Functional analysis of jasmonate-responsive transcription factors in Arabidopsis thaliana

Zarei, A.

Citation

Zarei, A. (2007, December 11). *Functional analysis of jasmonate-responsive transcription factors in Arabidopsis thaliana*. Retrieved from https://hdl.handle.net/1887/12484

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/12484

Note: To cite this publication please use the final published version (if applicable).

Chapter 4

Jasmonate-responsive *Allene Oxide Cyclase* gene expression in Arabidopsis is regulated by the AP2/ERF-domain transcription factor ORA47

Adel Zarei, Martial Pré, Antony Champion[a] and Johan Memelink[1]

Institute of Biology, Leiden University, Clusius Laboratory, Wassenaarseweg 64, 2333 AL, Leiden, The Netherlands

[a] current address: Institut de Recherche pour le Développement, UMR Résistance des Plantes aux Bioagresseurs BP 64501 - 34394 Montpellier Cedex 5, France

[1] To whom correspondence should be addressed. E-mail: j.memelink@biology.leidenuniv.nl; tel: 31-71-5274751; fax: 31-71-5275088.

Abstract

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of bioactive oxylipins that regulate plant responses to wounding, herbivory and pathogen infection. Genes encoding JA biosynthesis enzymes are themselves responsive to JA. Here we studied the mechanism of JA-responsive expression of a small gene family in Arabidopsis encoding the JA biosynthesis enzyme allene oxide cyclase (AOC). Previously it was shown that the AP2/ERF-domain transcription factor ORA47 activates the expression of a whole suite of JA biosynthesis genes including *AOC2* when overexpressed (Pré, 2006). Here we show that the expression of all four members of the *AOC* gene family was induced by overexpression of ORA47. A GCC-like box in the *AOC2* promoter interacted specifically with ORA47 in vitro and in vivo, and this GCC box is important for ORA47-mediated activity of the *AOC2* promoter. In addition we show that ORA47 interacted with the *AOC1* promoter in vivo and that ORA47 can trans-activate the *AOC1* promoter in a transient assay.

Introduction

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues. Jasmonates are linolenic acid (18:3)-derived cyclopentanone-based compounds of wide distribution in the plant kingdom which play crucial roles in responses to mechanical and insect wounding and pathogen infection (Turner et al., 2002). The defense response involving JAs is a two-step process. First, perception of the external stress induces JA biosynthesis. Then, JA production results in signal transduction leading to the expression of a large number of defense-related genes (Turner et al., 2002; Atallah and Memelink, 2004).

Several molecular players in the jasmonate signaling network have been characterized (Lorenzo and Solano, 2005). The transcription factors ORA59 (Pré, 2006; Chapter 2), ERF1 (Lorenzo et al., 2003) and AtMYC2 (Lorenzo et al., 2004) were shown to regulate the expression of subsets of JA-responsive genes. Whereas it starts to be relatively well understood how JAs regulate defense genes, next to nothing is known about the signal transduction pathway leading to JA biosynthesis.

Most of the enzymes involved in the so-called octadecanoid pathway leading to JA biosynthesis have now been identified by a combination of biochemical and genetic

approaches (Creelman and Mulpuri, 2002; Turner et al., 2002). The enzymes are located in two different subcellular compartments (Vick and Zimmerman, 1987; Schaller, 2001; Wasternack and Hause, 2002). The first part of the pathway directs the conversion of α-linolenic acid to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The second part of the pathway takes place in peroxisomes, where OPDA is reduced by OPDA reductase (OPR3) followed by three rounds of beta-oxidation to yield (+)-*7-iso*-JA which equilibrates to the more stable (-)-JA.

The expression of all JA biosynthesis genes is induced by wounding or treatment with exogenous JA or MeJA (Turner et al., 2002; Sasaki et al., 2001; Stenzel et al., 2003). The observation that wounding induces the expression of JA biosynthesis genes suggests that, at least partly, the wound-induced production of JA is a result of the increased transcription of genes encoding the pathway enzymes. In addition, transcriptional activation of the JA biosynthesis genes by JA indicates that JA signaling is amplified by a positive feedback loop initiated by JA (Turner et al., 2002). However, it is not clear how the JA biosynthesis genes are regulated at the transcriptional level. WIPK, a mitogen-activated protein kinase, and CEV1, a cellulose synthetase protein, have been implicated as regulatory components of JA production. JA and its methyl ester accumulate in wounded tobacco plants, but do not accumulate in wounded transgenic plants, in which expression of *WIPK* is genetically suppressed (Seo et al., 1995), indicating that WIPK is a positive regulator of wound-induced JA biosynthesis. In Arabidopsis, the *cev1* mutant shows constitutive production of JA and ethylene and constitutive expression of JA-responsive defense-related genes (Ellis and Turner, 2001; Ellis et al., 2002). The CEV1 protein is thought to act as a negative regulator of stress perception or signal transduction, upstream of JA production. A similar function seems to be affected in the *cet1* mutant which exhibits constitutive elevation of JA and constitutive expression of *THIONIN* (Hilpert et al., 2001), but the corresponding *CET* gene has not yet been cloned. The AP2/ERF-domain transcription factor ORA47 was shown to activate the expression of a whole suite of JA biosynthesis genes when overexpressed (Pré, 2006). Plants constitutively overexpressing the *ORA47* gene showed a strong dwarf phenotype and produced anthocyanins, a phenotype similar to that observed with plants exposed to JA. Oxylipin measurements in plants overexpressing *ORA47* revealed strong alterations in the oxylipin profile. These results suggested that ORA47 is responsible for the regulation of the auto-stimulatory loop in JA biosynthesis.

Here we studied the mechanism of JA-responsive expression of a small gene family in Arabidopsis encoding the JA biosynthesis enzyme allene oxide cyclase (AOC). Three *AOC* genes are arranged in tandem on chromosome 3 (*AOC1-3*) and 1 separate *AOC4* gene is

present on chromosome 1 (Stenzel et al., 2003). The specific aims of the studies described here were to determine whether ORA47 affects the expression of all four *AOC* family members, and whether the *AOC* genes are direct target genes of ORA47. In our studies we concentrated on the *AOC2* gene, which is the most highly expressed family member that shows the strongest response to wounding (Stenzel et al., 2003). The results show that the expression of all four members of the *AOC* gene family was induced by overexpression of ORA47. In addition we show that a GCC-like box in the *AOC2* promoter interacted specifically with ORA47 in vitro and in vivo, and that this GCC box is important for ORA47-mediated activity of the *AOC2* promoter.

Results

ORA47 overexpression increases the expression of all four *AOC* gene family members

The biosynthesis of JA occurs through the octadecanoid pathway (Turner et al., 2002), and involves a series of well-characterized enzymatic steps. Previously we showed that ORA47 overexpression caused changes in the profile of JAs and a JA-related phenotype due to the induction of genes involved in JA biosynthesis including *LOX2*, *AOS*, *AOC2*, *OPR3*, *ACX1*, *KAT5* and *MFP2* (Pré, 2006). To test whether ORA47 might control other members of the *AOC* gene family, we examined the expression of the *AOC1-4* genes as well as other JA biosynthesis genes in transgenic plants that inducibly express ORA47 in response to estradiol treatment due to the presence of an *XVE-ORA47* expression module (Zuo et al., 2000). The coding regions of *AOC*1, 2 and 3 possess 81-90% nt identity, and the *AOC4* coding region shows 71-76% nt identity with the other 3 *AOC* sequences. To measure the expression level of each individual *AOC* gene we used probes consisting of 150 bp from the 3' untranslated regions, which were shown to hybridize specifically to each corresponding gene (Stenzel et al., 2003). RNA gel blot analysis revealed that expression of *AOC1* and *AOC2* and to a lower extent *AOC3* and *AOC4* was induced in XVE-*ORA47* plants treated with estradiol (Figure 1). Similar results were obtained with three independent XVE-*ORA47* transgenic lines. The expression level of the JA biosynthesis genes was tightly correlated with *ORA47* gene induction in each XVE-*ORA47* transgenic line. No expression of the biosynthetic genes was observed in the XVE-*ORA47* lines without inducer, except for line 19 where *AOC2* and *OPR3* mRNA accumulated to slightly elevated levels in the absence of estradiol. In this line, *ORA47* mRNA also showed a slightly elevated level in the non-induced condition, suggesting leaky expression of the transgene in the absence of inducer. In contrast,

