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Functional analysis of jasmonate-responsive transcription factors in *Arabidopsis thaliana*

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Functional analysis of jasmonate-responsive transcription factors in *Arabidopsis thaliana*

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Functional analysis of jasmonate-responsive transcription factors in *Arabidopsis thaliana*

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To Azita, Parna and my parents

Contents	Page
Chapter 1	General introduction
	9
Chapter 2	A role for the AP2/ERF-domain transcription factors ORA59 and ERF1 in jasmonate-ethylene mediated activation of the <i>PDF1.2</i> gene in Arabidopsis
	33
Chapter 3	Jasmonic acid induces stabilization and nuclear localization of ORA59, an AP2/ERF-domain transcription factor essential for defense responses in Arabidopsis
	53
Chapter 4	Jasmonate-responsive <i>Allene Oxide Cyclase</i> gene expression in Arabidopsis is regulated by the AP2/ERF- domain transcription factor ORA47
	79
Chapter 5	A jasmonate-responsive promoter element from <i>Catharanthus roseus</i> is active in <i>Arabidopsis thaliana</i> and is controlled by the transcription factor AtMYC2
	103
Chapter 6	Summary
	123
Samenvatting	
	131
Curriculum vitae	
	137

Chapter 1

General introduction

Plants are exposed to many forms of stress, including pathogen and herbivore attack, or adverse light, water, temperature, nutrient or salt conditions. Due to their sessile life style, plants are only able to survive by the ability to build up fast and highly adapted responses to these diverse environmental stresses. Perception of stress signals often results in the biosynthesis of one or more of the major secondary signaling molecules jasmonic acid (JA; Turner et al., 2002), ethylene (ET; Wang et al., 2002; Guo and Ecker, 2004) and salicylic acid (SA; Shah, 2003). Production of these hormones generates a signal transduction network that leads to a cascade of events responsible for the physiological adaptation of the plant to the external stress. The JA, ET and SA signal transduction pathways act synergistically or antagonistically in a variety of responses, leading to fine-tuning of the complex defense response (Kunkel and Brooks, 2002).

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 (Turner et al., 2002; Devoto and Turner, 2003). These signaling molecules affect a variety of plant processes, including wounding and abiotic stresses, and defense against insects (McConn et al., 1997), and necrotrophic pathogens (Thomma et al., 1999). Among developmental processes which are known to be influenced by JAs are root growth, pollen maturation and dehiscence, ovule development and senescence as evidenced by various *Arabidopsis* mutants in JA biosynthesis and JA signaling (Turner et al., 2002; Wasternack 2006).

Stress-induced JA biosynthesis

Endogenous JA levels increase in response to external stress stimuli. In *Arabidopsis thaliana*, mutants that are impaired in JA production, such as the *fatty acid desaturase fad3/fad7/fad8* (*fad*) triple mutant, or JA perception, such as the *coronatine insensitive1* (*coi1*) mutant, exhibit enhanced susceptibility to a variety of pathogens (Vijayan et al., 1998; Thomma et al., 1998;

Norman-Setterblad et al., 2000) and insects (McConn et al., 1997; Ellis et al., 2002). This demonstrates that JA production and sensing are required for resistance against certain pathogens and insects. JA also plays an important role in the establishment of induced systemic resistance (ISR), a mechanism of defense that is induced by root colonization of the host plant by certain strains of non-pathogenic *Pseudomonas* species (Pieterse et al., 1998; 2000).

Jasmonates are synthesized via the octadecanoid pathway. Most of the enzymes of this pathway leading to JA biosynthesis have now been identified by a combination of biochemical and genetic approaches (Figure 1; Creelman and Mulpuri, 2002; Turner et al., 2002). The enzymes leading to JA biosynthesis are located in two different subcellular compartments (Vick and Zimmerman, 1984; Schaller, 2001; Wasternack and Hause, 2002). The octadecanoid pathway starts in the chloroplasts with phospholipase-mediated release of α -linolenic acid from membrane lipids. The fatty acid α -linolenic acid is then converted 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. OPDA is transported from the chloroplasts to the peroxisomes where it is reduced by OPDA reductase (OPR3) to give 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), followed by three rounds of beta-oxidation involving three enzymes to yield (+)-7-*iso*-JA which equilibrates to the more stable (-)-JA. Subsequently, JA can be metabolized in the cytoplasm by at least seven different reactions (Schaller et al., 2005). Well-characterized reactions include methylation to methyl-jasmonate (MeJA) by S-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase (JMT; Seo et al., 2001), conjugation to amino acids by JA amino acid synthase (JAR1; Staswick and Tiryaki, 2004) or hydroxylation to 12-hydroxyjasmonic acid (12-OH-JA; Wasternack and Hause, 2002).

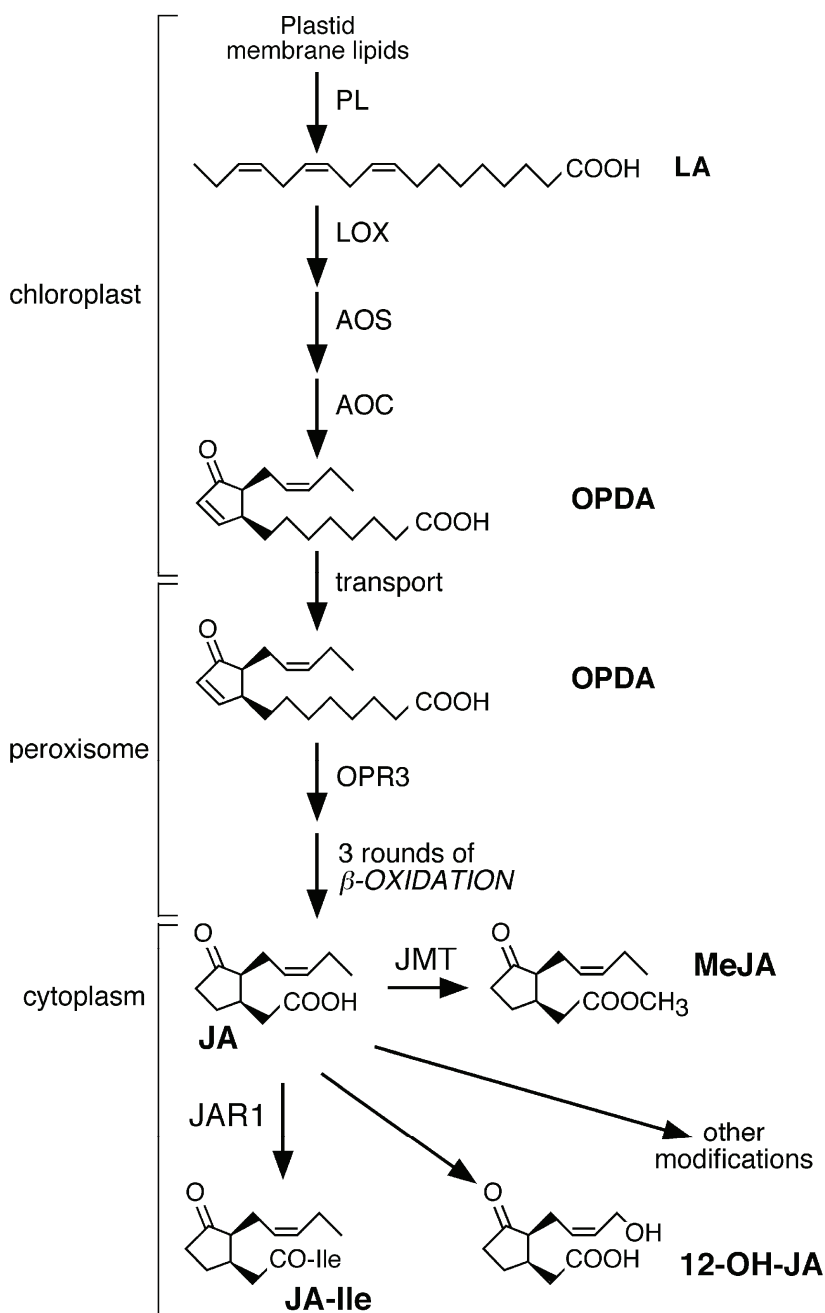


Figure 1. Schematic representation of the octadecanoid pathway leading to jasmonic acid biosynthesis. 12-OH-JA, 12-hydroxy-jasmonic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; JAR1, enzyme responsible for the conjugation of JA with isoleucine (JA-Ile); JMT, S-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase; LA, α-linolenic acid; LOX, lipoxygenase; MeJA, methyl jasmonate; OPDA, 12-oxo-phytodienoic acid; OPR3, OPDA reductase3; PL, phospholipase. Figure is taken from Pré (2006).

How stress signals induce JA biosynthesis is still unclear and the molecular components involved in the perception of the initial stimulus and in subsequent signal transduction resulting in JA production are largely unknown. The control points that govern the synthesis and accumulation of jasmonates remain to be identified. Timing and control of JA biosynthesis suggest several ways in which JA signaling might be modulated during stress perception. One level of control in JA biosynthesis and/or signaling might be the sequestration of biosynthetic enzymes and substrates inside the chloroplasts (Stenzel et al., 2003). In this way, JA biosynthesis and signaling would only be activated by the availability of substrate upon cellular decompartmentalization during wounding or pathogen attack. However, wounding induces the expression of several JA biosynthesis genes (Turner et al., 2002), suggesting that, at least partly, the wound-induced production of JA is a result of the increased transcription of genes encoding the JA biosynthesis pathway enzymes and their subsequent de novo protein synthesis. In addition, cDNA macro-array analysis revealed that MeJA treatment induced the expression of several genes involved in JA biosynthesis, such as *AOC*, *OPR1*, *OPR3*, *LOX2* and *AOS* (Sasaki et al., 2001). This analysis confirms the results presented in other reports, which show that JA induces transcription of the (Me)JA biosynthesis genes *LOX2*, *AOS*, *OPR3*, *DAD1*, *JMT*, and *AOC* (Bell and Mullet, 1993; Laudert and Weiler, 1998; Mussig et al., 2000; Ishiguro et al., 2001; Seo et al., 2001; Stenzel et al., 2003).

Together, these results indicate the existence of a positive feedback regulatory mechanism for JA biosynthesis in which JA stimulates its own production (Figure 2).

JA-responsive promoter elements

The expression of a gene is determined by the cis-acting DNA elements located in the vicinity of the gene and the trans-acting factors that interact with them. In general, these cis-acting elements are concentrated in a relatively small promoter region of a few hundred to a few thousand nucleotides upstream of the transcriptional start site.

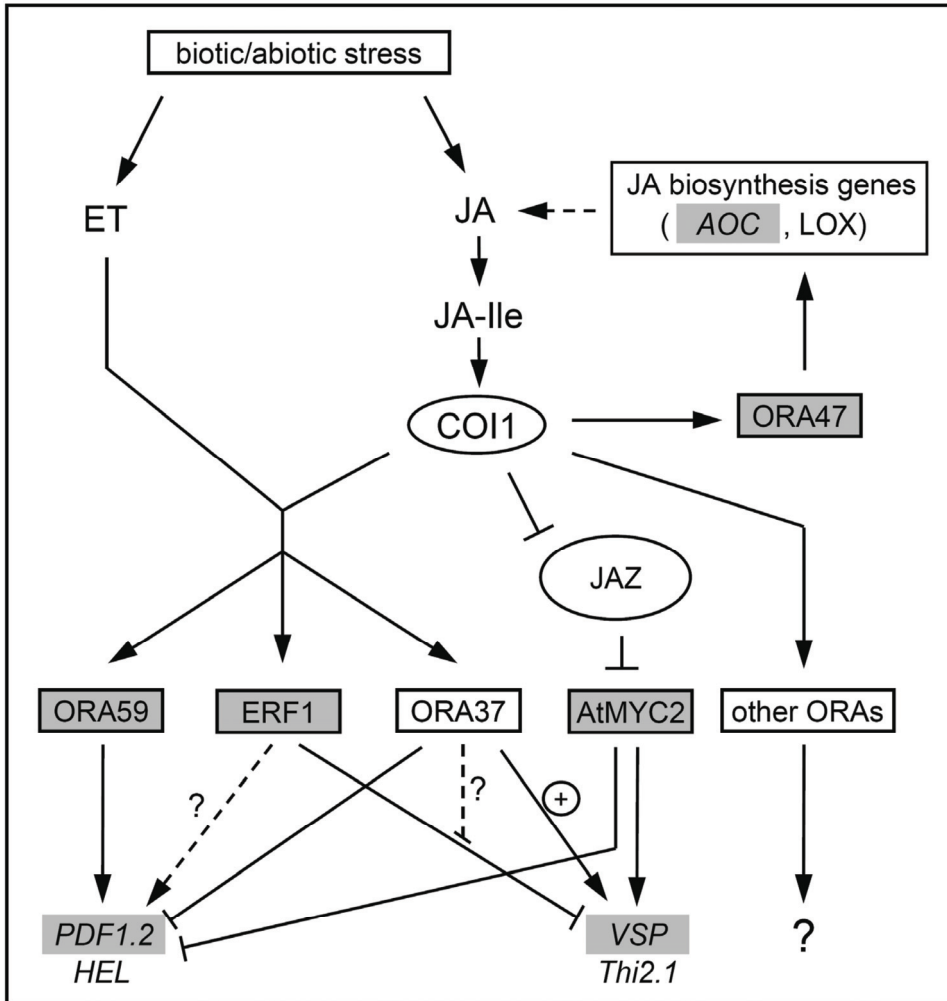


Figure 2. Role of transcription factors in the stress-responsive network involving the JA and ET signaling pathways. Different types of biotic or abiotic stress, including wounding, herbivore attack and infection with necrotrophic pathogens, induce the synthesis of JA and related oxylipins such as the biologically active JA-Ile. Some stress signals simultaneously induce ET biosynthesis. JAs induce the expression of several genes encoding transcription factors, including the ORAs, ERF1 and AtMYC2, via COI1, an F-box protein that is the receptor for JA-Ile. Binding of JA-Ile results in COI1-mediated degradation of JAZ repressors via the ubiquitin/proteasome pathway, thereby releasing AtMYC2 from repression. The transcription factor ORA47 acts as a regulator of the positive feed-back loop of JA biosynthesis. The bHLH-type transcription factor AtMYC2 positively regulates the expression of wound-responsive genes (i.e. *VSP* and *Thi2.1*) and represses other genes, including *PDF1.2* and *HEL*. The JA and ET signals cooperate to induce the expression of the *ORA59*, *ERF1* and *ORA37* genes. *ORA59* is the key regulator of the *PDF1.2* and *HEL* genes in response to ET and JA, whereas the role of *ERF1* in the regulation of these genes remains unclear (represented by a dashed arrow and a question mark). Conversely, *ORA37* represses the induction of the *PDF1.2* and *HEL* genes in response to JA and/or ET. *ORA37* also enhances the JA-induced expression of *VSP* genes (circled plus), presumably by repressing the negative effect of ET operated through *ERF1* (dashed bar line). The functions of other ORAs remain to be characterized. Genes used in the studies described in this thesis are shown against a grey background. Figure is adapted from Pré (2006).

Several cis-acting elements in various gene promoters that mediate the JA responsiveness have been identified. In the promoter of the terpenoid indole alkaloid biosynthesis gene *strictosidine synthase* (*STR*) from *Catharanthus roseus* a jasmonate- and elicitor-responsive element (JERE) has been identified (Menke et al., 1999). Mutation or removal of this JERE results in an inactive and unresponsive *STR* promoter derivative. A tetramer of the JERE fused to a minimal promoter confers JA-responsive gene expression on a reporter gene, showing that the JERE is an autonomous JA-responsive sequence (Menke et al., 1999). Within this JERE a GCC-box-like sequence is present. In Arabidopsis, a GCC-box (AGCCGCC) plays a role in conferring JA-responsiveness to the *PDF1.2* promoter (Brown et al., 2003). The GCC-box has also been shown to function autonomously as an ethylene-responsive element (Ohme-Takagi and Shinshi, 1995; Fujimoto et al., 2000). The *PDF1.2* gene is synergistically induced by a combination of JA and ethylene (Penninckx et al., 1998), which is likely caused by a convergent action of both signals on the GCC box.

G-box sequences (CACGTG) or G-box-like sequences (AACGTG) that are essential for the JA response were found in the promoters of the potato *PIN2* gene (Kim et al., 1992), the soybean *VSPB* gene (Mason et al., 1993), the Arabidopsis *VSP1* gene (Guerineau et al., 2003), the tomato *LAP* gene (Boter et al., 2004) and the *Catharanthus ORCA3* gene (Vom Endt et al., 2007). Also, analysis of the promoters of JA-responsive genes showed that the G-box element was statistically significantly over-represented in these promoters (Mahalingam et al., 2003). In the tomato *LAP* promoter, the G-box-like sequence is flanked by another sequence characterized by a GAGTA repeat, which is also essential for JA-responsive expression (Boter et al., 2004). In the *ORCA3* promoter the G-box-like sequence is flanked by an A/T-rich sequence that is important for the expression level (Vom Endt et al., 2007).

