3′ exoribonuclease required for regulation of the EIN3-targeting F-box proteins EBF1/2. Proc Natl Acad Sci USA 103:13286–13293

Pasquali G, Ouwerkerk PBF, Memelink J (1994) Versatile transformation vectors to assay the promoter activity of DNA elements in plants. Gene 149:373–374

Penninckx IAMA, Thomma BPHJ, Buchala A, Metraux JP, Broekaert WF (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell 10:2103–2113

Pieterse CMJ, Leon-Reyes A, van der Ent S, van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nature Chem Biol 5:308–316

Pré M, Atallah M, Champion A, De Vos M, Pieterse CMJ, Memelink J (2008) The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol 147:1347–1357

Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E, Wilm M, Seraphin B (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods 24:218–229

Schirawski J, Planchais S, Haenni AL (2000) An improved protocol for the preparation of protoplasts from an established *Arabidopsis thaliana* cell suspension culture and infection with RNA of turnip yellow mosaic tymovirus: a simple and reliable method. J Virol Methods 86:85–94

Solano R, Stepanova A, Chao Q, Ecker JR (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev 12:3703–3714

Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, van Pelt JA, Mueller MJ, Buchala AJ, Métraux JP, Brown R, Kazan K, van Loon LC, Dong X, Pieterse CMJ (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell 15: 760–770

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature 448:661–665

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res 15:5890

van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. Plant Mol Biol 33:943–946

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot 100:681–697

Zander M, La Camera S, Lamotte O, Métraux JP, Gatz C (2009) *Arabidopsis thaliana* class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. Plant J 61:200–210

Zuo JR, Niu QW, Chua NH (2000) An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J 24:265–273