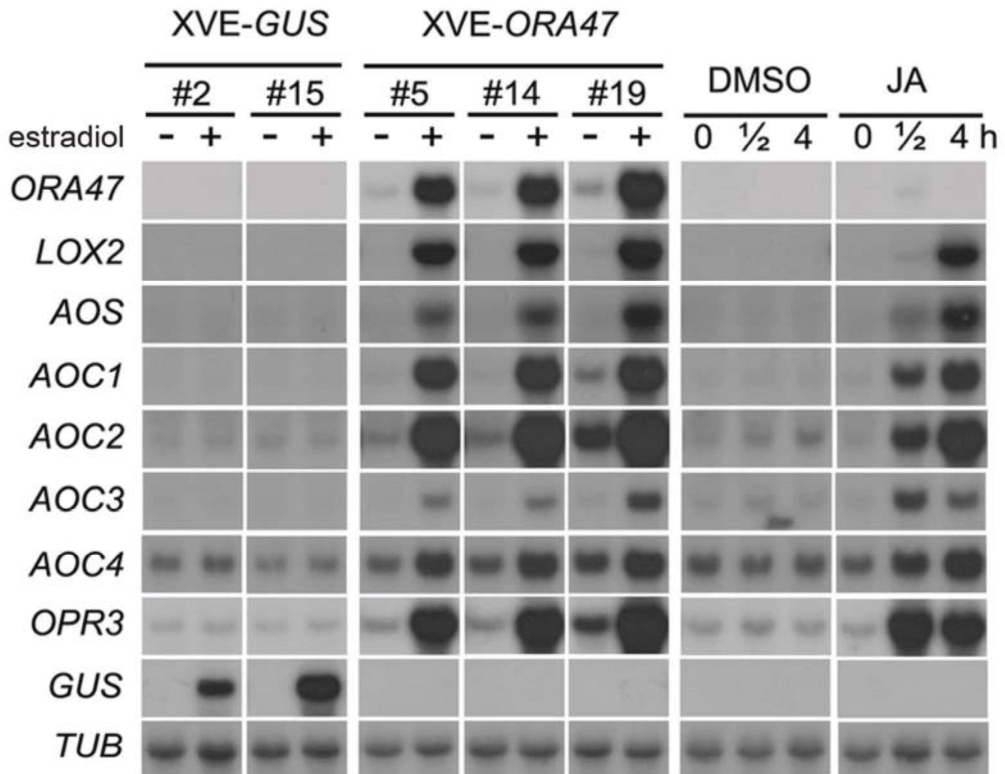


Figure 1. *ORA47* overexpression increases the expression of all four *AOC* gene family members. RNA gel blot analyses with two-weeks-old seedlings from three independent XVE-*ORA47* and two independent XVE-*GUS* lines treated for 24 hrs with 2 μM estradiol (+) or with 0.1% v/v of the solvent DMSO (-). To study JA-responsive gene expression, two-weeks-old wild type plants were treated with 50 μM JA or the solvent for the number of hrs (h) indicated. The *TUB* probe was used to verify RNA loading.

no *ORA47* or JA biosynthesis gene expression was detected in the *XVE-GUS* control plants after estradiol treatment. *GUS* mRNA accumulation on the other hand was strongly induced, demonstrating the effectiveness of estradiol treatment. Hybridization with a *TUBULIN* (*TUB*) probe showed equal RNA loading. This experiment demonstrates that overexpression of ORA47 induces the expression of all four *AOC* family members with the strongest effect on *AOC1* and *AOC2*.

ORA47 trans-activates the *AOC1* and *AOC2* promoters

We wanted to determine whether ORA47 activates the expression of the *AOC* genes via direct binding to their promoters. As a first step, the ability of ORA47 to trans-activate the *AOC* promoters in a transient assay was tested. Arabidopsis protoplasts or *Catharanthus* cells were co-transformed with the β-glucuronidase (*GUS*) reporter gene fused to 600 bp of

one of the *AOC* promoters and an expression vector carrying the *ORA47* open reading frame (ORF) under the control of the CaMV 35S promoter (Figure 2A). In addition a similar experiment was performed with a 300 bp fragment of the *AOC2* promoter. In all experiments the *chloramphenicol acetyltransferase* (*CAT*) gene controlled by the CaMV 35S promoter was included to be able to correct for differences in transformation and protein extraction efficiencies.

ORA47 strongly trans-activated the *AOC1* and *AOC2* promoters in Arabidopsis protoplasts as well as *Catharanthus* cells (Figure 2). In *Catharanthus* cells a very weak activating effect was observed with the *AOC3* and *AOC4* promoters, whereas in Arabidopsis protoplasts no activation was measured. Interestingly, *AOC1* and *AOC2* were strongly induced in the XVE-*ORA47* lines, whereas *AOC3* and *AOC4* were only weakly induced. ORA47 did not have a significant effect on the activity of the 300 bp *AOC2* promoter in Arabidopsis protoplasts (Figure 2B), suggesting that cis-acting elements interacting with ORA47 are located between positions -600 and -300.

The *AOC2* promoter is trans-activated by ORA47 but not by other JA-responsive AP2/ERF-domain transcription factors

The previous results showed that ORA47 activated the *AOC1* and *AOC2* promoters in transactivation assays. It can be envisaged that many AP2-domain transcription factors can activate the *AOC* promoters when overexpressed. Therefore we wanted to determine how specific the activation of the *AOC* promoters by ORA47 was. For these studies we used the *AOC2* promoter, because *AOC2* showed the strongest response to ORA47, and a set of previously identified JA-responsive AP2/ERF genes (Atallah, 2005).

Arabidopsis protoplasts or *Catharanthus* suspension cells were co-transformed with a 600 bp *AOC2* promoter-*GUS* construct, and an overexpression vector carrying *ORA1* (At4g17500), *ORA2* (At5g47220), *ORA4* (At2g44840), *ORA19* (At2g22200), *ORA31* (At5g47230), *ORA37* (At3g15210), *ORA44* (At1g43160), *ORA47 (At1g74930)*, *ORA59* (At1g06160), or *ERF1* (At3g23240).

In *Catharanthus* cells only ORA47 activated the *AOC2* promoter (Figure 3B). In Arabidopsis cells ORA47 also had a strong activating effect on the *AOC2* promoter (Figure 3A). In addition ORA19 activated the *AOC2* promoter. In both cell types ORA37 had a negative effect on *AOC2* promoter activity, consistent with its reported activity as a repressor (McGrath et al., 2005) due to the presence of the ERF-associated amphiphilic repression (EAR) motif (Ohta et al., 2001) in the protein. These results show that the ability to activate the *AOC2* promoter in transient trans-activation assays is a relatively specific property of ORA47.

Figure 2. ORA47 trans-activates the *AOC1* and *AOC2* promoters. **(A)** Schematic representation of *AOC* promoter fragments fused to the *GUS* reporter gene used in transient trans-activation assays. Numbers indicate the position relative to the translation start site. Also depicted are the effector plasmids which contain the CaMV 35S promoter with or without the *ORA47* open reading frame, and the *chloramphenicol acetyltransferase* (*CAT*) reference gene driven by the CaMV 35S promoter. **(B)** Arabidopsis protoplasts or **(C)** *C. roseus* cells were co-transformed with reporter plasmids carrying different *AOC* promoters fused to *GUS* and effector plasmids as indicated together with the reference plasmid. GUS activities were related to CAT activities to correct for differences in transformation and protein extraction efficiencies. Bars represent means ± SE (n=3).