TGACG (*as-1*-type) sequences were found to be essential for JA inducibility of the promoter of the *Agrobacterium tumefaciens* T-DNA nopaline synthase (*nos*) gene (Kim et al., 1993; 1994), the CaMV 35S promoter (Xiang et al., 1996) and the barley *LOX1* gene (Rouster et al., 1997).

Two JA-responsive elements, JASE1 (5'-CGTCAATGAA-3') and JASE2 (5'-CATACGTCGTCAA-3'), were identified in the promoter of the *OPR1* gene in *Arabidopsis* (He and Gan, 2001). JASE1 is a new motif without any signature sequence so far reported, whereas JASE2 possesses an ACGT core which is also found in the G-box and in *as-1*-type elements.

In the LTR promoter of the tobacco retrotransposon *Tto1* a 13-bp element, which contains a box L/AC-I or H-box-like motif, is involved in responsiveness to MeJA (Takeda et al., 1998).

In conclusion, a variety of JA-responsive elements appear to exist. The best characterized elements are the G-box and closely related variants, which respond to JA and are negatively affected by ET, and the GCC box which responds in a synergistic manner to JA combined with ET.

Transcription factors and JA responses

Several members of APETALA2/Ethylene-Response-Factor (AP2/ERF)-domain transcription factor super family have emerged as important players in JA-responsive gene expression. ERF proteins are a family within the AP2/ERF-domain transcription factor super family, which is characterized by the presence of a single conserved 58-60 amino acid DNA-binding domain of the AP2/ERF type. ERF proteins from different subfamilies have been shown to bind to two similar cis-elements. Proteins from the ERF subfamily bind to the GCC box, which is found in several defense gene promoters, whereas proteins belonging to the CBF/DREB subfamily bind to the C-repeat (CRT)/dehydration-responsive element (DRE) motif, which is present in the promoters of dehydration and low-temperature-responsive genes.

In *C. roseus*, expression of the *ORCA2* and *ORCA3* genes, encoding ERF proteins, is rapidly induced by MeJA (Menke et al., 1999; van der Fits and Memelink, 2001). The ORCA proteins interact with the JERE in the *STR* promoter. This was the first evidence for a link between JA signaling and members of the ERF family of transcription factors.

In *Arabidopsis*, the ERF transcription factor family comprises 122 proteins. In a family-wide screening, Atallah (2005) characterized 14 genes called *Octadecanoid-Responsive Arabidopsis AP2/ERF (ORA)* genes, which were rapidly induced by JA treatment.

ORA59 gene expression is induced by JA or ET, and synergistically induced by both hormones (Atallah, 2005). Genome-wide microarray analysis showed that overexpression of the *ORA59* gene resulted in increased expression of a large number of JA- and ET-responsive defense genes, including genes encoding plant defensin1.2 (*PDF1.2*) and hevein-like protein (*HEL*). Plants overexpressing *ORA59* were more resistant to infection by the necrotrophic fungus *Botrytis cinerea*. Plants overexpressing *ERF1*, a closely related member of the ERF family, were previously shown to have elevated expression levels of the *PDF1.2* and *HEL* genes (Solano et al., 1998; Lorenzo et al., 2003) and to be more resistant to *B. cinerea* (Berrocal-Lobo et al., 2002). Similar to *ORA59* expression, the *ERF1* gene is synergistically induced by JA and ET (Lorenzo et al., 2003). These similarities in gene expression patterns and in target gene sets, as well as the fact that they are close homologues in the ERF family, suggest that *ORA59* and *ERF1* have redundant functions in JA and ET signal transduction. However, an essential role of *ORA59* as an integrator of the JA and ET signals leading to regulation of defense genes was demonstrated with plants where the *ORA59* gene was silenced via an RNAi approach. In response to JA and/or ET, or after infection with the necrotrophic fungi *B. cinerea* or *Alternaria brassicicola*, expression of *PDF1.2* and other defense genes was blocked in *ORA59*-silenced plants. As expected from the dramatic effect on defense gene expression, the silenced plants were also more susceptible to *B. cinerea* infection. The results demonstrate that *ORA59* integrates JA and ET signal inputs to coordinate the appropriate gene expression response directed against pathogen attack. An evaluation of whether *ERF1* has essential roles or whether it is an expendable functionally redundant transcription factor awaits analysis of *ERF1* knock-out mutants.

Constitutive overexpression of the *ORA47* gene in *Arabidopsis* results in an extreme dwarf phenotype with production of anthocyanins at the shoot apex (Pré, 2006), which is reminiscent of the phenotype exhibited by JA-treated plants. Overexpression of *ORA47* led to the activation of a large number of genes encoding JA biosynthetic enzymes, including AOC2, AOS and LOX2. Consistent with this finding, plants overexpressing *ORA47* contained high levels of hydroxylated derivatives of JA, indicating that *ORA47* overexpression leads to JA biosynthesis but that JA is inactivated by hydroxylation (Pré, Miersch, Wasternack, Memelink, unpublished results).

In addition to the JA biosynthesis genes, induction of *ORA47* expression led to increased expression of several JA-responsive defense genes, most likely indirectly as a consequence of JA production. The results suggest that *ORA47* controls oxylipin biosynthesis via direct transcriptional regulation of the JA biosynthesis genes, although this remains to be demonstrated.

The expression of all JA biosynthesis genes, including *LOX2*, *AOS* and *AOC*, is induced by treatment with exogenous JA or MeJA (Turner et al., 2002; Pré, 2006), indicating the existence of a positive feedback regulatory mechanism for oxylipin biosynthesis. *ORA47* appears to be a key regulator of this auto-stimulatory loop.

The transcription factor *ORA37* differs from the other JA-responsive ORAs by the presence of an ERF-associated amphiphilic repression (EAR) motif in the C-terminal part of the protein. The EAR motif has been shown to function as an active repressor of transcription (Otha et al., 2001). The *ORA37* gene, also referred to as *AtERF4*, is induced by JA (Atallah, 2005), ET (Fujimoto et al., 2000) or wounding (Cheong et al., 2002). Overexpression of *ORA37* had no effect on the basal transcript level of several JA-responsive genes in untreated plants. However, upon JA and/or ET treatment, *ORA37*-overexpressing plants showed significantly lower induction of a subset of JA- and ET-responsive genes, including the defense genes *PDF1.2*, *HEL* and *ChiB*, compared to control plants treated similarly (McGrath et al., 2005; Pré, 2006). On the other hand, plants in which *ORA37* expression was

silenced via T-DNA insertion (McGrath et al., 2005) or via RNAi (Pré, 2006) showed increased *PDF1.2*, *HEL* and *ChiB* transcript levels after JA- and/or ET-treatment compared to control plants, corroborating the complementary results obtained with *ORA37*-overexpressing plants. This demonstrates that *ORA37* plays a role in JA and ET signaling by repressing the expression of a number of genes in response to JA and/or ET. The same genes were shown to be positively regulated by *ORA59*.

In addition, overexpression of the *ORA37* gene resulted in enhanced JA-induced expression of a distinct subset of JA-responsive genes, including *VSP1* and *CYP79B2* (Pré, 2006). This indicated that the presence of *ORA37* positively regulated the expression of these genes in response to JA treatment. It is not clear how the positive effect of *ORA37* overexpression on JA signaling for this gene subset is operating at the molecular level, but assuming that *ORA37* always acts as a repressor, the positive effect is hypothesized to be caused by the repression of a repressor. The ET signaling pathway was shown to repress the wound-induced expression of several wound-responsive genes, including the *VSP1* and *CYP79B2* genes (Rojo et al., 1999; Mikkelsen et al., 2000). Overexpression of the ET-responsive *ERF1* gene has been shown to inhibit the expression of the *VSP2* gene in response to JA (Lorenzo et al., 2004). JA-induced expression of the *VSP2* gene is controlled by the basic helix-loop-helix (bHLH)-type transcription factor *AtMYC2* (Figure 2; Lorenzo et al., 2004). It was therefore suggested that the negative regulation of the *VSP2* gene by ET is executed through *ERF1*, although the molecular relationships between the activator *AtMYC2* and the repressor *ERF1* on JA-responsive *VSP2* expression remains to be characterized. It is possible that *ORA37* antagonizes the *ERF1*-mediated negative effect of ET on the expression of a subset of JA-responsive genes, including *VSP* genes. *ORA37* and *AtMYC2* seem to positively regulate the same subset of JA-responsive genes. However, overexpression of *AtMYC2* is sufficient to activate *VSP2* expression (Lorenzo et al., 2004), which is not the case in *ORA37*-overexpressing plants (Pré, 2006).

Therefore, JA and ET induce both activators (e.g. ORA59, AtMYC2 and ERF1) and repressors (e.g. ORA37) of gene expression. The functional importance of the simultaneous induction of both positive and negative regulators by JA and ET remains unclear. The balance between activators and repressors on common target promoters may provide a mechanism for switch-like transcriptional control.

JA perception and signaling

To identify molecular components of JA signal transduction, screenings for mutants that are insensitive to (Me)JA or to coronatine (a bacterial toxin which is a structural and functional analog of JA) or that show constitutive JA responses have been performed (Lorenzo and Solano, 2005). Several mutants were characterized.

The *coronatine insensitive1* (*coi1*) mutant was isolated in a screen for Arabidopsis mutants insensitive to root growth inhibition by coronatine (Feys et al., 1994). The *coi1* mutant is also insensitive to MeJA (Feys et al., 1994), is defective in resistance to certain insects and pathogens and fails to express JA-regulated genes (Benedetti et al., 1995; McConn et al., 1997; Thomma et al., 1998). The *COI1* gene encodes an F-box protein (Xie et al., 1998). F-box proteins associate with cullin, Skp1 and Rbx1 proteins to form an E3 ubiquitin ligase known as the SCF complex, where the F-box subunit functions as the specificity determinant targeting proteins for ubiquitin-mediated proteolysis by the 26S proteasome. Co-immunoprecipitation experiments showed that COI1 associates in vivo with SKP1, cullin and Rbx1 proteins to form the SCF^{COI1} complex (Devoto et al., 2002; Xu et al., 2002). Therefore, the requirement for COI1 in JA-dependent responses indicates that ubiquitin-mediated protein degradation is involved in JA signaling. Plants that are deficient in other components or regulators of SCF complexes, including AXR1, COP9 and SGT1b, also show impaired JA responses (Feng et al., 2003; Lorenzo and Solano, 2005; Tiryaki and Staswick, 2002). The existence of a *COI1* function that is conserved in species other than

Arabidopsis was demonstrated by the identification of a *COI1* homologue in tomato (*LeCOI1*; Li et al., 2004).

Putative targets of COI1-dependent proteasome degradation have been identified using yeast two-hybrid screening. RPD3b, a histone deacetylase, was identified as a COI1-interacting protein (Devoto et al., 2002). Since histone deacetylation decreases the accessibility of chromatin to the transcription machinery (Lusser et al., 2001), COI1-dependent proteasome degradation of RPD3b could be a mechanism for derepression of JA-dependent transcription. However, constitutive overexpression of an RPD3b-related histone deacetylase in Arabidopsis (i.e. RPD3a/HD19) has the opposite effect, increasing transcription of the transcription factor ERF1 and its target genes (Zhou et al., 2005). Therefore assessment of the involvement of histone deacetylation in JA signaling requires further studies.

COI1 is a component that is specific to the JA pathway, whereas SGT1b and AXR1 are shared by other signaling pathways (Gray et al., 2003; Tiryaki and Staswick, 2002; Xu et al., 2002). AXR1 is also a positive regulator of auxin responses, and it modulates the activity of SCF^{TIR1} by modifying cullin through the addition of the ubiquitin-like protein Nedd8/Rub1 (del Pozo et al., 2002; Schwechheimer et al., 2002). The conserved function of SGT1 in mediating SCF activity in plants is supported by the complementation of the yeast *sgt1* mutation by either of the two highly related Arabidopsis SGT1 genes, and by the observation that the SGT1 proteins HvSGT1 and NbSGT1 co-immunoprecipitate with core SCF subunits in extracts from barley (*Hordeum vulgare*) and *Nicotiana benthamiana* respectively (Azevedo et al., 2002; Liu et al., 2002). Like mutations in *AXR1*, mutations in *SGT1b* have pleiotropic effects that impair plant responses not only to JA but also to auxin and pathogens, suggesting that both SGT1b and AXR1 are regulators of SCF complexes and are involved in several different signaling pathways (Austin et al., 2002; Azevedo et al., 2002; Gray et al., 2003).

A particularly effective screen for JA signaling mutants is described by Lorenzo et al. (2004). Screening for mutants affected in JA-induced root growth inhibition in an *ethylene-insensitive3* (*ein3*) background resulted in the identification of five complementation groups identifying 5 loci called *JA-insensitive* (*JAI*) 1-5. The *JAI1* locus corresponds to the *AtMYC2* gene (Lorenzo et al., 2004). The *JAI2* locus corresponds to the previously characterized (Staswick et al., 1992; Staswick and Tiryaki, 2004) *JAR1* gene. The *JAI4* locus corresponds to the *SGT1b* gene (Lorenzo and Solano, 2005). The *JAI5* locus corresponds to the *COI1* gene (Lorenzo et al., 2004).

Recently, the gene affected in the *jai3* mutant was identified. It encodes a protein with a zinc finger-like ZIM motif (Chini et al., 2007). There are several related genes in *Arabidopsis* forming a gene family called ZIM or TIFY (Vanholme et al., 2007). The members that are induced at the gene expression level by JA are called Jasmonate ZIM domain (JAZ) proteins (Chini et al., 2007; Thines et al., 2007). In the *jai3* mutant an aberrant protein is expressed with a deletion of a C-terminal domain conserved in the JAZ proteins. The wild-type JAI3 protein is rapidly degraded in response to JA in a COI1-dependent manner, whereas the *jai3* mutant protein is stable. The JAI3 protein interacts in vitro and in yeast with COI1. It also interacts with AtMYC2. Based on these findings it is postulated that JAI3 is a repressor of AtMYC2 which is rapidly degraded in response to JA thereby activating AtMYC2 (Figure 2; Chini et al., 2007).

In independent studies, members of the JAZ gene family were characterized as being predominant among genes induced in anthers after 30 minutes of JA treatment (Mandaokar et al., 2006). Subsequent study of the family member JAZ1 demonstrated that it is rapidly degraded in response to JA in a COI1-dependent manner (Thines et al., 2007). On the other hand a deletion derivative of JAZ1 lacking the C-terminal domain is stable. Interestingly, these authors did not detect interaction between JAZ1 and COI1 in yeast or in in vitro pull-down assays in the absence of a biologically active jasmonate. They could detect interaction in the presence of JA conjugated to Ile in the yeast growth medium or in the in

vitro pull-down assay, but not with OPDA, JA, MeJA or JA conjugated to Trp or Phe, whereas JA-Leu was about 50-fold less effective than JA-Ile. JA-Ile and JA-Leu are products of the JAR1-mediated conjugation reaction (Staswick and Tiryaki, 2004).

The picture that emerges for JA signal transduction is highly reminiscent of auxin signal transduction, which involves auxin-responsive degradation of AUX/IAA repressor proteins via the F-box protein TIR1 (Guilfoyle, 2007). TIR1 is the auxin receptor (Kepinski and Leyser, 2005; Dharmasiri et al., 2005) with auxin acting as the molecular glue between TIR1 and AUX/IAA proteins (Tan et al., 2007). Interestingly, COI1 is the closest relative to TIR1 that is not related to auxin perception among the about 700 members of the Arabidopsis F-box protein family. Therefore it appears that JA-Ile forms the molecular glue between COI1 and JAZ1 and possibly other JAZ family members. It was also proposed that distinct biologically active JAs could form the molecular glue between COI1 and specific JAZ family members, and that these family members could act as repressors of specific downstream targets, presumably transcription factors such as AtMYC2 (Thines et al., 2007).

The challenges are now to determine which JAs can act as molecular glues with which specific JAZ family members, and to find out what are the specific targets of each member of the JAZ family of repressors.

Outline of the thesis

Jasmonic acid is a plant signaling molecule that plays an important role in defense against wounding, insects and necrotrophic pathogens. Depending on the stress situation and on the simultaneous induction of ET and SA biosynthesis, JA induces the expression of a specific set of genes encoding defense-related proteins and/or enzymes involved in biosynthesis of protective secondary metabolites. Many aspects concerning the mode of action of JA on the regulation of gene expression are poorly understood. Several transcription factors have been identified that appear to be involved in JA-responsive gene expression, including ORA59, ERF1, ORA47 and AtMYC2. Identification of the mechanisms whereby these transcription

factors are activated by JA at the protein level and of the interaction between these transcription factors and the binding sites in the promoters of their target genes is of major importance to understand how JA acts.