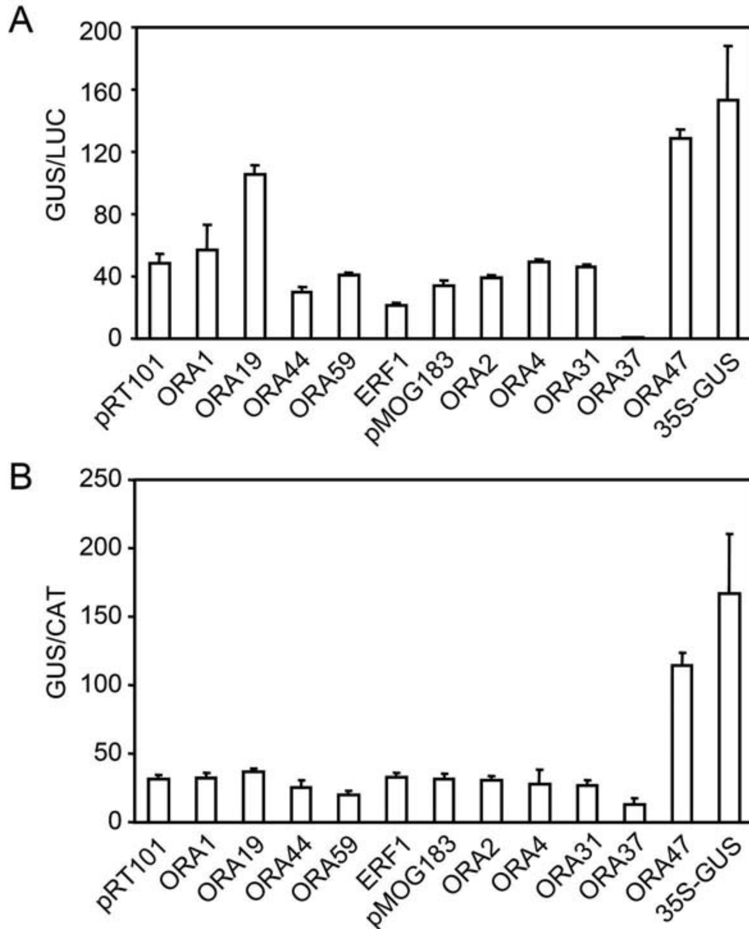


Figure 3. The *AOC2* promoter is trans-activated by ORA47 but not by other JA-responsive AP2/ERF-domain transcription factors. **(A)** Arabidopsis protoplasts or **(B)** *C. roseus* cells were co-transformed with a *GUS* reporter plasmid carrying the 600 bp *AOC2* promoter, and an effector plasmid expressing an AP2/ERF-domain transcription factor as indicated. As reference the *CAT* gene was used for *C. roseus* transformations and the *LUC* gene for Arabidopsis transformations. The negative control contained the empty effector plasmid and as a positive control the *GUS* gene fused to the CaMV 35S promoter was used. GUS activities were related to the corresponding reference enzyme activities to correct for differences in transformation and protein extraction efficiencies. Bars represent means ± SE (n=3).

ORA47 protein interacts with a GCC-like box in the *AOC2* promoter

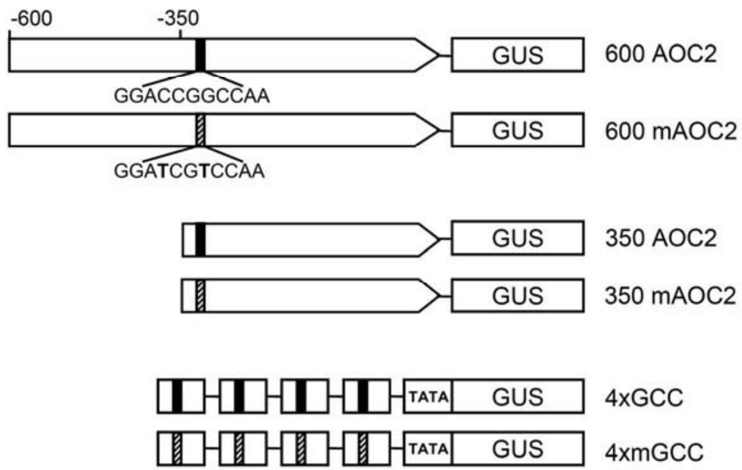
Since the 600 bp, but not the 300 bp *AOC2* promoter was activated by ORA47, it can be concluded that one or more binding sites for ORA47 must be located between positions -600 and -300. Therefore we inspected this region for the presence of GCC boxes, which are the consensus binding sites for AP2-domain proteins. We found two potential GCC-like boxes, one at positions -548 to -543 (GCC-1; GCAGCC) and one at positions -313 to -307 (GCC-2; ACCGGCC). We constructed mutant derivatives of the 600 bp and the 350 bp versions of the

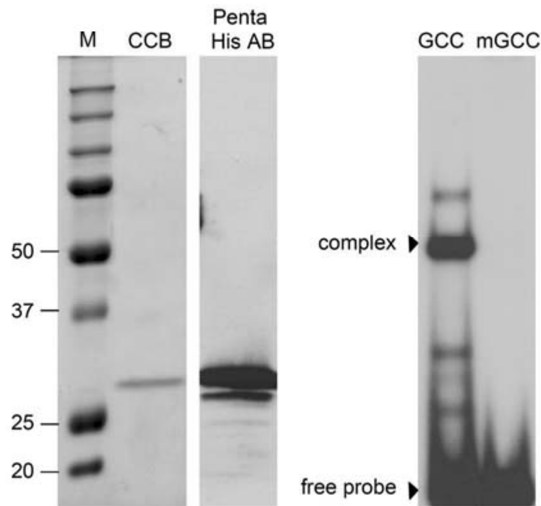
AOC2 promoter (Figure 4A), with two nucleotide substitutions in the GCC-2 box. In addition, we constructed artificial promoters consisting of 4 copies of the wild-type and mutated GCC-2 box fused head-to-tail to the TATA box from the CaMV 35S promoter.

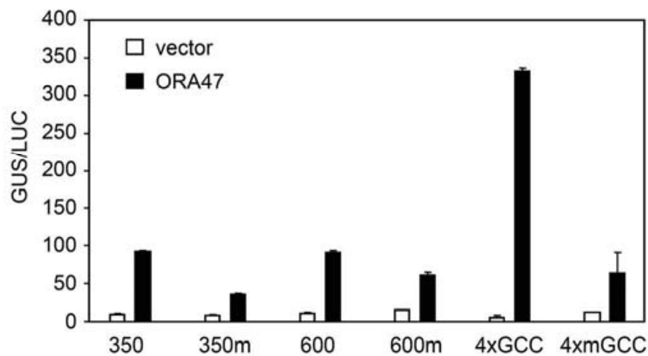
We first tested whether ORA47 could bind to the GCC-2 box in vitro, and whether the two nucleotide mutations affected binding. Recombinant ORA47 protein with an N-terminal Strep-tag and a C-terminal His-tag was produced in *Escherichia coli* (Figure 4B). Coomassie brilliant blue staining of an ORA47 protein gel detected a single band of the expected size. Immunoblot analysis with anti-His-tag antibodies detected the same band as the major immuno-reactive material with a minor additional band with slightly smaller size. Electrophoretic mobility shift assays (EMSA) with recombinant ORA47 protein and the wild-type GCC-2 box showed that ORA47 was able to interact in vitro with this sequence (Figure 4 B). The EMSA also demonstrated that the two nucleotide substitutions in the GCC-2 box abolished in vitro binding of ORA47.

Next we tested whether the GCC-2 box was involved in trans-activation of the *AOC2* promoter by ORA47. Arabidopsis protoplasts were co-transformed with one of the *AOC2* promoter-*GUS* constructs shown in Figure 4A and an overexpression vector carrying the ORA47 ORF. ORA47 induced the activities of the 600AOC2 and the 350AOC2 promoters about 5-fold (Figure 4c). ORA47 induced the artificial 4xGCC-2 promoter about 30-fold. Mutation of the GCC-2 box had a strong negative effect on the activity of the 350 bp *AOC2* promoter, but had only a minor effect on the 600 bp promoter. This indicates that the GCC-1 box located between positions -600 and -350 is also a functional binding site for ORA47. Mutation of the GCC-2 box in the context of the artificial tetrameric promoter caused a 5-fold reduction in activity.

We also analyzed the effect of the two nucleotide substitution in the GCC-2 box on JA-responsive activity of the *AOC2* promoter in stably transformed plants. Transgenic plants contained a T-DNA carrying the wild-type or mutated 600 bp *AOC2* promoter fused to *GUS* and the *CAT* gene driven by the CaMV 35S promoter. For each construct seedling samples of twelve independent transformed lines were analyzed for GUS and CAT activities. Figure 4D shows the average fold induction of GUS activity levels in the twelve lines by JA. The results show that the effect of the two-nt substitution on JA-responsive expression in stably transformed plants was very similar to the effect on ORA47 trans-activation in transient assays. The relatively weak negative effect of the GCC-2 box mutation on the activity of the 600 bp *AOC2* promoter also indicates that additional elements such as the GCC-1 box are operating in JA-responsive expression. Taken together these results establish the GCC-2 box as a binding site for ORA47, which is important for the expression level of the *AOC2* promoter.


A
-600
-350
GUS
600 AOC2
GGACCGGCCAA
GUS
600 mAOC2
GGATCGTCCAA
GUS
350 AOC2
GUS
350 mAOC2
TATA
GUS
4xGCC
TATA
GUS
4xmGCC


B
M
CCB
Penta His AB
GCC
mGCC
50
37
25
20
complex
free probe


C
GUS/LUC
vector
ORA47
400
350
300
250
200
150
100
50
0
350
350m
600
600m
4xGCC
4xmGCC

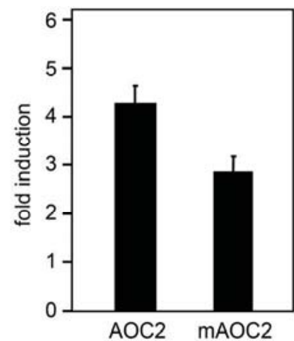

D
fold induction
6
5
4
3
2
1
0
AOC2
mAOC2

Figure 4. ORA47 protein interacts with a GCC-like box in the *AOC2* promoter. **(A)** Schematic diagram of reporter plasmids used in transient assays. *AOC2* promoter derivatives of 350 or 600 bp in length were fused to *GUS*. The position of the GCC-like box as well as its wild-type and mutated sequences are indicated. Head-to-tail tetramers of the wild-type and mutant GCC-like box were fused to a minimal TATA box from the CaMV 35S promoter and the *GUS* gene. **(B)** Gel shift assay of ORA47 protein incubated with the radio-labeled wild-type or mutated GCC-like box. The left panel shows the recombinant ORA47 protein used in the assay stained with coomassie brilliant blue R-250 (CBB) or upon detection with antibodies directed against the N-terminal His tag. **(C)** Arabidopsis protoplasts were co-transformed with one of the reporter plasmids shown in (A), one of the effector plasmids shown in Figure 2A and the *LUC* reference plasmid. **(D)** Mutation of the GCC-2 box reduces JA-responsive expression of the 600 bp *AOC2* promoter in stably transformed plants. Average induction values were generated from pools of 10 seedlings from twelve independent transgenic lines for each construct treated with 50 μM JA for 24 hrs. Bars represent means ± SE (n=12).

ORA47 binds to the *AOC1* and *AOC2* promoters in vivo

The trans-activation experiments as well as the in vitro binding studies suggested that ORA47 binds directly to the *AOC2* promoter in vivo to regulate gene expression. We wanted to confirm this directly using chromatin immunoprecipitation analysis (ChIP). Therefore we constructed plants expressing ORA47 with the tandem affinity purification (TAP) 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 *ORA47-TAP* mRNA level, line #4 was selected for further analysis.

We first verified that the ORA47-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 5). Whereas *ORA47-TAP* mRNA amounts increased over the 48 hrs sampling period (Figure 5B), maximum levels of ORA47-TAP protein were observed after 16 to 24 hours (Figure 5A). Estradiol treatment also induced the ORA47 target genes *AOC2*, *LOX2* and *OPR3*, showing that the ORA47-TAP fusion protein is functionally active. DMSO-treated transgenic plants did not express ORA47-TAP or the ORA47 target genes including *AOC2*. Estradiol treatment had no effect on *AOC2* expression in control plants (Figure 6C).

Based on the results from the expression analysis, seedlings treated with 4 μM estradiol or 0.1% DMSO for 16 hrs were used for ChIP analyses. Transgenic seedlings expressing the unrelated AP2-domain transcription factor ORA59 fused to the TAP tag under control of the estradiol-inducible XVE system (Chapter 2) were similarly treated as controls. Protein and mRNA analysis of the harvested samples prior to formaldehyde cross linking showed that the TAP fusion products were induced by estradiol treatment at the mRNA (Figure 6C) and protein level (Figure 6D). The ORA47-TAP and ORA59-TAP proteins were also functional as judged by the induction of the target genes *AOC2* and *PDF1.2*, respectively (Figure 6C). PCR analysis with primers specific for a part of the *AOC2* promoter containing

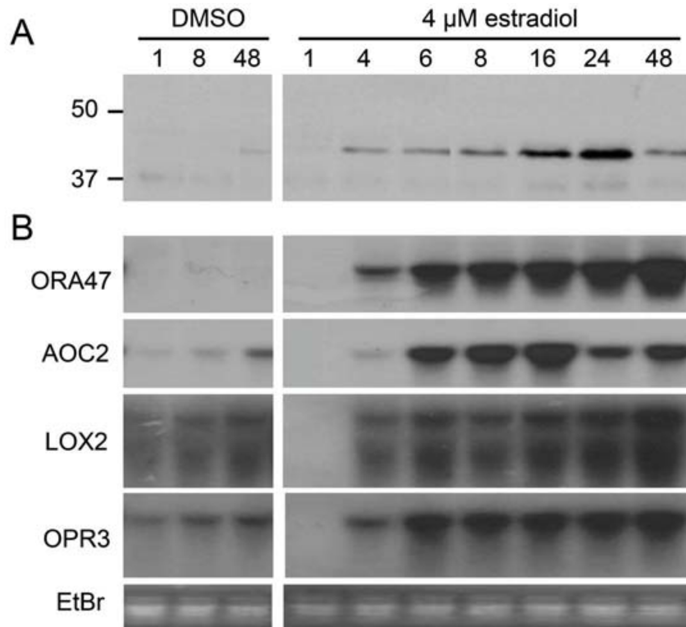


Figure 5. The ORA47-TAP protein is inducibly expressed and functional. Fourteen days old T2 seedlings from XVE-*ORA47-TAP* line #4 cultured in liquid medium were treated for varying times in hrs as indicated with 4 µM estradiol or the solvent DMSO at a final concentration of 0.1% and protein and RNA was extracted. **(A)** Western blot analysis. The protein samples separated by SDS-PAGE followed by Western blotting were immuno-probed with the peroxidase anti-peroxidase antibody (PAP) which recognizes the TAP tag. Sizes of two marker bands are indicated in k Dalton. (B) Northern blot analysis with probes as indicated. Bottom panel shows an ethidium bromide (EtBr) stained RNA gel prior to blotting.