The studies described in this thesis are focused on the functional analysis of JA-responsive transcription factors in *Arabidopsis* with an emphasis on the interaction with the promoters of their target genes.

Chapter 2 describes studies aiming at the dissection of the interaction of ORA59 and the related transcription factor ERF1 with the *PDF1.2* promoter. Two GCC boxes in the *PDF1.2* promoter are equally important for trans-activation by ORA59 and ERF1 in transient assays and for in vitro binding. Application of the chromatin immunoprecipitation technique showed that ORA59 binds to the *PDF1.2* promoter in vivo. Interestingly, mutation of only one of the GCC boxes at positions -256 to -261, previously reported by others to be important for JA-responsive expression, completely abolished the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ET-releasing agent ethephon.

The aim of the studies reported in **Chapter 3** was to determine whether JA has an activating effect on ORA59 at the protein level. The results show that JA caused stabilization as well as nuclear localization of ORA59 protein. Interestingly, nuclear localization of ORA59 did not require a functional COI1 protein. Based on the findings it is postulated that there is a jasmonate receptor distinct from COI1, an F-box protein that targets ORA59 for degradation, and a repressor protein that sequesters ORA59 in the cytoplasm.

Chapter 4 describes studies on the role of the transcription factor ORA47 in the regulation of the AOC genes encoding the JA biosynthesis enzyme allene oxide cyclase with emphasis on the highest expressed member AOC2. 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 a transient assay. In addition

ORA47 interacted with the *AOC1* promoter in vivo, and ORA47 can trans-activate the *AOC1* promoter in a transient assay.

In **Chapter 5** studies on the activity of a JA-responsive element (JRE) from the promoter of the *Catharanthus* gene encoding the JA-responsive AP2/ERF-domain transcription factor ORCA3 are reported. It turns out that the JRE from the *ORCA3* promoter is active in Arabidopsis. It interacts in vitro and in vivo with the bHLH transcription factor AtMYC2. Analysis of JRE-mediated reporter gene expression in an *atmyc2-1* mutant background showed that the activity was strictly dependent on AtMYC2.

Finally, in **Chapter 6** a summary of the thesis work is presented.

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Chapter 2

A role for the AP2/ERF-domain transcription factors ORA59 and ERF1 in jasmonate-ethylene mediated activation of the *PDF1.2* gene in Arabidopsis

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Abstract

Plant defense against pathogens and herbivores depends on the action of several endogenously produced hormones, including jasmonic acid (JA) and ethylene. In certain defense responses in *Arabidopsis thaliana*, JA and ethylene signaling pathways synergize to activate a specific set of defense genes including *plant defensin1.2* (*PDF1.2*). The AP2/ERF domain transcription factor *ORA59* acts as the integrator of the JA and ethylene signalling pathways and is the key regulator of JA- and ethylene-responsive *PDF1.2* expression. In this chapter we describe studies aimed at dissecting the interaction of *ORA59* and the related transcription factor *ERF1* with the *PDF1.2* promoter. We show that two GCC boxes in the *PDF1.2* promoter are important for trans-activation by *ORA59* and *ERF1* in transient assays and for in vitro binding. Using the chromatin immunoprecipitation technique we were able to show that *ORA59* binds to the *PDF1.2* promoter in vivo. Interestingly, mutation of a single GCC box at positions -256 to -261 previously reported by others to be important for JA-responsive expression completely abolished the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ethylene-releasing agent ethephon.

Introduction

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

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

Thomma et al., 1999a; Thomma et al., 1999b), demonstrating that JA and ET are important signal molecules for resistance against these pathogens.

A crucial step in the JA/ET-dependent defense response is the rapid transcription of genes coding for antimicrobial proteins (Penninckx et al., 1998) or enzymes involved in the biosynthesis of secondary metabolites (Memelink et al., 2001). Studying the mechanism whereby the expression of these defense-related genes is regulated is of major importance to understand signal transduction pathways and plant responses to environmental stress.

Transcription factors belonging to a family of the AP2/ERF-domain protein superfamily known as the ethylene response factors (ERF) have emerged as important players in plant defense responses (Gutterson and Reuber, 2004). Proteins from the ERF subfamily are characterized by a single AP2/ERF-type DNA-binding domain with a conserved amino acid sequence, and several members were shown to bind specifically to the sequence AGCCGCC (Hao et al., 1998). This so-called GCC box is found in the promoters of several pathogen-responsive genes including *plant defensin1.2* (*PDF1.2*).

Constitutive overexpression of ERF1 (At3g23240) or AtERF2 (At5g47220) was shown to cause high levels of expression of the *PDF1.2* gene and other defense genes in *Arabidopsis* (Brown et al., 2003; Lorenzo et al., 2003; Solano and Ecker, 1998) and caused resistance to several fungi (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). It has been shown that the *ERF1* gene is synergistically induced by ET and JA and it was suggested that this transcription factor is a key element in integration of both signals for the regulation of defense genes (Lorenzo et al., 2003). Atallah, (2005) characterized the AP2/ERF-domain transcription factor ORA59 (At1g06160), which was also transcriptionally induced by JA and ET in a synergistic manner. Overexpression of *ORA59* activated the expression of several JA- and ET-responsive defense-related genes including *PDF1.2*, and caused increased resistance against *B. cinerae* (Pré, 2006). Although several AP2/ERF-domain transcription factors have been suggested to be positive regulators of *PDF1.2* gene expression (Brown et al., 2003; Lorenzo et al., 2003; Pré, 2006), a recent study showed that only ORA59 and ERF1 were able to function as transcriptional activators of *PDF1.2* gene expression, whereas AtERF2 and the related AtERF1 (At4g17500) were not (Pré, 2006). Analysis of transgenic plants in which *ORA59* gene expression was silenced by RNAi, whereas the *ERF1* gene was normally expressed, showed that ORA59 is strictly required for *PDF1.2* gene expression in response to JA, JA/ET, and infection with necrotrophic fungi (Pré, 2006). Studies of the *PDF1.2* gene promoter (Brown et al., 2003; Manners et al., 1998) identified a GCC-box at positions -256 to -261 which is involved in the JA response. However interactions of the *PDF1.2* promoter with the relevant transcription factors ERF1 and

especially ORA59 have not been reported, which prompted us to undertake the studies described in this chapter.

In the present study, we show that ORA59 and ERF1 transactivate the *PDF1.2* promoter in transient assays via binding to two GCC boxes. Using the chromatin immunoprecipitation technique we were able to show that ORA59 binds the *PDF1.2* promoter in vivo. Interestingly, mutation of a single GCC box at positions -256 to -261 previously reported by others to be important for JA-responsive expression completely abolished the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ethylene-releasing agent ethephon.

Results

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

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

To study the dose-response relationship for trans-activation of the *PDF1.2* promoter by ORA59 and ERF1, Arabidopsis protoplasts were co-transformed with the SF promoter derivative fused to *GUS*, and variable amounts of effector plasmids carrying the *ORA59*, *ERF1* or *ORA47* (At1g74930) genes fused to the CaMV 35S promoter (Figure 1B). ORA59 and ERF1 activated the *SF-GUS* reporter gene 40 or 10 fold respectively, whereas the unrelated AP2/ERF-domain transcription factor ORA47 (Pré, 2006; Chapter 4) had no effect. As shown in Chapter 4, ORA47 trans-activated the promoters of the *allene oxide cyclase* 1 and 2 genes in the protoplast assay, demonstrating that ORA47 is expressed and active. Previously we have shown that AtERF1 and AtERF2 did not significantly trans-activate the SF promoter derivative in a similar experimental set up (Pré, 2006). 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 μ g of effector plasmid, where after the response saturated.

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

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

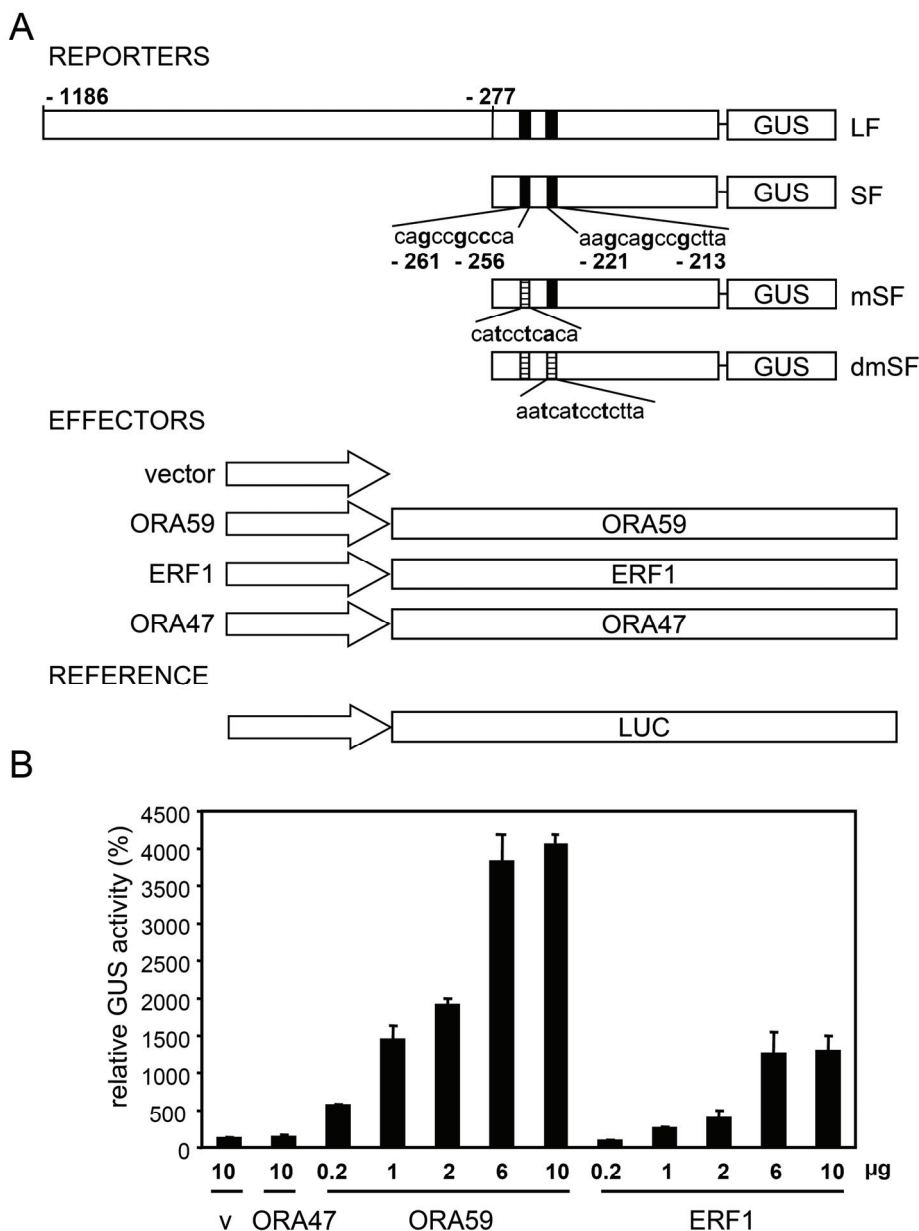


Figure 1. ORA59 and ERF1 trans-activate the *PDF1.2* promoter in a dose-dependent manner. **(A)** Constructs used in trans-activation assays in Figures 1 and 2. Reporter constructs consisted of the *GUS* gene driven by wild-type or mutated LF (long fragment) or SF (short fragment) *PDF1.2* promoter derivatives.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

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

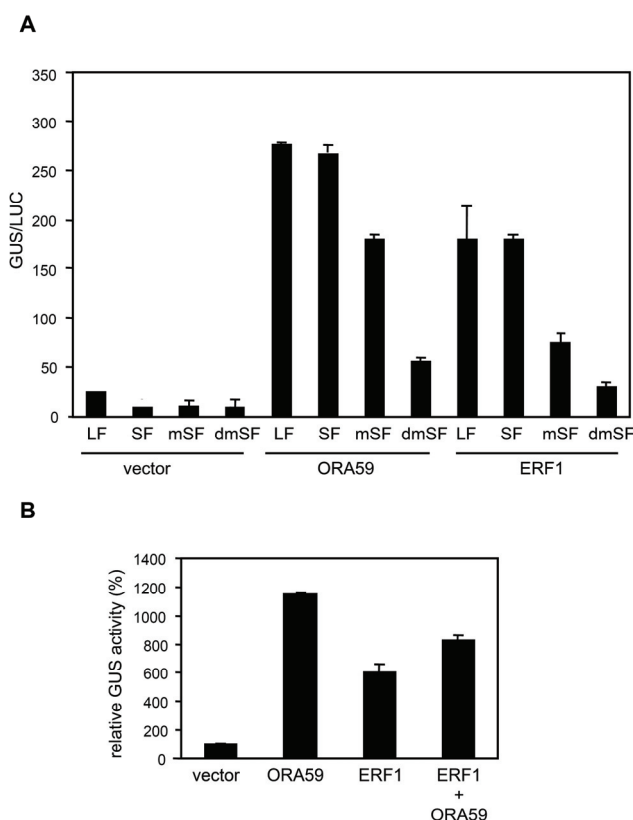


Figure 2. *ORA59* and *ERF1* trans-activate the *PDF1.2* promoter independently via two GCC boxes. **(A)** Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying different versions of the *PDF1.2* promoter shown in Figure 1A fused to *GUS* and overexpression vectors containing the *ORA59* or *ERF1* ORFs driven by the CaMV 35S promoter. Values represent means $\pm$ SE of triplicate experiments. **(B)** The SF-*GUS* reporter plasmid was co-transformed with 1 μ g of overexpression vectors carrying *ORA59* or *ERF1*, or with a combination of 0.5 μ g of each overexpression plasmid. Values represent means $\pm$ SE of triplicate experiments and are expressed relative to the vector control.

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

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

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

Next, the binding of *ORA59* and *ERF1* proteins to radiolabeled SF, mSF, and dmSF fragments was studied in electrophoretic mobility shift assays (EMSA). The unrelated AP2/ERF-domain transcription factor *ORA47* was used as a control at an amount that gave clear complex formation with a binding site from the promoter of the target gene *allene oxide cyclase 2* (*AOC2*; Chapter 4). As shown in Figure 3B, *ORA59* and *ERF1* were able to bind to the SF fragment, in contrast to *ORA47*. *ERF1* formed a single complex, whereas with *ORA59* two complexes were observed. These two complexes do not seem to reflect the occupation of a single GCC box or both GCC boxes, since both complexes were still formed after mutation of one of the GCC boxes. Binding of *ORA59* and *ERF1* was partially decreased when the GCC box at positions -261 to -256 was mutated, and completely abolished when both GCC boxes were mutated. 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 transactivation experiments in combination with 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).