the GCC-2 box using the chromatin prepared following formaldehyde cross linking of the harvested samples showed that equivalent amounts of DNA were present (input; Figure 6B). 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 the *AOC2* primer set revealed that this promoter region was overrepresented in the preparation from XVE-*ORA47-TAP* seedlings treated with estradiol, but not in the chromatin prepared from estradiol-treated XVE-*ORA59-TAP* seedlings (Figure 6B). In contrast primers specific for the promoter of the unrelated *plant defensin 1.2* (*PDF1.2*) gene showed preferential amplification of a fragment in XVE-*ORA59-TAP* samples but not in XVE-*ORA47-TAP* preparations. PCR amplification with primer set 1 specific for a region of the *AOC1* promoter containing a GCC-like box (Figure 6A) resulted in a PCR product that was overrepresented in affinity-purified chromatin from estradiol-treated XVE-*ORA47-TAP* seedlings (Figure 6B). PCR with *AOC1* primer set 2 covering a promoter region lacking GCC-like sequences did not show preferential amplification of a fragment in any of the affinity-purified chromatin samples. The fact that the

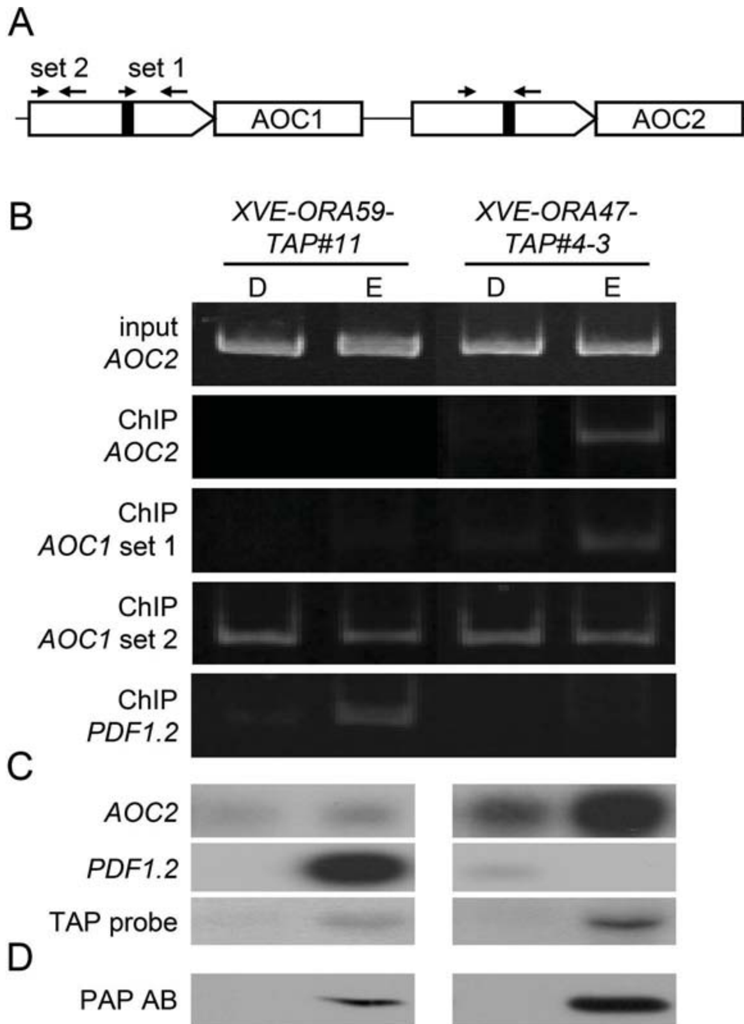


Figure 6. ORA47 binds to the *AOC1* and *AOC2* promoters in vivo. Fourteen days-old seedlings transformed with XVE-*ORA47-TAP* or XVE-*ORA59-TAP* were treated with 4 µM estradiol (E) or DMSO (D) for 16 hrs. RNA and protein was extracted for Northern and Western blot analysis of transgene expression. 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)** Schematic representation of the location of primer sequences relative to the *AOC1* and *AOC2* genes located in tandem on chromosome 3. Black boxes represent GCC-like sequences. **(B)** ChIP analysis. Input chromatin or recovered chromatin was used as template in PCR for 35 cycles with promoter-specific primers as indicated. **(C)** Northern blot analysis with the *PDF1.2*, *AOC2* and TAP probes. **(D)** Western blot analysis with peroxidase anti-peroxidase (PAP) antibody.

region covered by primer set 2 was not overrepresented in affinity-purified chromatin shows that the *AOC1* promoter region covered by primer set 1 really interacted with ORA47-TAP and was not immuno-precipitated due to binding of ORA47-TAP to the more distant *AOC2*

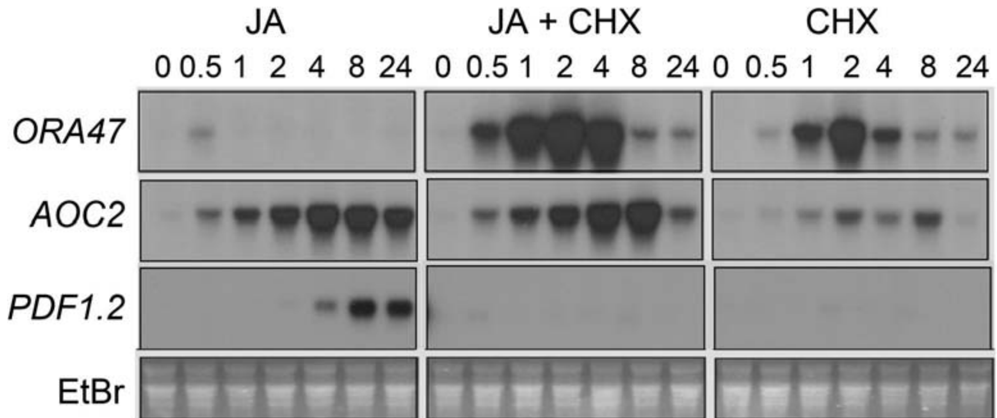


Figure 7. *AOC2* and *ORA47* are immediate-early JA-responsive genes. Fourteen days-old wild-type Arabidopsis seedlings were treated with 50 µM JA, 100 µM cycloheximide (CHX) or both for number of hrs as indicated. Blots were hybridized with probes corresponding to the complete open reading frames of *ORA47*, *AOC2* and *Plant defensin 1.2* (*PDF1.2*). The ethidium bromide (EtBr) stained gel is shown as a control for RNA loading.

promoter. Taken together, these results show that the ORA47-TAP fusion protein directly interacts with the *AOC1* and *AOC2* promoters in vivo.

***AOC2* and *ORA47* are immediate-early JA-responsive genes.**

The previous results establish *AOC2* as a direct target gene of ORA47. In these experiments ORA47 was overexpressed either transiently from the CaMV 35S promoter, or stably from the XVE expression module. We asked the question whether in the normal JA-responsive expression of the *AOC2* gene there is a need for de novo synthesis of ORA47 protein in response to JA.

Wild-type Arabidopsis seedlings were treated with the inhibitor of protein synthesis cycloheximide (CHX), JA or both. Figure 7 shows that *ORA47* expression was weakly and transiently induced by JA at the 0.5 hr time point, strongly induced by CHX and superinduced by the combination in a less transient manner. The *AOC2* gene was weakly induced by CHX alone and CHX did not inhibit JA-responsive expression. No synergism between CHX and JA was observed for *AOC2* expression. The *plant defensin 1.2* (*PDF1.2)* gene showed a totally different expression pattern without a response to CHX alone and strong inhibition of JA-responsive expression by CHX. This shows that the CHX treatment was effective. These results show that JA-responsive *AOC2* expression did not require de novo protein synthesis of ORA47 or any other protein.

Discussion

Previously it was shown that the AP2-domain transcription factor ORA47 activates the expression of a whole suite of JA biosynthesis genes including *AOC2* when overexpressed (Pré, 2006).

Here we show that the expression of all four members of the *AOC* gene family was induced by overexpression of ORA47. A GCC-like box in the *AOC2* promoter interacted specifically with ORA47 in vitro and in vivo, and this GCC box is important for ORA47-mediated activity of the *AOC2* promoter. In addition we show that ORA47 interacted with the *AOC1* promoter in vivo.

In transient trans-activation assays using Arabidopsis protoplasts ORA47 trans-activated the *AOC1* and *AOC2* promoters but not the *AOC3* and *AOC4* promoters. In *Catharanthus* cells weak activation of the *AOC3* and *AOC4* promoters was observed. The weak activation of these promoters is consistent with the relatively weak effect of ORA47 overexpression in stably transformed plants on *AOC3* and *AOC4* mRNA accumulation.

In the *AOC2* promoter a GCC-like box at positions -313 to -307 relative to the translational start point was found to be critical for regulation by ORA47 in transient trans-activation assays. Mutation of this GCC-like box also affected the response of the 600 bp *AOC2* promoter to jasmonic acid in stably transformed plants. The sequence of this element is ACCGGCC, which differs from the GCC box (AGCCGCC) reported as a binding site for AP2/ERF-domain proteins (Ohme-Takagi and Shinshi, 1995). There are other AP2/ERF-domain proteins for which different binding sites have been reported. The DRE/CRT element (ACCGAC) is a binding site for the DREB class of proteins (Yamaguchi-Shinozaki and Shinozaki, 1994; Sakuma et al., 2002). The JA-responsive ORCA2 and ORCA3 proteins from *Catharanthus* bind to the sequence ACCGCC in the promoter of the target gene *Strictosidine synthase* (Menke et al., 1999; van der Fits and Memelink, 2001). While these different sequences have some similarity to the GCC box, two tobacco AP2/ERF-domain proteins called WRAF1 and WRAF2 bind to the wound-responsive cis-element GAAAAGAAAATTTC lacking any similarity with the other binding sites (Sasaki et al., 2007). If we compare the GCC-like box in the *AOC2* promoter, the DRE/CRT and the ORCA binding site, these 3 sequences resemble each other and share the sequence ACCGNC. In this common core sequence, the G and the last C are essential for the highly specific interactions with both DRE-binding DREB proteins and ERF proteins (Sakuma et al., 2002). Other nucleotides are responsible for specific recognition by either DREB proteins or ERF proteins (Sakuma et al., 2002). As our results show, the GCC-2 box in the *AOC2* promoter was specifically recognized by ORA47 among the set of eleven JA-responsive AP2/ERF-domain transcription

factors tested. It remains to be determined which nucleotides in the common core sequence of the GCC-2 box are responsible for specific recognition by ORA47.

The ChIP analysis shows most conclusively that the *AOC1* and *AOC2* genes are direct target genes of ORA47. JA-responsive expression of the *AOC2* gene is not inhibited by the protein synthesis inhibitor cycloheximide. Together these findings indicate that pre-existing signal transduction components including ORA47 are activated post-translationally in response to JA. Activation of ORA47 could occur via posttranslational modifications such as phosphorylation and/or changes in protein-protein interactions between ORA47 and unknown regulators. ORA47 is therefore an attractive target for studies aiming to identify modifications in transcription factor activity as a result of JA signal transduction.

Material and Methods

Growth conditions and treatments

Arabidopsis thaliana ecotype Columbia (Col-0) is the genetic background for all wild type and transgenic plants. Seeds were surface sterilized by incubation for 1 min in 70% ethanol, 15 minutes in 4-fold diluted household bleach and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for three hrs (http://plantpath.wisc.edu/~afb/vapster.html). Surface-sterilized seeds were grown on plates containing MA medium (Masson and Paszkowski, 1992) supplemented with 0.6% agar. Transgenic plants from T1 and T2 generations were selected on MA medium containing 20 mg/L hygromycin. Following stratification for 3 days at 4ºC, seeds were first germinated at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) on solid MA medium with hygromycin for 10 days, where after 15-20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml liquid MA without antibiotic and incubated on a shaker at 120 rpm for 4 additional days before treatments. Seedlings were treated for 24 hrs with 50 µM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO; 1% v/v final concentration), As control, seedlings were treated with 0.1% DMSO. In the cycloheximide (CHX) experiment, seedlings were first treated for 10 min with 100 µM CHX dissolved in DMSO (0.1% final concentration) and then with JA dissolved in DMSO at a final concentration of 50 µM for number of hrs as indicated. Transgenic seedlings carrying an XVE expression module containing the *ORA47* gene fused to the TAP tag were treated with 4 µM estradiol. As control, seedlings were treated with 0.1% DMSO. After 16 hours incubation on the shaker, seedlings were harvested and used for further experiments.

AOC2 promoter and constitutive overexpression constructs

Arabidopsis genomic DNA was used as template for amplification of 600 bp *AOC*1, 2, 3, 4 promoters with primer sets 5’-GC TCT AGA CGT TAT CCT AAT AGT TTT AGA-3’ and 5’-TGG TCG ACT GCT CAA TAA AAG AAA GAC TAC-3’; 5’-GCT CTA GAA TAA AAA TCA GTG TTC TAT CC-3’ and 5’-TGG TCG ACT GAT AAA AAT AAA ATA AAA AG-3’; 5’-GCT CTA GAA CAA AAA TGA TGA CCG GAG ATT G-3’

and 5'-TGG TCG ACT GAT CAA GAA GAG TTT AAA TTA AA-3'; 5'-GCT CTA GAA TTG GAA CCA CAG AAT GTG TAA C-3' and 5'-TGG TCG ACA GAC TTG CCT GAG TTA GTT TAA TTG-3' respectively. All PCR amplified products were digested with XbaI and SalI and cloned in plasmid GusSH (Pasquali et al., 1994). Mutated promoter derivative 600mAOC2 was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and the primer 5'-GTA GTT TAG GGA ATC GTC CAA AAG TAT TTC AAC TTA AAT CCA AGA C-3' and its reverse complementary primer. Promoter derivatives 350AOC2 and the mutant version 350mAOC2 were generated with the primer set 5'-GCT CTA GAG ATT CAT TAC ATT TAG AAG-3' and 5'-CGG ACC ATG GTC GAC TGA T-3' and plasmids carrying 600AOC2 and 600mAOC2 respectively as templates in PCR reactions. The amplified fragments digested with XbaI and SalI were cloned in GusSH. The *ORA1* (At4g17500) open reading frame (ORF) PCR-amplified on Arabidopsis genomic DNA using the primer set 5'-CGG GAT CCA TAT GAC GGC GGA TTC TCA AAT C-3' and 5'-CGG GAT CCT TAT AAA ACC AAT AAA CGA TC-3' was digested with BamHI and cloned in pRT101 (Töpfer et al., 1987). The *ORA2* (At5g47220) ORF amplified with the primer set 5'-CGG GAT CCA TAT GTA CGG ACA GTG CAA TAT AG -3' and 5'-CGG GAT CCT TAT GAA ACC AAT AAC TCA TC-3' was digested with BamHI and cloned in pRT101. The *ORA4* (At2g44840) ORF amplified with the primer set 5'-GAA GAT CTC ATA TGA GCT CAT CTG ATT CC -3' and 5'-GAA GAT CTT TAT ATC CGA TTA TCA GAA TAA G -3' was digested with BglII and cloned in pMOG181 (Mogen International, Leiden, The Netherlands) digested with BamHI. The *ORA19* (At2g22210) ORF amplified with the primer set 5'-CGG AAT CCA TAT GGA AAC TGC TTC TCT TTC TTT C-3' and 5'-GAA GAT CTT TAA GAA TTG GCC AGT TTA C-3' was digested with BamHI and BglII and cloned in pRT101 digested with BamHI. The *ORA31* (At5g47230) ORF amplified with the primer set 5'-CGG GAT CCA TAT GGC GAC TCC TAA CGA AGT ATC-3' and 5'-CGG GAT CCT CAA ACA ACG GTC AAC TGG GAA TAA C-3' was digested with BamHI and cloned in pMOG183. The *ORA37* (At3g15210) ORF amplified with the primer set 5'-CGG GAT CCA TAT GGC CAA GAT GGG CTT GAA ACC C-3' and 5'-CGG GAT CCT CAG GCC TGT TCC GAT GGA GG-3' was digested with BamHI and cloned in pMOG183. The *ORA44* (At1g43160) ORF amplified with the primer set 5'-CGG GAT CCA TAT GGT GTC TAT GCT GAC TAA TGT TGT C-3' and 5'-CGG GAT CCA CAA GAC TTT GAT CAC AAA TT-3' was digested with BamHI and cloned in pRT101. The *ORA47* (At1g74930) ORF amplified with 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' was digested with BglII and cloned in BamHI-digested pMOG183. The *ORA59* (At1g06160) ORF amplified with 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' was digested with BamHI and cloned into pRT101. The *ERF1* ORF amplified with 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' was digested with BglII and cloned into BamHI-digested pRT101.