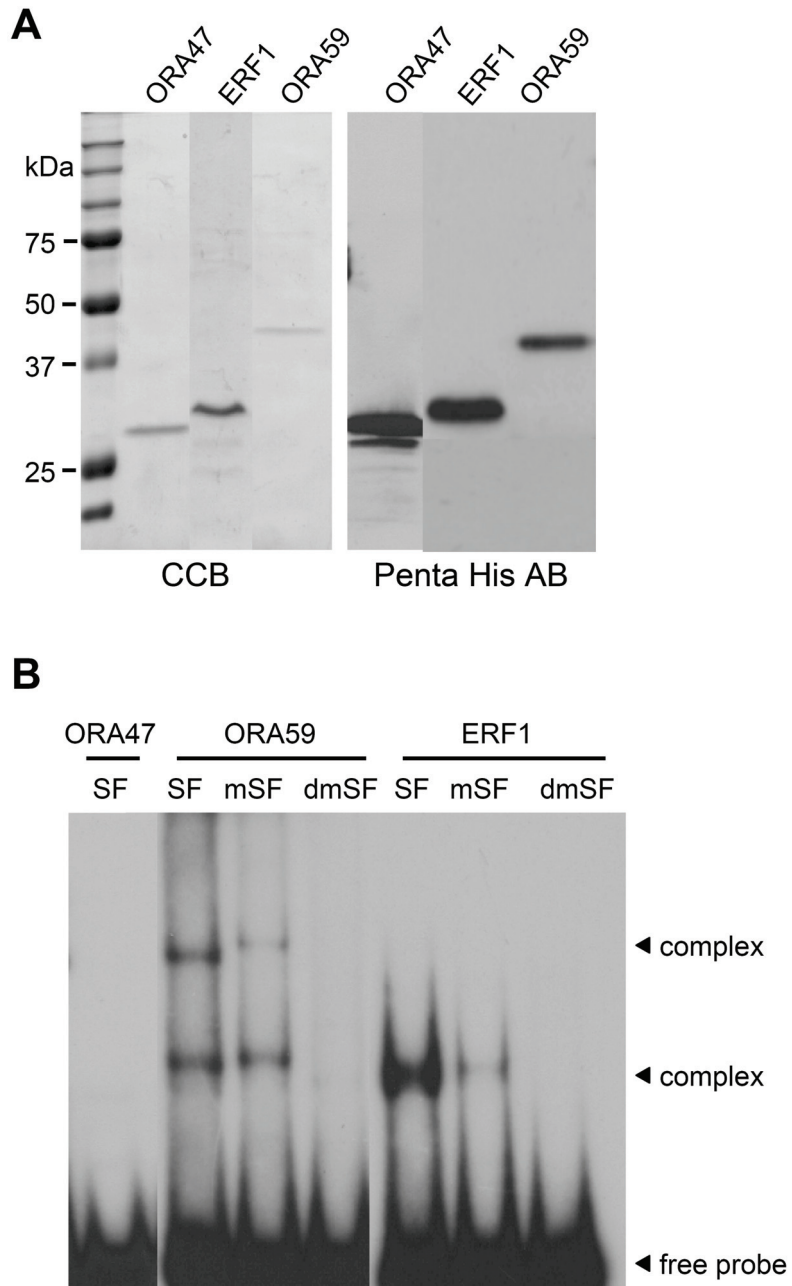


Figure 3. ORA59 and ERF1 bind to two GCC boxes in the *PDF1.2* promoter in vitro. **(A)** After SDS-PAGE recombinant proteins were detected with Coomassie Brilliant Blue R-250 (CBB; left), or with Penta-His HRP antibody conjugate following Western blotting (right). Protein size markers are indicated in k Dalton. **(B)** EMSAs were performed with recombinant ORA59 and ERF1 proteins and radio-labeled SF, mSF or dmSF fragments. ORA47 protein was used as a control. The arrow heads mark the positions of protein-DNA complexes and free probes.

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 μ M estradiol or the solvent DMSO the kinetics of mRNA and protein accumulation were followed (Figure 4). Maximum levels of ORA59-TAP mRNA and protein were observed after 16 to 24 hours. Estradiol treatment also induced the ORA59 target gene *PDF1.2*, but with slower kinetics, showing that the *ORA59-TAP* fusion protein is functionally active. DMSO-treated transgenic plants did not express ORA59-TAP or *PDF1.2*. Estradiol treatment had no effect on *PDF1.2* expression in control plants (Figure 5B).

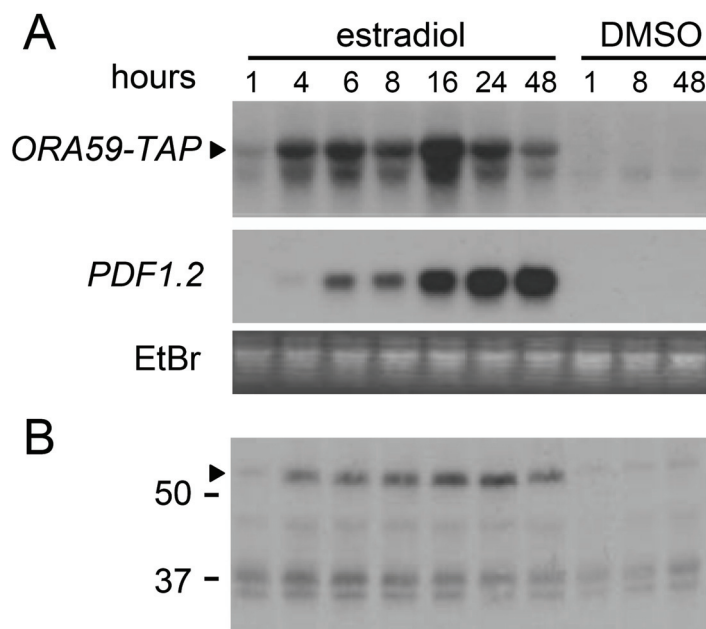


Figure 4. The ORA59-TAP protein is inducibly expressed and functional. Fifteen days-old T2 seedlings from XVE-ORA59-TAP line #4 cultured in liquid medium were treated for varying times in hrs with 4 μ M estradiol or the solvent DMSO at a final concentration of 0.1%. **(A)** Northern blot analysis. Gel blots were hybridized with the indicated probes. The arrowhead indicates the position of the *ORA59-TAP* mRNA. The ethidium bromide (EtBr) stained gel is shown as a control for RNA loading. **(B)** Western blot analysis. The protein samples were separated by SDS-PAGE followed by Western blotting and immuno-probing with the peroxidase anti peroxidase (PAP) antibody, which has affinity for the protein A part of the TAP tag. The arrowhead indicates the position of the ORA59-TAP fusion protein. Positions of protein size markers are indicated in k Dalton. The 37 kDa background band is shown as a control for protein loading.

Based on the results from the expression analysis, seedlings treated with 4 μ M estradiol or 0.1% DMSO for 16 hours were used for ChIP analysis. Transgenic seedlings expressing only the TAP tag under control of the CaMV 35S promoter were similarly treated as controls. Protein and mRNA analysis of the harvested samples prior to formaldehyde cross linking showed that the *ORA59*-TAP fusion protein was induced by estradiol treatment and was functional as judged by the induction of *PDF1.2* expression (Figures 5B and C).

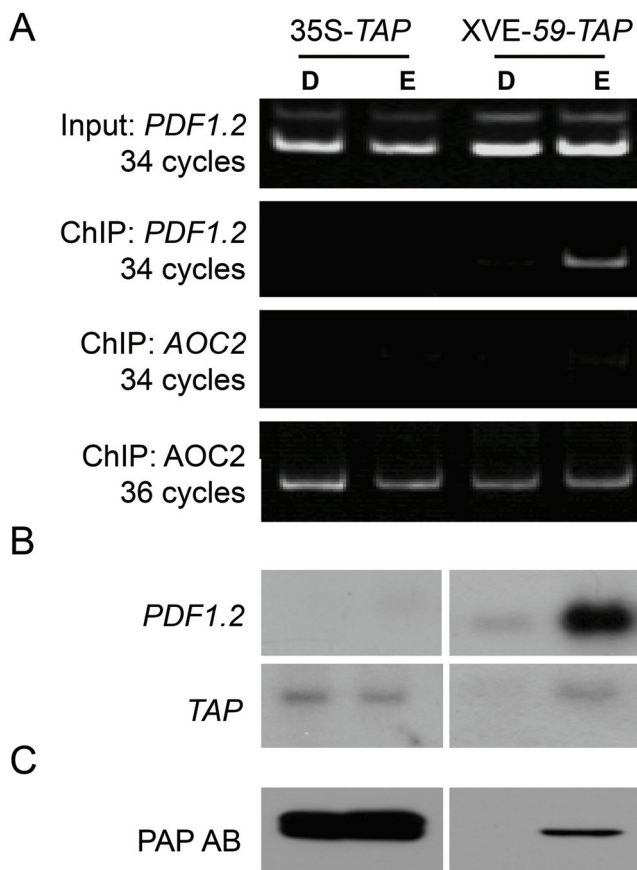


Figure 5. *ORA59* binds to the *PDF1.2* promoter in vivo. Seedlings from XVE-*ORA59*-TAP line #4 and 35S-TAP line #7 were treated with 4 μ M estradiol (E) or 0.1% DMSO (D). RNA and protein was extracted for Northern and Western blot analysis of transgene expression. Sonicated chromatin prepared from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

The 35S-*TAP* seedlings expressed the *TAP* mRNA and protein, but as expected did not express the *PDF1.2* gene. PCR analysis using *PDF1.2* primers of the chromatin prepared following formaldehyde cross linking of the samples showed that equivalent amounts of DNA were present (Figure 5A). 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 (Figure 5A). The results therefore show that the ORA59-*TAP* fusion protein binds directly to the *PDF1.2* promoter in vivo.

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

The expression of the *PDF1.2* gene is synergistically induced by a combination of JA and ethylene (Penninckx et al., 1998). To study the contribution of the two GCC boxes to JA- and ethylene-responsive activity of the *PDF1.2* promoter derivative SF, we generated stably transformed plants containing the *GUS* fusion constructs shown in Figure 1A via *Agrobacterium*-mediated transformation. The T-DNA also contains the *chloramphenicol acetyltransferase* (CAT) reporter gene driven by the CaMV 35S promoter. T2 seedlings from eight independent transgenic lines for each construct were treated with JA, ethephon or both for 24 hrs. *GUS* activities were normalized with CAT activities in the same samples. Comparison of the average *GUS* values in JA- or JA/ethephon-treated seedlings containing the wild-type SF construct with control-treated seedlings showed 5-6 fold induction. Mutation of the GCC box at positions -261 to -256 (mSF) dramatically decreased *PDF1.2* promoter activity in response to JA or JA/ethephon down to the level observed with the wild-type promoter after control treatment (Figure 6). Mutation of both GCC boxes at positions -261 to -256 and -221 to -213 (dmSF) had a similar effect as the single GCC box mutation. Whereas both GCC boxes were important for transient trans-activation and in vitro binding by ORA59 and ERF1, it turns out that mutation of a single GCC box reduced JA- and JA/ethephon-responsive expression of *PDF1.2* promoter derivative SF to background levels.

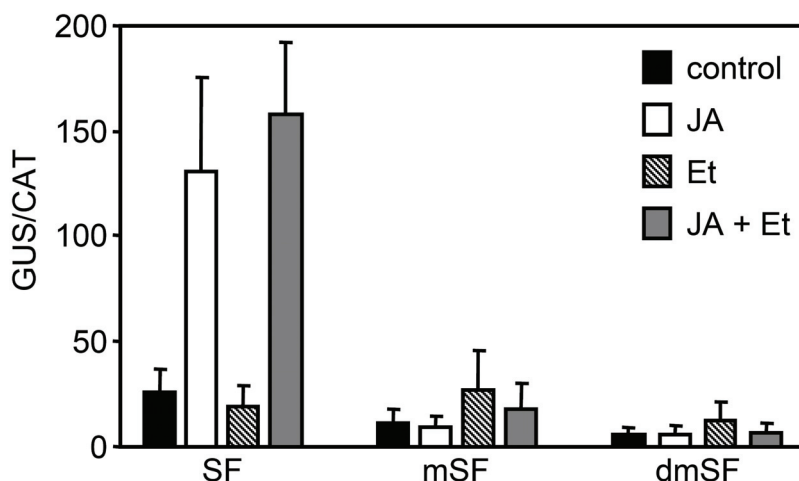


Figure 6. Effects of GCC box mutations on JA- and ethephon-responsive expression of *PDF1.2* promoter derivative SF in stably transformed *Arabidopsis* plants. Each bar represents average GUS activity values determined in pools of 10 T2 seedlings from 8 independent transformed lines for each construct corrected for chloramphenicol acetyltransferase (CAT) values in the same samples $\pm$ SE. Absolute CAT activities were in a similar range for all transgenic lines (data not shown). Seedlings were control-treated or treated with 50 μ M JA, 1 mM of the ethylene-releasing agent ethephon or both for 24 hrs.

Discussion

In certain defense responses, JA and ethylene signaling pathways synergize to activate a specific set of defense genes including *PDF1.2* (Penninckx et al., 1998). The AP2-domain transcription factor *ORA59* acts as the integrator of the JA and ethylene signalling pathways and is the key regulator of JA- and ethylene-responsive *PDF1.2* expression (Pré, 2006). Here we aimed at dissecting the interaction of *ORA59* and the related transcription factor *ERF1* with the *PDF1.2* promoter. We show that two GCC boxes in the *PDF1.2* promoter are important for trans-activation by *ORA59* and *ERF1* in transient assays and for in vitro binding. Using the chromatin immunoprecipitation technique we were able to show that *ORA59* binds the *PDF1.2* promoter in vivo. Interestingly, mutation of a single GCC box at positions -256 to -261 previously reported to be important for JA-responsive expression (Brown et al., 2003) completely abolished the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ethylene-releasing agent ethephon.

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

second GCC box at positions -221 to -213 knocked out in vivo and in vitro interaction of ORA59 and ERF1 with *PDF1.2* promoter derivative SF.

The function of ERF1 is somewhat mysterious at the current level of understanding. ERF1 can induce the expression of defense genes when overexpressed, but is not able to support JA- or JA/ethephon-responsive expression of defense genes when *ORA59* expression is knocked out by RNAi (Pré, 2006). One option could be that ERF1 acts synergistically with ORA59 on gene expression. We tested this idea by comparing *PDF1.2* promoter activity levels in response to ORA59 and ERF1 separately or combined, but we did not find evidence for synergism.

Quite surprising in view of the other results presented here was the observation that mutation of the single GCC box at positions -261 to -256 completely abolished the response of the *PDF1.2* promoter derivative SF in stably transformed plants in response to JA or JA/ethephon. Although this finding is consistent with the previous report of Brown et al. (2003), it was unexpected since the other assays highlighted the importance of both GCC boxes. There are several explanations for this apparent inconsistency. One likely option is that the *PDF1.2* promoter in the context of a chromatin structure in stably transformed plants requires two GCC boxes to be activated in response to JA whereas a single GCC box is not sufficient for opening up the chromatin structure for transcription. According to this explanation the GCC boxes would be functionally equivalent which can be tested by generating transgenic plants with the *PDF1.2* promoter derivative SF carrying a mutation in the GCC box at positions -221 to -213.

Materials 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 2.5% sodium hypochlorite and five rinses with sterile water. Alternatively seeds were surface-sterilized in a closed container with chlorine gas for three hours (<http://plantpath.wisc.edu/~afb/vapster.html>). Surface-sterilized seeds were grown on plates containing MA medium (Masson and Paszkowski, 1992) with 0.6% agar supplemented with 20 mg/L hygromycin for selection of transgenic plants. Following stratification for 3 days at 4°C, seeds were first germinated at 21°C in a growth chamber (16 h light/8 h dark, 2500 lux) on solid MA medium supplemented with hygromycin for 10 days, where after batches of 15-20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml liquid MA medium without antibiotic and the tubes were incubated on a shaker at 120 rpm for 4 additional days before treatments. Transgenic plants carrying an XVE expression module containing the *ORA59* gene

fused to the TAP tag were treated for 16 hrs with 4 μ M estradiol. As control, seedlings were treated with 0.1% DMSO. Transgenic seedlings carrying *PDF1.2* promoter derivatives SF, mSF or dmSF fused to *GUS* were treated for 24 hrs with 50 μ M JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (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 LF and SF fragments with forward primers 5'-CGG GAT CCA TGC AGC ATG CAT CGC CGC ATC-3' or 5' CGG GAT CCC CAT TCA GAT TAA CCA GCC GCC C-3', respectively, and the reverse primer 5'-GCG TCG ACG ATG ATT ATT ACT ATT TTG TTT TCA ATG-3'. Amplified products were digested with BamHI and Sall and cloned in plasmid GusXX (Pasquali et al., 1994). Mutations mSF and dmSF were generated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and primers 5'-CCA TTC AGA TTA ACC ATC CTC ACC TGT GAA CGA TG-3' or 5'-CAT TAG CTA AAA GCC GAA TCA TCC TCT TAG GTT ACT TTA GAT ATC G-3', respectively, and their respective reverse complementary primers. 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 into pRT101 (Töpfer et al., 1987). The *ERF1* ORF was PCR-amplified using the primer set 5'-GAA GAT CTT CAT CAC CAA GTC CCA CTA TTT TC-3' and 5'-GAA GAT CTC ATA TGG ACC CAT TTT TAA TTC AGT CC-3', digested with BglII and cloned into 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 pMOG183 (Mogen International, Leiden, The Netherlands) digested with BamHI.

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*- Δ stop) 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 Sall/XhoI and cloned into pC1300intB-35SnosBK-TAP. The *ORA59*-TAP fusion was excised with Sall/Spel from pC1300intB-35SnosBK-*ORA59*-TAP and introduced into the binary vector pER8 (Zuo et al., 2000) digested with XhoI/Spel. The *PDF1.2* promoter derivatives SF, mSF and dmSF fused to *GUS* were cloned into binary vector pMOG22 λ CAT (Menke et al., 1999) with XbaI/XhoI. The pMOG22 λ CAT 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

ORA59 and ERF1 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 Sall and cloned in pASK-IBA45 (IBA Biotagology, 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 Sall and cloned in pASK-IBA45. 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-Aldrich 1:10000) antibody and 5% nonfat dry milk as blocking agent. Plant proteins were extracted by grinding frozen tissue samples (0.2 g) in liquid nitrogen and thawing the powder in 0.25 ml protein extraction buffer (PBS buffer; 137 mM NaCl, 27 mM KCl, 100 mM NaHPO₄, 2 mM K₂HPO₄, pH 7.4, 1x Complete protease inhibitor Cocktail (Roche) and 0.5% Triton X100). After centrifugation at 15000 x g for 10 min at 4°C, supernatants were transferred into 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 (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% H₂O₂) 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, mSF and dmSF were isolated from the GusXX plasmid with BamHI and Sall and labeled by filling in the overhangs with the Klenow fragment of DNA polymerase I and α -³²P-dCTP. DNA binding reactions containing 0.1 ng of end-labeled DNA probe, 500 ng of poly(dAdT)-poly(dAdT), binding buffer (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% glycerol), and protein extract in a 10 µl volume, were incubated for 30 min at room temperature before loading on 5% w/v acrylamide/bisacrylamide (37:1)-0.5x Tris-Borate-EDTA gels under tension. After electrophoresis at 125 V for 1 hour, gels were dried on Whatman DE81 paper and exposure to Fuji X-ray films.

Transient expression assays

Protoplasts prepared from *Arabidopsis thaliana* cell suspension ecotype Col-0 were co-transformed with plasmids carrying one of the *PDF1.2*-promoter-*GUS* versions, effector plasmids carrying *ORA59*, *ERF1* or *ORA47* fused to the CaMV 35S promoter and the p2rL7 plasmid (De Sutter et al., 2005) carrying the *Renilla reniformis luciferase* (*LUC*) gene under the control of the CaMV 35S promoter. As controls, co-

transformations of *PDF1.2*-promoter-*GUS* with the empty pRT101 expression vector and the p2rL7 plasmid were carried out. Protoplasts were transformed using 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 hrs after transformation and were frozen in liquid nitrogen. GUS and LUC activity assays were performed as described (van der Fits and Memelink, 1997; Dyer et al., 2000). GUS activities were related to LUC activities in the same samples to correct for differences in transformation and protein extraction efficiencies. In experiments using a single reporter gene average GUS/LUC ratios from triplicate experiments were expressed relative to the respective vector controls.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed according to Bowler et al. (2004) with some modifications. Two grams of 2 weeks-old seedlings constitutively overexpressing TAP (line #7) or seedlings from XVE-ORA59-TAP transgenic line #4 treated with 0.1% DMSO or 4 μM estradiol for 16 hours in liquid MA medium were harvested. A small part of the samples was used for mRNA and protein detection. The rest was infiltrated with 1% formaldehyde to crosslink protein and DNA and chromatin sonicated to an average size of 400 bp was prepared. IgG Sepharose 6 fast flow (GE Healthcare) preabsorbed with salmon sperm DNA (0.1 mg/ml) and BSA (1 mg/ml) in ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl) was added to chromatin preparations and the mixtures were rotated at 4 °C for 6 hrs to bind TAP or TAP-fusion 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. 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 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.

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Chapter 3

Jasmonic acid induces stabilization and nuclear localization of ORA59, an AP2/ERF-domain transcription factor essential for defense responses in Arabidopsis

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Abstract

Plant defense against pathogens and herbivores depends on the action of several endogenously produced hormones, including jasmonic acid (JA) and ethylene. In certain defense responses, JA and ethylene signaling pathways synergize to activate a specific set of defense genes. The AP2/ERF-domain transcription factor ORA59 acts as the integrator of the JA and ethylene signalling pathways. How JA and ethylene affect the activity of ORA59 is not known. The aim of the studies reported here was to determine whether JA has an activating effect on ORA59 at the protein level. The results show that JA caused stabilization as well as nuclear localization of ORA59. Domain mapping of ORA59 showed that stabilization and nuclear localization were conferred by more than one single domain. Interestingly, nuclear localization of ORA59 did not require a functional COI1 protein. We postulate that there is a jasmonate receptor distinct from COI1, an F-box protein that targets ORA59 for degradation, and a repressor protein that keeps ORA59 in the cytoplasm.

Introduction

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

JA is one of a group of related signalling molecules called jasmonates which are involved in defense against wounding, herbivores and necrotrophic pathogens (Turner et al., 2002). Several components of the JA signal transduction pathway have been characterized. The JA-insensitive *coi1-1* mutant is affected in a gene encoding an F-box protein that forms part of an SCF-type E3 ubiquitin ligase complex (Xu et al., 2002; Devoto et al., 2002). COI1 interacts with members of a family of repressor proteins called JAZ (Thines et al., 2007; Chini et al., 2007). Several members of this family were shown to also interact with the JA-responsive transcription factor AtMYC2 (Chini et al., 2007). In response to biologically active jasmonates the repressor proteins are rapidly degraded (Thines et al., 2007; Chini et al., 2007), which is proposed to lead to derepression of AtMYC2 activity.

Another set of genes distinct from those regulated by AtMYC2 is synergistically induced by JA in combination with the stress hormone ethylene (Penninckx et al., 1996; Lorenzo et al., 2003, 2004). The transcription factors ORA59 and ERF1 have been suggested to act as integrators of JA and ethylene signaling pathways in Arabidopsis to

control this gene subset (Lorenzo et al., 2003; Pr , 2006). Overexpression of *ORA59* as well as *ERF1* activates the expression of several defense-related genes including *plant defensin1.2* (*PDF1.2*; Lorenzo et al., 2003; Pr , 2006) and confers resistance to the necrotrophic fungus *Botrytis cinerae* (Berrocal-Lobo et al., 2002; Pr , 2006). Analysis of plants where *ORA59* expression is knocked out by RNAi shows that the JA- and ethylene-responsive expression of defense genes including *PDF1.2* is not controlled by *ERF1* as previously reported (Lorenzo et al., 2003), but instead by the related transcription factor *ORA59* (Pr , 2006). Expression of *ORA59* (Atallah, 2005; Pr , 2006) and the subset of genes controlled by *ORA59* including *PDF1.2* is also dependent on *COI1* (Lorenzo et al., 2003; Pr , 2006). However *ORA59* is not known to interact with members of the JAZ family of repressors.

The aim of the studies reported here was to determine whether JA has an activating effect on *ORA59* at the protein level. The results show that JA caused stabilization as well as nuclear localization of *ORA59*. Interestingly, nuclear localization of *ORA59* did not require a functional *COI1* protein.

Results

***PDF1.2* is not an immediate-early JA-responsive gene**

As a first step, we wanted to get some indication that JA induces *PDF1.2* gene expression by activating the transcription factor *ORA59*, for example via covalent modifications or protein-protein interactions. Therefore we determined whether *PDF1.2* is a primary JA-responsive gene. Primary response genes generally do not require de novo protein synthesis, because the signal activates pre-existing regulatory proteins including transcription factors active in the signal transduction pathway (Pauw and Memelink, 2005). Fourteen-days old seedlings were treated with JA alone or in combination with the ethylene releasing compound ethephon in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX). As shown in Figure 1, *PDF1.2* expression was induced by JA alone and super-induced by JA and ethephon consistent with a previous report (Penninckx et al., 1998). CHX completely abolished this response, indicating that the expression of *PDF1.2* in response to JA and ethephon requires de novo protein expression. *PDF1.2* is therefore not an immediate-early response gene. *ORA59* on the other hand is an immediate-early response gene, since its expression in response to JA or JA/ethephon treatment was not negatively affected by CHX. In fact CHX alone induced *ORA59* mRNA accumulation, and in combination with JA or

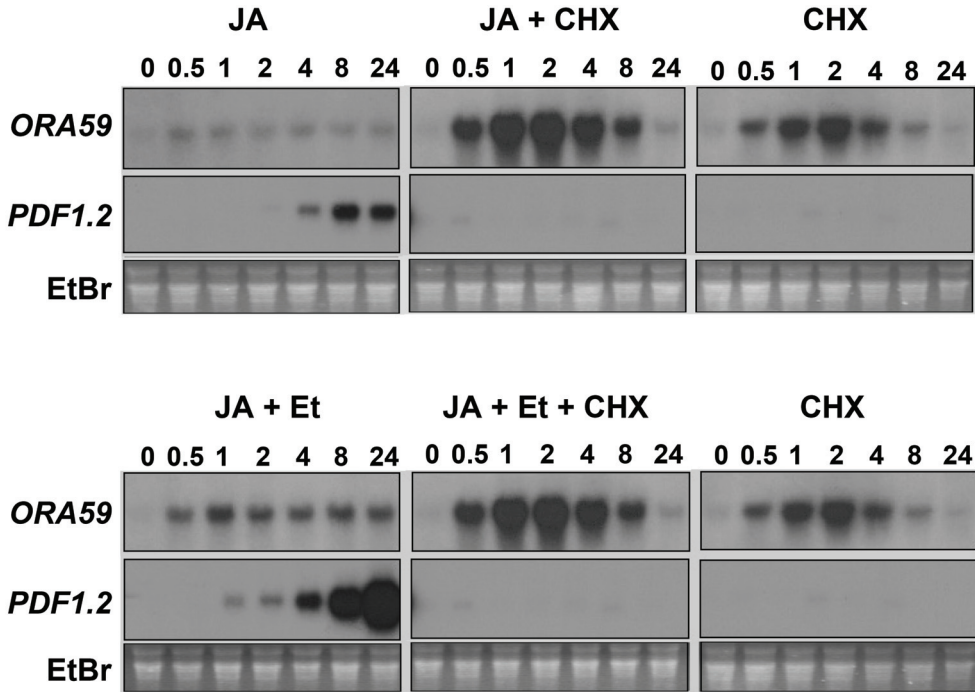


Figure 1. *PDF1.2* is not an immediate-early JA-responsive gene. Fourteen-days old seedlings were treated with JA alone or in combination with the ethylene-releasing agent ethephon (Et) in the presence or absence of cycloheximide (CHX) for number of hrs as indicated. All panels hybridized with the same probe were on the same blot and exposed to film for the same time, therefore signal intensities can be directly compared. The ethidium bromide (EtBr) stained gel is shown as a control for RNA loading.

JA/ethephon super-induction of mRNA accumulation was observed. (Super)-induction by CHX is commonly observed with immediate-early response genes in mammalian cells (Edwards and Mahadevan, 1992), and is usually attributed to decreased mRNA degradation.

ORA59 accumulates in the nucleus in response to JA

The previous result is not in favour of a mechanism where ORA59 activity is affected at the post-translational level by JA. In fact, the CHX experiments indicate that JA switches on *PDF1.2* expression by inducing *ORA59* gene expression resulting in an increase in ORA59 protein abundance. However such a scenario does not exclude that JA also affects ORA59 activity at the protein level. Of all possible changes in transcription factor activity we decided to study nuclear localization and protein stability since these are two prominent mechanisms whereby transcription factor activity is regulated (Vom Endt et al., 2002). Nuclear localization was studied by expressing ORA59 fused N-terminally or C-terminally to green fluorescent protein (GFP) in Arabidopsis cell suspension protoplasts and observing localization by confocal laser scanning microscopy.

The first remarkable observation was that N- (Figure 2A) and C-terminal (data not shown) ORA59-GFP fusions showed a similar localization as GFP alone in untreated protoplasts in both the cytoplasm as the nucleus. In contrast, several other AP2-domain transcription factors tested (data not shown) including ORA37 (Figure 2A) were nuclear localized. Even ERF1, which is closely related to ORA59, and which can also switch on *PDF1.2* expression when overexpressed (Lorenzo et al., 2003; Pr , 2006), was constitutively localized in the nucleus (Figure 2A). With ERF1, about 30% of the cells showed a homogeneous nuclear localization (top ERF1 panel in Figure 2A), whereas 70% of the protoplasts showed a spotted nuclear localization with at least one spot (bottom ERF1 panel in Figure 2A).

The second remarkable observation was that when protoplasts transformed with either GFP-ORA59 (Figure 2A) or ORA59-GFP (Figure 2B) expression plasmids (Figure 2A) were treated with JA for 4 hrs, the GFP fusion protein was nuclear localized in 20-30 % of the transformed cells. Nuclear localization was never observed in untreated or DMSO-treated protoplasts. Nuclear localization was relatively slow with most of the fusion protein in the nucleus after 1-2 hrs (Figure 2B).

To show unequivocally that the GFP-ORA59 fusion protein accumulated inside the nucleus and not outside around the nuclear membrane, the plasmid was co-transformed with a plasmid carrying a fusion between *Discosoma sp.* red fluorescent protein (DsRFP) and the nuclear tobacco (*Nicotiana tabacum*) protein NtKIS1a (Jasinski et al., 2002). As shown in Figure 2C NtKIS1a-DsRFP and ORA59-GFP showed complete overlap in nuclear localization when co-expressed in protoplasts that show nuclear localization of ORA59-GFP in response to JA.

To study whether this nuclear relocation is a peculiarity of Arabidopsis protoplasts or a more general phenomenon, the localization of the GFP-ORA59 fusion was compared to GFP fusions with ORA37 or ERF1 in bombarded *Catharanthus roseus* suspension cells with and without treatment with methyl-jasmonic acid (MeJA). As in Arabidopsis protoplasts, the GFP-ORA59 fusion protein showed a similar localization as GFP alone in *Catharanthus* cells, and the ORA37 and ERF1 fusion proteins were constitutively nuclear (Figure 3). Treatment with MeJA for 2 hrs resulted in nuclear localization of the GFP-ORA59 fusion in the transformed cells (Figure 3). This shows that nuclear localization of ORA59 in response to jasmonates occurs both in protoplasts and in cells via a mechanism that is conserved across plant species.

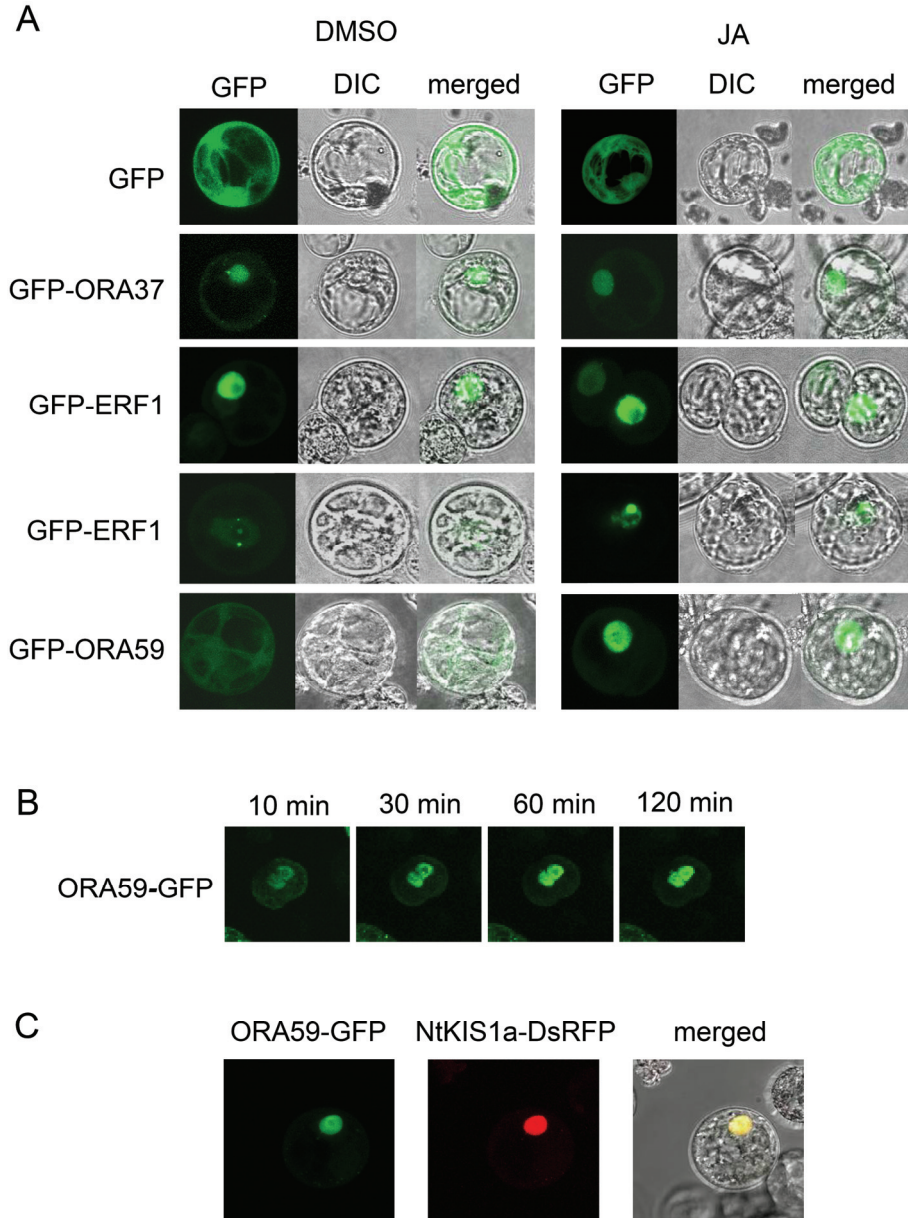


Figure 2. ORA59 accumulates in the nucleus of Arabidopsis protoplasts in response to JA. **(A)** GFP, GFP-ORA37, GFP-ERF1, or GFP-ORA59 constructs were transformed to Arabidopsis cell suspension protoplasts and examined by confocal laser scanning microscopy after treatment with 50 μ M JA or 0.1% (v/v) DMSO as indicated for 4 hrs. Confocal microscopic images are shown at the left (GFP), the corresponding differential interference contrast (DIC) images are in the middle and the merged images are at the right. **(B)** Time-lapse confocal laser scanning microscopy of ORA59-GFP in individual protoplasts. Projections of series of confocal optical sections are shown at each time point. Protoplasts were treated with 50 μ M of JA. **(C)** Confocal laser scanning microscopic images of Arabidopsis protoplasts transformed simultaneously with NtKIS1a-DsRed and ORA59-GFP expression plasmids taken after treatment with 50 μ M JA or 0.1% DMSO control for 4 hr.