Binary constructs and plant transformation

The *ORA47* ORF with the tandem affinity purification (TAP) tag at its C-terminal end was placed under the control of the estradiol-inducible XVE system (Zuo et al., 2000a). 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 *ORA47* ORF lacking the stop codon was amplified by

PCR with the primer set 5'-ACG CGT CGA CAA AAT GGT GAA GCA AGC GAT GAA GGA A-3' and 5'-CCG CTC GAG CCT TGA AAA TCC CAA AGA ATC AAA GAT TC-3' and cloned in pGEM-T Easy (Promega, Madison, WI). The *ORA47*Δstop fragment was excised from pGEM-T Easy with SalI/XhoI and cloned into pC1300intB-35SnosBK-TAP. The *ORA47-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. Construction of the pER8 vector carrying the *ORA59-TAP* fusion is described in Chapter 2. For the construction of transgenic lines containing the promoter derivatives 600AOC2 and 600mAOC2 fused to *GUS,* the promoter-*GUS* fusions were excised from GusSH with XbaI/XhoI and cloned into binary vector pMOG22λCAT (Menke et al., 1999). The binary vectors were introduced into *Agrobacterium tumefaciens* strain LBA1119. 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

ORA47 protein was produced with N and C terminal Strep and His tags. The *ORA47* ORF 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' was digested with EcoRI/SalI and cloned in pASK-IBA45 (IBA Biotagnology, Göttingen, Germany). The proteins were expressed in *Escherichia coli* strain BL21(DE3)pLysS (Novagen) and purified by sequential Ni-NTA agarose (Qiagen) and Strep-Tactin sepharose (IBA) chromatography according to the manufacturer's instructions. 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 1:10000) antibody and 5% nonfat dry milk as blocking agent. To extract proteins frozen plant tissues (0.2 g) were ground in liquid nitrogen and the tissue powder was thawed 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% v/v Triton X100). After centrifugation at 15000xg for 10 min at 4ºC, supernatants were transferred into clean tubes, frozen in liquid nitrogen, and stored at -80ºC. Protein concentrations were determined using the Bio-Rad protein assay reagent with bovine serum albumin (BSA) as the standard. Detection was carried out by incubating the blots in 10 ml luminol solution (250 µM sodium luminal (Sigma-Aldrich), 0.1 M Tris-HCl pH 8.6, 0.01% v/v 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

Wildtype and mutated versions of the GCC-2 box from the *AOC2* promoter region 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 BamHI/BglII (Marsh et al., 1984). Fragments were excised with SalI and HindIII and 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)-0.5xTris-Borate-EDTA gels under tension. After electrophoresis at 125 V for 1 hour, gels were dried on Whatman DE81 paper and exposed to Fuji X-ray films.

Transient expression assays

Protoplasts were prepared from *Arabidopsis thaliana* cell suspension ecotype Col-0 with some modifications (Axelos et al., 1992). A one week-old cell suspension culture was diluted 10-fold in 250 ml Erlenmeyer flasks containing 50 ml medium (3.2 g/L Gamborg B5 basal medium with minimal organics (Sigma-Aldrich), 3% sucrose, 1 µM 1-naphtalene acetic acid (NAA), pH 5.8) and incubated overnight at 25 ºC at 150 rpm. A total of 150 ml of cell culture were then left to sediment. After removal of most of the medium, cells were centrifuged in a 50 ml tube at 600 rpm for 5 min at room temperature and the supernatant was removed. Cell walls were digested by addition of 20 ml of enzyme mix (0.4% macerozyme R-10 (Yakult), 1.5% cellulase Onozuka R-10 (Yakult), 12% sorbitol pH 5.8) for 3 hrs at 37 ºC. The protoplasts were filtrated through a 70 µm nylon sieve (BD Biosciences) and transferred to a 50 ml tube in a total volume of 30 ml of Proto medium (Gamborg B5 basal medium, 0.1 M glucose, 0.25 M mannitol, 1 µM 1-NAA, pH 5.8). The protoplasts were centrifuged at 80 g for 5 min, washed with 50 ml of Proto medium and re-centrifuged. After addition of 15 ml of Proto medium, the number of protoplasts was determined using a Mallasez cell. Finally, the volume of the protoplast suspension was adjusted to $4x10^6$ cells/ml. Protoplasts were co-transformed with plasmids carrying one of the *AOC2*-promoter-*GUS* versions, effector plasmids carrying *ORA1*, *ORA2*, *ORA4*, *ORA19*, *ORA31*, *ORA37*, *ORA44*, *ORA47*, *ORA59*, or *ERF1* fused to the CaMV 35S promoter and the reference plasmids p2rL7 (De Sutter et al., 2005) carrying the *Renilla reniformis luciferase* (*LUC*) or *chloramphenicol acetyl transferase* (*CAT*) gene under the control of the CaMV 35S promoter . As a control, co-transformation of *AOC2*-promoter-*GUS* with the corresponding empty expression vector (pRT101 or pMOG183) and the p2rL7 plasmid was carried out. Protoplasts were transformed using polyethylene glycol as described previously (Schirawski et al., 2000) with the three constructs in a ratio of 2:2:6 (µg GUS:LUC:effector plasmid). The protoplasts were harvested 18 hours after transformation and frozen in liquid nitrogen. For trans-activation assays, *Catharanthus roseus* cell suspension line MP183L was grown as described (Pasquali et al., 1992), and co-transformed with the same constructs as Arabidopsis protoplasts by particle bombardment as described before (van der Fits and Memelink, 1997). GUS and LUC activity assays were performed as described (van der Fits and Memelink, 1997; Dyer et al., 2000) with minor modifications. GUS activities from triplicate experiments were related to corresponding LUC activities to correct for differences in transformation and protein extraction efficiencies.

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 100 mM LiCl, 10 mM EDTA, 1% w/v sodium dodecyl sulfate, 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. *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. For probe preparation, DNA fragments corresponding to the complete ORF of the following genes were PCR amplified using the following primer sets: 5'-CGG GAT CCG TGC GGA ACA TAG GCC ACG G-3' and 5'-CGG GAT CCG GAA CAC CCA TTC CGG TAA C-3' for *Lipoxygenase2* (*LOX2, At3g45140*); 5'-ATG GCT TCT ATT TCA ACC CC-3' and 5'-CTA AAA GCT AGC TTT CCT TAA CG-3' for *Allene oxide synthase* (*AOS*, *At5g42650*); 5'-GTC GAC AAT AAA ATT AGT CTT ATC TTT CTC-3' and 5'-GTC GAC ATT CGA TTA CAA GTA TTT TTC-3' for *Allene oxide cyclase1* (*AOC1, At3g25760*); 5'-GTC GAC TTC ATG AAA TTA AAA TGT TTC TC-3' and 5'-GTC GAC CCA AAA GAT TAC AAA GAC TTT TC-3' for *AOC2 (At3g25770)*; 5'-GTC GAC GTA ATT AGC TGT TTA ATA GTT GTT TTG-3' and 5'-GTC GAC TTT GAA ATC GAG TAC AAC AGT TCC-3' for *AOC3 (At3g25780)*; 5'-GTC GAC AAT CTA AAT CTT TGT GGG TGT-3' and 5'-GTC GAC GGA GTT CAC GCG CTT AAA TCC A-3' for *AOC4 (At1g132800)*; 5'-ATG ACG GCG GCA CAA GGG AAC-3 and 5'-TCA GAG GCG GGA AGA AGG AG-3' for *OPDA reductase3* (*OPR3, At2g06050*); 5'-CGG AAT TCA TGA GAG AGA TCC TTC ATA TC-3' and 5'-CCC TCG AGT TAA GTC TCG TAC TCC TCT TC-3' for *β-tubulin* (*TUB, At5g44340*). For *ORA47* (*At1g74930*), a specific DNA fragment that shows little homology with other AP2/ERF genes was PCR amplified from Arabidopsis genomic DNA using the primer set 5'-GGG GTA CCG GAT CCT CTC CTT CTA CAT CTG CAT CTG TTG-3' and 5'-GCT CTA GAC TCG AGT CCC AAA GAA TCA AAG ATTC-3'.