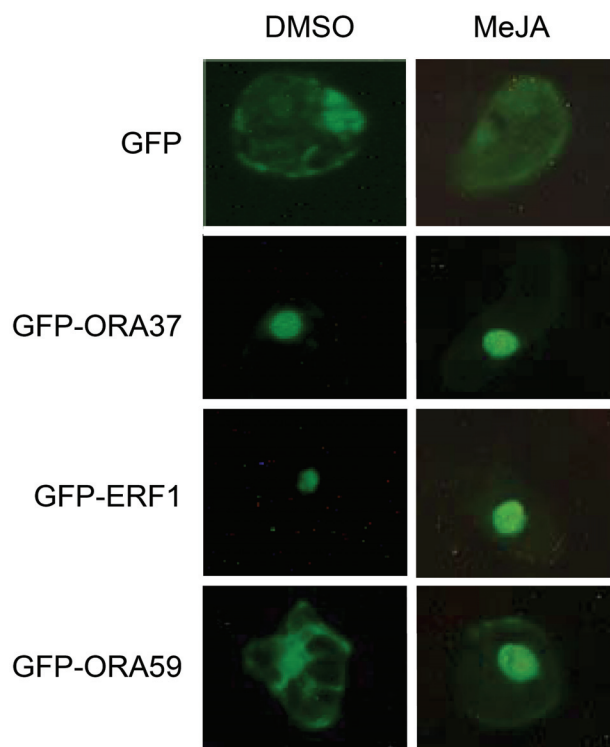


Figure 3. ORA59 accumulates in the nucleus of *Catharanthus* cells in response to MeJA. GFP, GFP-ORA37, GFP-ERF1 and GFP-ORA59 expression plasmids were transformed to cell suspension cells of *Catharanthus roseus* and examined by confocal laser scanning microscopy after treatment with 100 μ M methyl-jasmonic acid (MeJA) or 0.1% DMSO for 2 hrs.

GFP-transcription factor fusions are functional in transcriptional activation assays

To demonstrate that the observed re-localization of ORA59 reflects a property of a functional transcription factor, we determined the ability of GFP fusion proteins to trans-activate the *PDF1.2* promoter in *Arabidopsis* cell suspension protoplasts. As shown in Figure 4, the fusion proteins of ORA59 and GFP trans-activated the *PDF1.2* promoter to a similar level as the unfused ORA59 protein. The GFP-ERF1 fusion was less active than the unfused protein, but still gave a considerable stimulation of *PDF1.2* promoter activity. These results show that the GFP fusion proteins were functionally active as transcription factors.

JA-responsive ORA59 nuclear localization is independent of COI1

We examined the role of COI1, an important component of JA signal transduction, in the JA-induced nuclear accumulation of ORA59. The ORA59-GFP construct was introduced in wildtype leaf protoplasts and protoplasts derived from the mutant *coi1-1*. In *coi1-1* leaf protoplasts, the ORA59-GFP fusion protein re-localized to the nucleus in response to JA in a similar percentage of the cells as in wild-type protoplasts (Figures 6A and 6B), indicating that JA-induced nuclear accumulation of ORA59 did not require the COI1 protein.

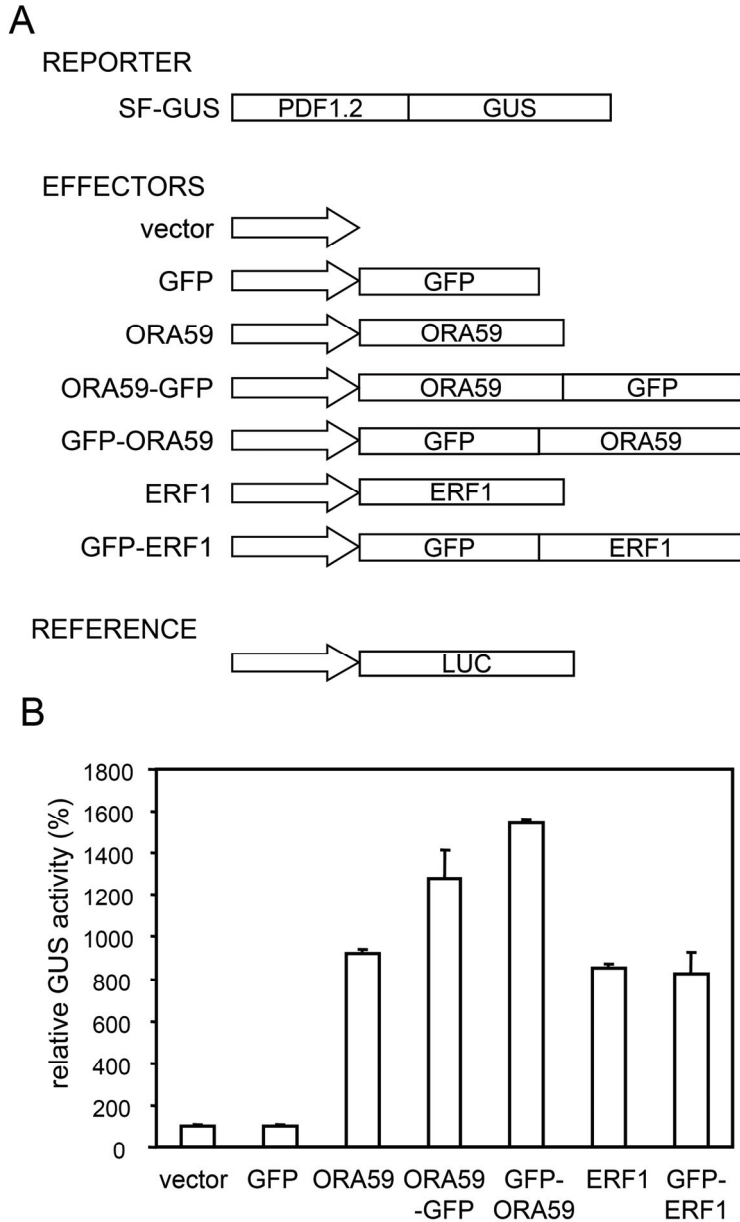


Figure 4. GFP-transcription factor fusions are functional in transcriptional activation assays in Arabidopsis protoplasts. **(A)** Constructs used in experiments with Arabidopsis cell suspension protoplasts. The reporter construct consisted of the *GUS* gene driven by *PDF1.2* promoter derivative SF. The effector constructs consisted of an expression vector carrying the CaMV 35S promoter (arrow) without or with the *ORA59* or *ERF1* cDNAs alone or fused to GFP. 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. **(B)** Arabidopsis protoplasts were co-transformed with the reporter construct, one of the effector plasmids and the reference plasmid. Bars represent average GUS/LUC ratios from triplicate experiments $\pm$ SE expressed relative to the vector control set at 100%.

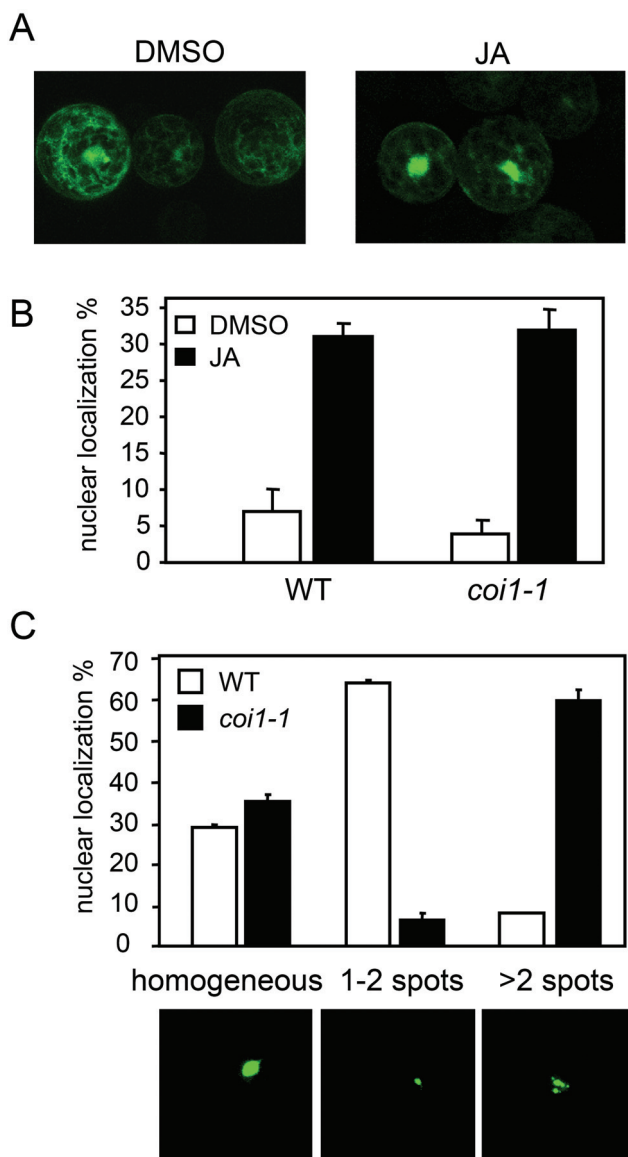
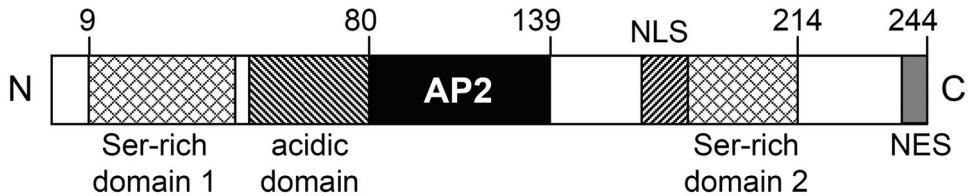


Figure 5. JA-responsive ORA59 nuclear localization is independent of COI1. **(A)** The ORA59-GFP expression plasmid was transformed to *coi1-1* leaf protoplasts and cells were examined by confocal laser scanning microscopy after treatment with 50 μ M JA or 0.1 % DMSO for 2 hrs. **(B)** Nuclear localization of ORA59-GFP in wild-type and *coi1-1* leaf protoplasts expressed as the percentage of cells showing nuclear localization relative to the total number of GFP-expressing cells analyzed by confocal laser scanning microscopy. For each data point at least 100 GFP-expressing protoplasts were analyzed. The experiment was repeated twice with similar results. **(C)** Localization of ERF1 in the nucleus is COI1-dependent. Distribution of GFP-ERF1 in wild-type and *coi1-1* leaf protoplasts as determined by confocal laser scanning microscopy. For each data point at least 100 GFP-expressing protoplasts were analyzed. The experiment was repeated twice with similar results. Below the graphs representative images of examples of the three different categories of nuclear localization of GFP-ERF1 are shown.

In contrast to ORA59, we observed that compartmentalization of GFP-ERF1 in the nucleus was changed in the *coi1-1* mutant protoplasts. In wild type protoplasts, 60% of the transformed cells showed one fluorescent spot in the nucleus and less than 10% displayed more than one spot (Figure 6C). In *coi1-1* mutant protoplasts the majority (60%) showed more than one fluorescent spot in the nucleus.

A



B

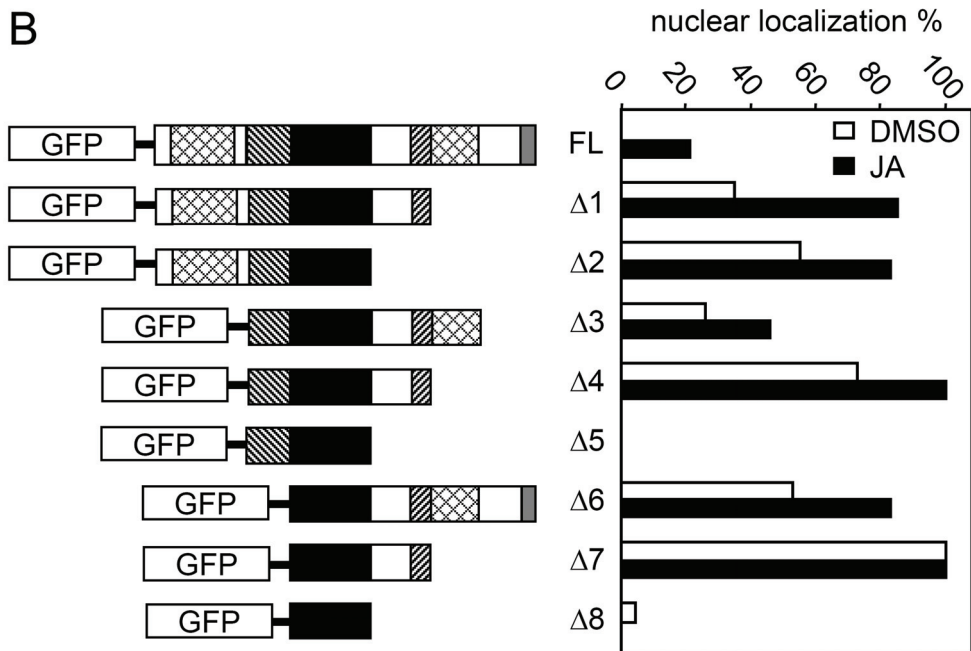


Figure 6. Mapping the ORA59 domain responsible for JA-responsive nuclear localization. **(A)** Schematic overview of domains in the ORA59 protein, which contains two Serine-rich domains, an acidic domain, the AP2 domain, a putative bipartite nuclear localization signal (NLS) and a putative nuclear export signal (NES). **(B)** Sixteen hrs after transformation Arabidopsis cell suspension protoplasts transformed with the indicated ORA59 deletion derivatives fused to GFP were treated with 0.1% DMSO or 50 μ M JA. Values represent cells with nuclear localization as a percentage of the total number of GFP-expressing cells analysed by confocal laser scanning microscopy. For each data point at least 100 GFP-expressing protoplasts were analyzed. The experiment was repeated twice with similar results.

Mapping the ORA59 domain responsible for JA-responsive nuclear localization

We attempted to map the domain responsible for JA-responsive nuclear localization by analyzing nuclear/cytoplasmic distribution of a range of deletion derivatives. Domains with distinct features derived from in silico analysis of the ORA59 protein are shown in Figure 6A. The full-length ORA59 protein with GFP fused at its N-terminal end localized to the nucleus in about 20 % of JA-treated cell suspension protoplasts, whereas nuclear localization was never observed in untreated protoplasts (Figure 6B). ORA59 contains a putative bipartite nuclear localization signal (NLS) flanking the C-terminal end of the AP2 DNA-binding domain. Derivatives $\Delta 5$ and $\Delta 8$ lacking the NLS showed a dramatic reduction in nuclear localization. Constructs $\Delta 1$, $\Delta 2$, $\Delta 3$ $\Delta 4$, and $\Delta 6$ showed a qualitatively similar pattern of nuclear localization with a dramatically enhanced nuclear localization without JA treatment in 20-75 % of the cells and some stimulation of nuclear localization by JA. This indicates that all these deletion derivatives lack a critical domain that keeps the protein in the cytoplasm in the absence of JA. In addition these deletion derivatives still contain a domain that can confer increased nuclear localization in response to JA. Deletion $\Delta 7$ was constitutively nuclear localized.

The conclusion from these experiments is that there is not a single domain responsible for cytoplasmic retention, since this function is present both in the N-terminal and the C-terminal part of the protein (compare for example $\Delta 1$ and $\Delta 6$). In addition there is also not a single domain responsible for JA-responsive nuclear localization since this function is also present both N-terminally and C-terminally. The Ser-rich domains could be candidates for cytoplasmic retention. A very strange observation was the localization of $\Delta 2$, which lacks the NLS but could still localize to the nucleus. To make it even stranger, this deletion derivative was inactive as a transcriptional activator of the *PDF1.2* promoter (Figure 10), although it was nuclear localized.