Chromatin immunoprecipitation

Chromatin immunoprecipitation experiments were performed according to Bowler et al. (2004) with some modifications. Two grams of 2 weeks-old seedlings from XVE-*ORA47-TAP* line # 4-3 or XVE-*ORA59-TAP* line # 11 treated with 0.1% DMSO or 4 µM estradiol for 16 hrs 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% v/v 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 ^{o}C for 6 hrs to bind TAP or TAP-fusion proteins. 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. PCR was performed for 35 cycles with the primer sets 5'-CAT GTA TTT TCA TTC CAA GAG CAG C -3' and 5'-GAT GCT TTG GGA GGA ATT TGG-3' (*AOC2*), 5'-TAT ACT TGT GTA ACT ATG GCT TGG-3' and 5'-TGT TGA TGG CTG GTT TCT CC -3' (*PDF1.2*), 5'-CCG GCC AAA AGT ACA

TCA AC-3’ and 5’-TGG GAC TTT GGT TTT ATG TGG-3’ (*AOC1* set 1), and 5’-ACA CAT GCA CTC GAG GAA AC-3’ and 5’-TGG AAG AAA CTC GAG AAT TTA GAC-3’ (*AOC1* set 2).

Acknowledgements

A.C. was supported by a Marie Curie Intra-European fellowship within the European Community 5th Framework Programme (contract QLK5-CT-2002-51650). M.P. was supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO; grant # 811.36.001). A.Z. was supported by a grant from the Ministry of Science, Research and Technology, Iran (grant no. 7911580). M.P. and A.Z. were also supported by a van der Leeuw grant from the Netherlands Organization for Scientific Research (NWO) awarded to J.M. The authors thank Dr.C. Wasternack for the reporter plasmids carrying the 600 bp and 300 bp *AOC* promoter derivatives and for the plasmids with the *AOC* gene-specific 3’ untranslated regions.

References

Atallah, M. (2005). Jasmonate-responsive AP2-domain transcription factors in Arabidopsis. Ph.D. thesis. Leiden, Leiden University.

Atallah, M. and Memelink, J. (2004) Plant response to stress: role of the jasmonate signal transduction pathway. In: Encyclopedia of Plant and Crop Science, RM Goodman (ed.), Marcel Dekker Inc., New York, USA, pp. 1006-1009.

Axelos, M., Curie, C., Mazzolini, L., Bardet, C., and Lescure, B. (1992). A protocol for transient gene expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures. Plant Physiol.Biochem. **30**, 123-128.

Bowler, C., Benvenuto, G., Laflamme, P., Molino, D., Probst, A.V., Tariq, M., and Paszkowski, J. (2004). Chromatin techniques for plant cells. Plant J. **39**, 776-789.

Clough, S.J. and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16**, 735-743.

Creelman, R.A., and Mulpuri, R. (2002). The oxylipin pathway in *Arabidopsis*. The Arabidopsis Book, eds., C.R. Somerville and E.M. Meyerowitz, American Society of Plant Biologists, Rockville, MD, pp 1-24, doi/10.1199/tab.0012, http://www.aspb.org/publications/arabidopsis/

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

Dyer, B.W., Ferrer, F.A., Klinedinst, D.K., and Rodriguez, R. (2000). A noncommercial dual luciferase enzyme assay system for reporter gene analysis. Anal. Biochem. **282**, 158-161.

Ellis, C., and Turner, J.G. (2001). The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell **13**, 1025-1033.

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J.G. (2002). The Arabidopsis mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. Plant Cell **14**, 1557-1566.

Hilpert, B., Bohlmann, H., op den Camp, R., Przybyla, D., Miersch, O., Buchala, A., and Apel, K. (2001). Isolation and characterization of signal transduction mutants of *Arabidopsis thaliana* that constitutively activate the octadecanoid pathway and form necrotic microlesions. Plant J. **26**, 435-446.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and 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.

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

Lorenzo, O. and Solano, R. (2005). Molecular players regulating the jasmonate signalling network. Curr. Opin. Plant Biol. **8**, 532-540.

Marsh, J.L., Erfle, M., and Wykes, E.J. (1984). The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**, 481-485.

Masson, J. and 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, K.C., Dombrecht, B., Manners, J.M., Schenk, P.M., Edgar, C.I., Maclean, D.J., Scheible, W.R., Udvardi, M.K., and 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., Swords, K.M.M., Staehelin, L.A., and Hoge, J.H.C. (1994). Southern, Northern and Western blot analysis. In Plant Molecular Biology Manual, S.B. Gelvin, R.A. Schilperoort and D.P.S. Verma, eds. (Dordrecht; Kluwer Academic Publishers), pp. F1-F23.

Menke, F.L.H, Champion, A., Kijne, J.W., and 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.

Ohme-Takagi, M. and Shinshi, H. (1995). Ethylene-inducible DNA-binding proteins that interact with an ethylene-responsive element. Plant Cell **7**, 173-182.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., Ohme-Takagi, M. (2001). Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell **13**, 1959-1968.

Pasquali, G., Ouwerkerk, P.B.F., and Memelink, J. (1994). Versatile transformation vectors to assay the promoter activity of DNA elements in plants. Gene **149**, 373-374.

Pré, M. (2006) Functional analysis of jasmonate-responsive AP2/ERF-domain transcription factors in *Arabidopsis thaliana*. Ph.D. thesis. Leiden University, Leiden, The Netherlands.

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

Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2002). DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem. Biophys. Res. Commun. **290**, 998-1009.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, K., Ohta, H., and Tabata, S. (2001). Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. DNA Res. 8, 153-161.

Sasaki, K., Mitsuhara, I., Seo, S., Ito, H., Matsui, H., Ohashi, Y. (2007). Two novel AP2/ERF domain proteins interact with *cis*-element VWRE for wound-induced expression of the tobacco *tpoxN1* gene. Plant J. **50**, 1079-1092.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. (1995). Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. Science **270**, 1988-1992.

Schaller, F. (2001). Enzymes of the biosynthesis of octadecanoid-derived signaling molecules. J. Exp. Bot. **52,** 11-23.

Schirawski, J., Planchais, S., and Haenni, A.L. (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.

Stenzel, I., Hause, B., Miersch, O., Kurz, T., Maucher, H., Weichert, H., Ziegler, J., Feussner, I., and Wasternack, C. (2003). Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. Plant Mol. Biol. **51**, 895-911.

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

Turner, J.G., Ellis, C., and Devoto, A. (2002). The jasmonate signal pathway. Plant Cell **14** Suppl, S153-S164.

van der Fits, L. and 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.

van der Fits, L., and Memelink, J. (2001). The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J. **25**, 43-53.

Vick, B.A. and Zimmerman, D.C. (1987). Pathways of the fatty acid hydroperoxide metabolism in spinach leaf chloroplast. Plant Physiol. 85, 1073-1078.

Wasternack, C. and Hause, B. (2002). Jasmonates and octadecanoids: signals in plant stress responses and development. Prog. Nucleic Acid Res. Mol. Biol. **72**, 165-221.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994). A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6**, 251-264.

Zuo, J.R., Niu, Q.W., and Chua, N.H. (2000). An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. **24**, 265-273.