Inhibition of nuclear export leads to nuclear localization of ORA59

Many nuclear proteins shuttle between the cytoplasm and the nucleus via interaction with nuclear import and export receptors which interact with NLS and nuclear export signals (NES), respectively. The presence of a putative NLS and a putative NES at the C terminal extremity suggest that ORA59 might also shuttle between the cytoplasm and the nucleus. To test this hypothesis we used leptomycin B (LMB), which is a specific inhibitor of the major nuclear export receptor CRM-1. Treatment of protoplasts transformed with ORA59-GFP with LMB for 3 hrs increased the proportion of cells showing nuclear localization to 100% (Figure 7A). Recompartmentalization was clearly visible after 30 min of incubation in the presence of

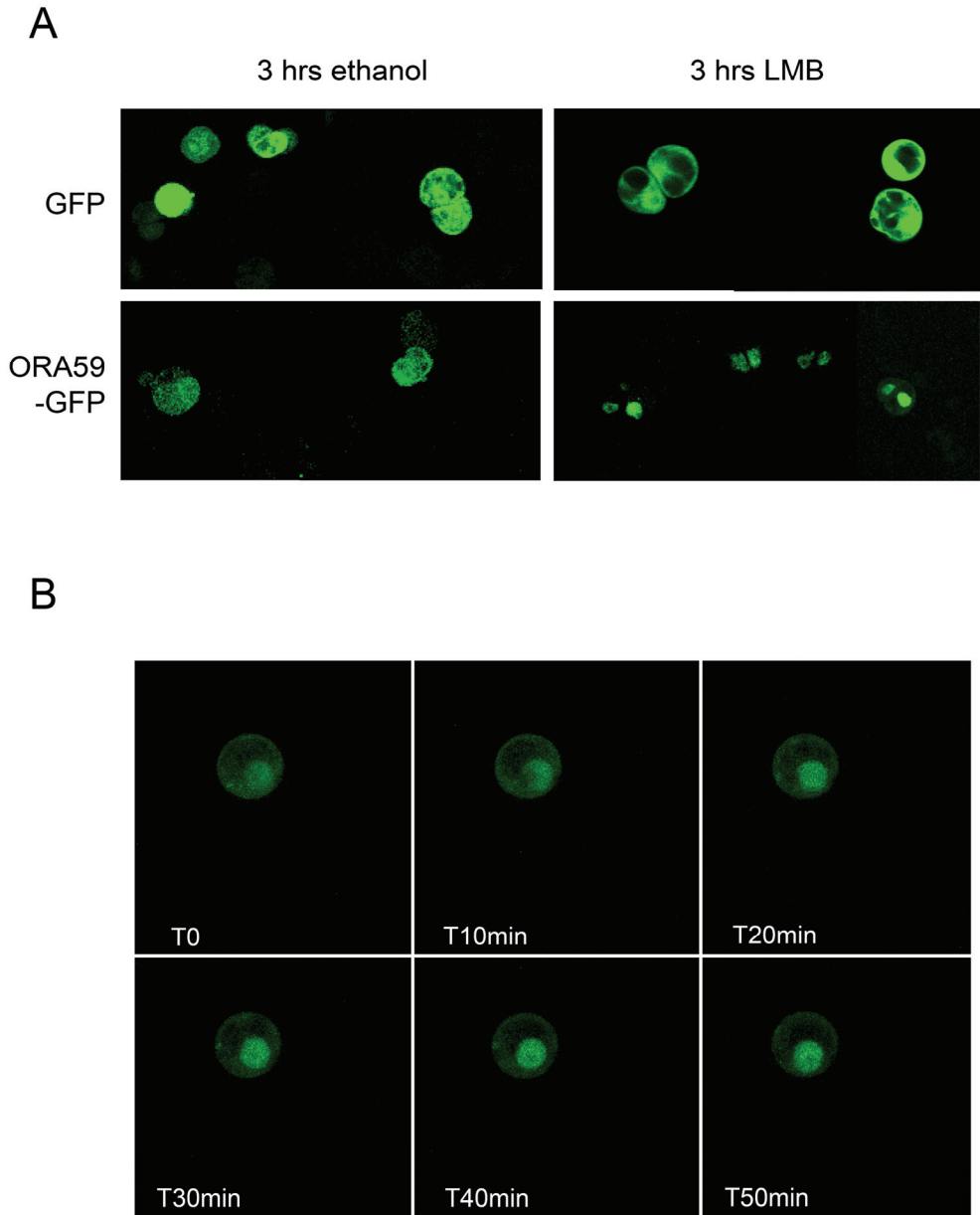


Figure 7. Inhibition of nuclear export leads to nuclear localization of ORA59. **(A)** Confocal laser scanning microscopy images of Arabidopsis cell suspension protoplasts expressing ORA59-GFP or GFP. Sixteen hrs after transformation with expression plasmids protoplasts were treated for 3 hrs with 2 μ M of the nuclear export inhibitor leptomycin B (LMB) or with the solvent ethanol at a final concentration of 0.5% (v/v). **(B)** Time-lapse confocal laser scanning microscopy of an individual protoplast expressing ORA59-GFP after treatment with 2 μ M LMB.

LMB and was nearly complete after 50 min (Figure 7B). This indicates that indeed ORA59 shuttles between cytoplasm and nucleus. JA could therefore cause re-localization of ORA59 either by unmasking the NLS or by hiding the NES.

JA stabilizes the ORA59 protein

A prominent mechanism of regulating the activity of a protein in a cell is by regulating its abundance. Especially ubiquitin/proteasome-mediated degradation emerged in the past decade as a predominant mechanism for regulating the activity of proteins including transcription factors (Bach and Ostendorff, 2003). Therefore we asked the question whether JA affected the level of ORA59 protein post-translationally. Protoplasts were co-transformed with a GFP expression plasmid and a plasmid expressing the ORA59-GFP fusion and were treated for 4 hrs with JA or the solvent DMSO. Immunoblot analysis of total cellular protein with anti-GFP antibodies revealed that JA caused an increase in the amount of ORA59-GFP protein (Figure 8A). The amount of GFP, expressed from the same version of the CaMV 35S promoter, was not affected, demonstrating that the effect of JA on ORA59-GFP protein abundance did not occur at the transcriptional level.

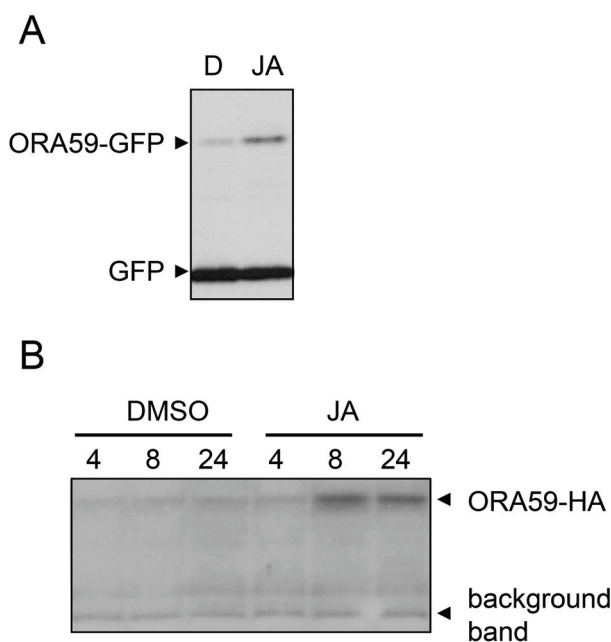


Figure 8. JA stabilizes the ORA59 protein. **(A)** Immunoblot analysis with anti-GFP antibodies of total protein extracts prepared from Arabidopsis cell suspension protoplasts co-expressing GFP and ORA59-GFP treated with 50 μ M JA or 0.1% DMSO for 4 hrs. **(B)** Immunoblot analysis with anti-HA antibodies of total protein extracts from 14-days-old plants containing the XVE-ORA59-HA construct (line #18) treated simultaneously with 2 μ M estradiol and 50 μ M JA or 0.1% DMSO. Intensities of the background band confirm equal protein loading.

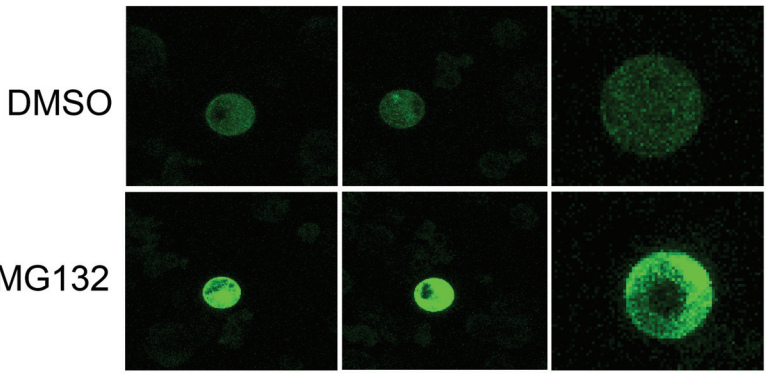
To connect this observation in protoplasts to processes occurring in whole plants we monitored the levels of ORA59 protein in transgenic plants treated with JA for different periods of time. Fourteen days-old T2 seedlings from two transgenic *Arabidopsis* lines expressing ORA59 tagged C-terminally with the influenza hemagglutinin (HA) epitope in an estradiol-inducible manner (XVE-ORA59-HA) were treated with estradiol for 24 hrs in presence or absence of JA. Control samples were treated with the solvent DMSO. Treatment with estradiol alone strongly induced the expression of *PDF1.2* (data not shown), demonstrating that the ORA59-HA fusion protein was functional as a transcriptional activator. As shown in Figure 8B, the level of ORA59-HA markedly increased after 8 and 24 hrs of JA treatment. Although *PDF1.2* expression was induced in DMSO-treated plants as a result of the simultaneous estradiol treatment (data not shown), a significant increase in ORA59-HA protein was not detected (Figure 8B). This also suggests that in the absence of JA ORA59 was rapidly turned over.

ORA59 is degraded by the 26S proteasome

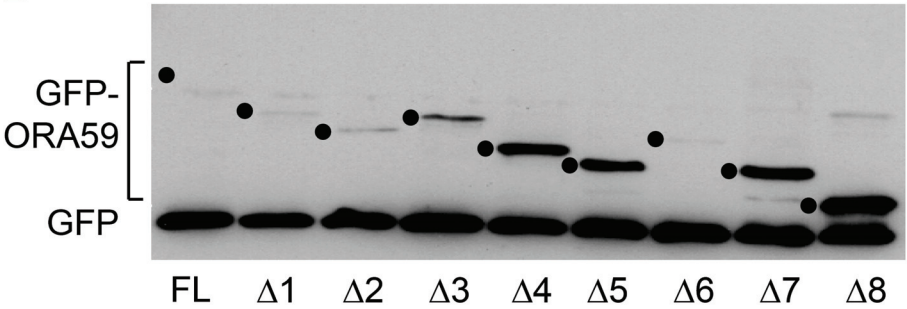
To test whether the low levels of ORA59 in DMSO treated protoplasts might be due to ORA59 degradation mediated by the 26S proteasome, we tested the effects of the proteasome inhibitor MG132 on ORA59 protein accumulation. *Arabidopsis* protoplasts co-transformed with GFP-ORA59 and GFP expression plasmids were incubated in the dark for 16 hrs and then treated with MG132 or with the solvent DMSO for 4 hrs. Fluorescence microscopy showed that MG132, but not DMSO, increased nuclear and cytosolic abundance of GFP-ORA59 in protoplasts (Figure 9A). Total protein was extracted and subjected to immunoblot analysis with anti-GFP antibodies. As shown in Figure 9C (upper left panel), MG132 treatment drastically increased GFP-ORA59 accumulation in protoplasts, indicating that GFP-ORA59 protein is subject to 26S proteasome-mediated degradation. Similar results were obtained with the stably transformed plant lines expressing ORA59-HA (data not shown).

We attempted to map the domain responsible for proteasome-mediated degradation (the “degron”) by analysing the stabilization of different deletion derivatives fused to GFP by treatment with MG132. In Figure 9B protein extracts from untreated protoplasts were run on the same gel allowing direct comparison of steady-state protein amounts. Full-length ORA59 was undetectable. All deletion derivatives were more stable than the full-length protein, indicating that there is not a single degron. Low amounts were detected of deletion derivatives $\Delta 1$, $\Delta 2$, and $\Delta 6$. Deletion derivatives $\Delta 4$, $\Delta 5$, $\Delta 7$ and $\Delta 8$ were almost as stable as GFP. This indicates that the Ser-rich domains are responsible for instability of ORA59. Next protein amounts were analyzed after treatment of transformed protoplasts with MG132

A



B



C

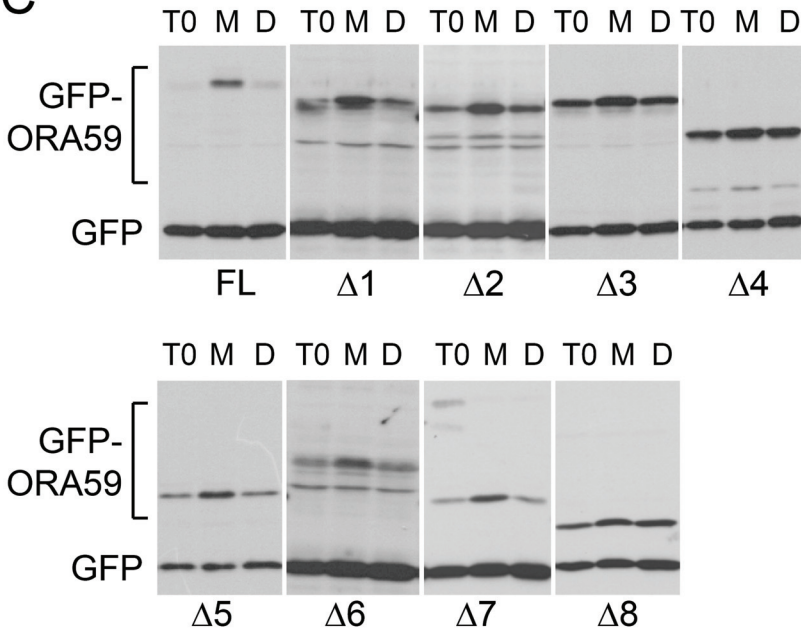


Figure 9. ORA59 is degraded by the 26S proteasome. **(A)** Confocal laser scanning microscopy images of Arabidopsis cell suspension protoplasts expressing GFP-ORA59. Sixteen hrs after transformation protoplasts were treated for 2 hrs with 50 μ M MG132 or 0.1% DMSO. Pictures taken at identical confocal laser scanning microscope settings of three representative protoplasts are shown for each treatment. **(B)** Steady-state protein amounts of various ORA59 deletion derivatives in Arabidopsis cell suspension protoplasts 16 hrs after co-transformation with a GFP expression plasmid. Proteins were detected with anti-GFP antibodies. Positions of the GFP-ORA59 fusion proteins are indicated with dots. **(C)** Mapping of the degron in ORA59. Immunoblot analysis with anti-GFP antibodies of total protein extracts from Arabidopsis cell suspension protoplasts co-expressing GFP and deletion derivatives of ORA59 fused at their N-terminus to GFP. Arabidopsis protoplasts were harvested 16 hrs after transformation (T0) or treated for 4 hrs with the solvent DMSO at 0.1% (v/v) final concentration (D) or with 50 μ M of the 26S proteasome inhibitor MG132 (M). The upper band is the correct GFP-ORA59 fusion protein in those gels where multiple bands are visible in the region indicated with GFP-ORA59. Panels with different deletion derivatives were run on different gels and band intensities cannot be directly compared. Use Figure 9B for direct comparison of steady-state protein levels without treatment or use GFP band intensities to estimate relative amounts of the fusion proteins.

(Figure 9C). All deletion derivatives except GFP- Δ 8 were stabilized by MG132 to some degree. This shows that the AP2 domain does not harbour a degron function in contrast to all other regions present in the various derivatives. The other conclusion is that degradation did not occur uniquely in the nucleus since also deletion derivative GFP- Δ 5 that does not contain an NLS and did not accumulate in the nucleus (Figure 6B) was to some degree stabilized by MG132.

Functional mapping of the activation domain in ORA59

In many mammalian transcription factors the degron overlaps with the activation domain (Salghetti et al., 2000). In the “suicide” model degradation is proposed to be crucial for the transcription activating activity of the activation domain (Bach and Ostendorff, 2003). To determine whether a similar mechanism may apply for ORA59, we mapped the transcription activating domain in parallel with the mapping of the degron. As shown in Figure 10, deletion derivatives lacking the putative NLS were inactive in the trans-activation assay, indicating that indeed this sequence is a functional NLS. Construct Δ 7 consisting of the AP2 domain and the NLS has a very low trans-activating activity, demonstrating that this part of ORA59 does not contain an activation domain. As with the degron we did not find a single domain responsible for transcription activation. Transcription activation functions were found to be present in the N-terminal region as well as in the C-terminal region. By comparing the activities of the GFP fusion constructs Δ 3 and Δ 4 it appeared that the C-terminal Ser-rich region functions as an activation domain (Figure 10B). By comparing the activities of the GFP fusion constructs Δ 1 and Δ 4 it appeared that the N-terminal Ser-rich region functions as an activation domain. The acidic region functioned only as an activation domain when deletion derivative Δ 4 was not fused to GFP (compare to Δ 7; Figure 10A).

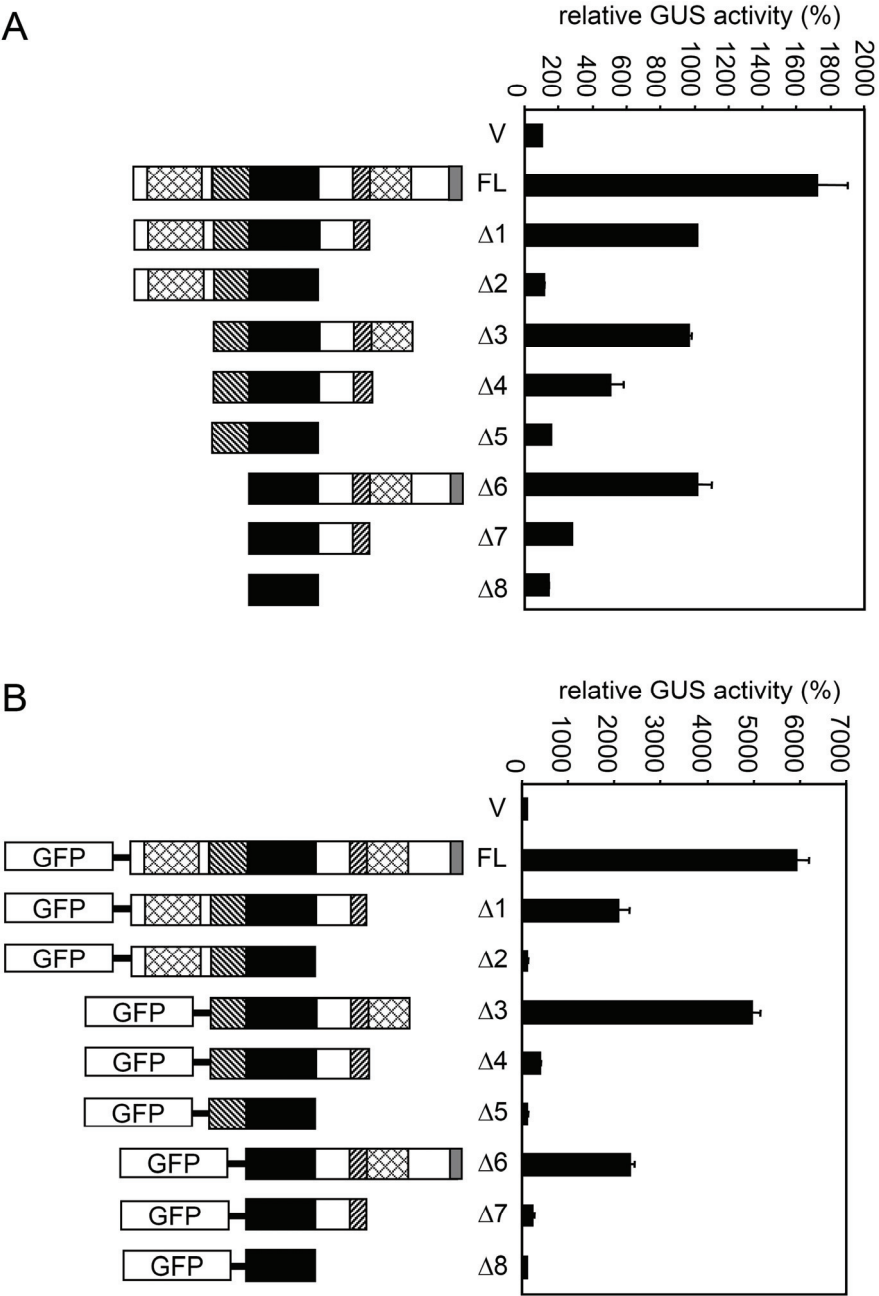


Figure 10. Functional mapping of the activation domain in ORA59. **(A)** Arabidopsis protoplasts were co-transformed with a *GUS* reporter gene driven by the SF derivative of the *PDF1.2* promoter and a CaMV 35S expression vector containing full-length (FL) *ORA59* or one of the deletion derivatives as indicated. A reference plasmid carrying the *Renilla LUC* gene fused to the CaMV 35S promoter was co-transformed in all experiments to correct for differences in transformation and protein extraction efficiencies. Bars represent average GUS/LUC ratios from triplicate experiments $\pm$ SE expressed relative to the vector control set at 100%. **(B)** Same as in (A) but with fusions between ORA59 deletion derivatives and GFP.

In general activities of GFP fusion proteins were similar to those of the unfused deletion derivatives, with the differences that $\Delta 3$ was relatively active as a GFP fusion, and that the activity of $\Delta 4$ was considerably lower as a GFP fusion. Interestingly the activation strength of ORA59 deletion derivatives was not related to their abundance in protoplasts. Full-length ORA59 fused to GFP for example was a strong activator (Figure 10B), whereas the protein was almost undetectable by Western blotting (Figure 9B). Deletion derivative $\Delta 4$ on the other hand was almost inactive as a transcription activator when fused to GFP (Figure 10B), whereas the protein was as abundant as the co-expressed GFP (Figure 9B).

Discussion

The goal of the studies described here was to get insight in JA signal transduction steps affecting ORA59 activity at the protein level. As a first step we determined whether the ORA59 target gene *PDF1.2* is an immediate-early JA-responsive gene. If so, it would indicate that pre-existing ORA59 protein is activated in response to JA. However it turned out that JA-responsive *PDF1.2* expression depended on de novo protein synthesis. It is possible that the missing protein which needs to be synthesized is ORA59 itself. If so, JA could regulate *PDF1.2* expression by simply increasing ORA59 abundance at the transcriptional level. In such a scenario ORA59 is formally not a component of JA signal transduction (Pauw and Memelink, 2005). However, our data show that JA directly affected ORA59 protein activity by inducing stabilization and nuclear localization, and this establishes ORA59 as a component of JA signal transduction. JA controls ORA59 at multiple levels via transcriptional induction and by acting on the de novo synthesized protein.

Domain mapping of ORA59 showed that stabilization and nuclear localization were conferred by more than one single domain. The conclusions from the different domain mapping experiments are summarized in Table 1. Transcriptional activation of the *PDF1.2* promoter required either the N-terminal or the C-terminal Ser-rich region. Similarly in untreated protoplasts either Ser-rich domain caused instability of the ORA59 deletion derivative resulting in low steady state protein levels. The full-length ORA59 protein containing both Ser-rich domains was by far the most unstable protein among all ORA59 derivatives analyzed.

For the interpretation of the nuclear localization studies comparison of ORA59 and ERF1 might be instructional. Both proteins act as transcriptional activators of the *PDF1.2* promoter (Pré, 2006). ERF1 was also stabilized by MG132 (data not shown), but was

Table 1. Summary of ORA59 domain mapping in different assays.

	act. <i>PDF1.2</i> unfused ^a	act. <i>PDF1.2</i> nGFP ^a	Stability ^b	stabilized by MG132 ^c	% nuclear DMSO	% nuclear JA
FL	+	+	-	+	0	21
Δ1	+	+	-	±	35	85
Δ2	-	-	-	±	55	83
Δ3	+	+	±	±	26	46
Δ4	±	-	+	-	73	100
Δ5	-	-	+	±	0	0
Δ6	+	+	-	±	53	83
Δ7	-	-	+	±	100	100
Δ8	-	-	+	-	5	0

^a Scores of transcriptional activating activity on the *PDF1.2* promoter as unfused proteins or as fusions with GFP at the N-terminal end.

^b Relative stability compared to co-expressed GFP in untreated protoplasts.

^c Scores whether MG132 caused an increase in protein amount compared to DMSO treatment.

constitutively nuclear localized. Comparison of the protein sequences shows that ORA59 and ERF1 share relatively high amino acid identity throughout their protein sequences except for the C-terminal Ser-rich domain, which is totally absent in ERF1. However in the nuclear localization studies the C-terminal Ser-rich domain did not emerge as a unique functional domain. Removal of either the N-terminal or the C-terminal region caused the constitutive nuclear accumulation of the deletion derivative in a significant fraction of the cells, something which was never observed for the full-length protein. The putative NES did also not play a unique role. Deletion derivative Δ6 contains the putative NES but was constitutively localized in the nucleus in more than half of the cells. This indicates that the N-terminal region is crucial for full cytoplasmic retention.

A highly surprising finding was that nuclear localization of ORA59 in response to JA did not require a functional COI1 protein. The COI1 protein is required for all known JA responses including induction of *ORA59* and *PDF1.2* gene expression (Lorenzo et al., 2003; Atallah, 2005; Pré, 2006). COI1 is very likely a jasmonate receptor which directly interacts with biologically active jasmonates (Thines et al., 2007). Upon binding of biologically active jasmonates COI1 interacts with the JAZ1 repressor and related JAZ proteins leading to their degradation (Chini et al, 2007; Thines et al., 2007) in a scenario which shows strong similarity with auxin-responsive degradation of AUX/IAA repressor proteins via the auxin receptor/F-box protein TIR1 (Guilfoyle, 2007). The fact that ORA59 relocalization is COI1-independent indicates that another JA receptor exists. The way in which this novel JA receptor functions could be similar to COI1 action on the JAZ family members. This receptor could direct the

degradation of a repressor protein which retains ORA59 in the cytoplasm by masking the NLS. Alternatively this putative receptor could activate a protein that masks the NES and due to disruption of the nuclear-cytoplasmic shuttling cycle ORA59 would accumulate in the nucleus.

Our favourite hypothesis is as follows. In the absence of JA signaling cellular ORA59 protein levels are kept low by recognition by a specific F-box protein and proteasome-mediated degradation. The low residual level of protein is retained in the cytoplasm by interaction with a repressor protein. Perception of a biologically active jasmonate by a receptor distinct from COI1 leads to disruption of the interactions between ORA59 and the repressor protein. JA perception by COI1 or the unknown receptor also disrupts the interaction with the F-box protein. Both events lead to increased amounts of ORA59 protein in the nucleus. One way to confirm this hypothesis is by identifying the F-box protein and the repressor protein, which might be feasible by yeast two-hybrid screening or TAP-tag purification of ORA59 complexes from plants.

Materials and Methods

Construct and transient expression assays

For the construction of GFP-ORA37 and GFP-ORA59, the *ORA37* and *ORA59* open reading frames (ORF) were excised from pIC20H-ORA37 and pBluescript SK+-ORA59 with BamHI and EcoRI/SpeI respectively and cloned into pTH2^{BN} digested with BamHI/BglII and EcoRI/SpeI respectively. pTH2^{BN} is a derivative of pTH2 (Niwa et al., 1999; Chiu et al., 1996) lacking the stop codon of GFP (Kuijt et al., 2004). For the construction of GFP-ERF1 the *ERF1* ORF was excised from pIC20H-ERF1 with BglII and cloned into pBluescript SK+ digested with BamHI, then *ERF1* was excised from pBluescript SK+-ERF1 with EcoRI/SpeI and cloned into pTH2^{BN} digested with EcoRI/SpeI to generate GFP-ERF1. ORA59 deletion derivatives Δ1 to Δ8 were amplified with pIC20H-ORA59 as template and primer sets 5'-CGG AAT TCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CGG GAT CCT TAT TTC TTC TTT CCT CTA GGA CG-3' for Δ1, 5'-CGG AAT TCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CGG GAT CCT TAG GGG AAA TTG AGT ACT GCG AGG-3' for Δ2, 5'-CGG AAT TCA AAA TGC CTA CTG ATA ACT ACT G-3' and 5'-CGG GAT CCT CAA GAA CAT GAT CTC ATA AG-3' for Δ3, 5'-CGG AAT TCA AAA TGC CTA CTG ATA ACT ACT G-3' and 5'-CGG GAT CCT TAT TTC TTC TTT CCT CTA GGA CG-3' for Δ4, 5'-CGG AAT TCA AAA TGC CTA CTG ATA ACT ACT G-3' and 5'-CGG GAT CCT TAG GGG AAA TTG AGT ACT GCG AGG-3' for Δ5, 5'-CGG AAT TCA AAA TGT CAT ACA GAG GAG TGA GG-3' and 5'-CGG GAT CCT CAA GAA CAT GAT CTC ATA AG-3' for Δ6, 5'-CGG AAT TCA AAA TGT CAT ACA GAG GAG TGA GG-3' and 5'-CGG GAT CCT TAT TTC TTC TTT CCT CTA GGA CG-3' for Δ7, 5'-CGG AAT TCA AAA TGT CAT ACA GAG GAG TGA GG-3' and 5'-CGG GAT CCT TAG GGG AAA TTG AGT ACT GCG AGG-3' for Δ8. All ORA59 deletion derivatives were cloned in pGEM-T Easy (Promega,

Madison, WI) and then inserts were excised with BamHI and EcoRI and and cloned in pRT101 (Töpfer et al., 1987). For N-terminal GFP fusions ORA59 deletion derivatives were excised from pGEM-T Easy with EcoRI and SpeI and cloned in pTH2^{BN}. The ORA59-GFP fusion was created by removal of the stop codon and in frame fusion with GFP. ORA59 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. The ORA59-ΔSTOP insert was excised with Sall/XhoI and cloned into pBluescript SK+ digested with Sall. Then ORA59-ΔSTOP was excised from pBluescript SK+ with Sall/EcoRI and cloned into pTH2^{SN} (another derivative of pTH2; Kuijt et al., 2004). ORA59-ΔSTOP was excised from pTH2^{SN} with Sall/NcoI and cloned into pTH2. Details about the cloning of ORA59 and ERF1 in pRT101 and about the fusion between the PDF1.2 promoter derivative SF and the GUS reporter gene are described in Chapter 2. Protoplasts were prepared from *Arabidopsis thaliana* cell suspension culture ecotype Col-0 as described (Axelos et al., 1992) with some modifications (Chapter 2) and were co-transformed with a reporter plasmid carrying PDF1.2-promoter-GUS, effector plasmids carrying ERF1, GFP-ERF1, ORA59, GFP-ORA59 or ORA59-GFP genes fused to the CaMV 35S promoter and the p2rL7 plasmid (De Sutter et al., 2005) carrying the *Renilla luciferase* (LUC) gene under the control of the CaMV 35S promoter. As controls, co-transformations of PDF1.2-promoter-GUS with the empty pRT101 or pTH2 vectors 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). Arabidopsis leaf protoplasts were prepared and transformed as described (Sheen, 2002). Protoplasts were harvested 18 hours after transformation and frozen in liquid nitrogen. GUS and LUC activity assays were performed as described by van der Fits and Memelink (1997) and Dyer et al. (2000) with minor modifications. GUS activities were related to LUC activities in the same samples to correct for differences in transformation and protein extraction efficiencies. Average GUS/LUC ratios from triplicate experiments were expressed relative to the respective vector controls.

Microscopy and treatments

Arabidopsis cell suspension protoplasts were transformed with 10 μg of GFP-fusion plasmid DNA, after which the protoplasts were incubated for at least 16 hours in the dark. *Catharanthus roseus* cell suspensions were grown and transformed by particle bombardment as described (van der Fits and Memelink, 1997). Confocal laser scanning microscopy was performed by placing the cells in a Lab-Tek II Chambered Coverglass (Nalge Nunc, International) and examination of GFP fluorescence using a Leica inverted microscope (IRBE) equipped with a Leica SP1 confocal scanhead with an argon laser. For visualization of GFP the excitation wavelength was 488 nm while the emitted fluorescence was collected between 500 to 550 nm. The resulting signal was amplified, digitalized and the consistent picture reconstituted using Leica software. Methyl-jasmonate (Bedoukian Research Inc.), JA (Sigma-Aldrich, St. Louis, MO), MG132 (Sigma-Aldrich) were diluted in dimethylsulfoxide (DMSO). Leptomycin B (Biomol) was diluted in ethanol.

Protein extraction

Protoplasts were ground in 50 µl of cold protein extraction buffer (50 mM HEPES-KOH pH 7.2, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 50 mM NaF, 1% Triton X-100, 1 mM Na₃VO₄, 5 µg/ml leupeptin, 5 µg/ml antipain, 5 mM DTT and 1 mM phenylmethylsulfonyl-fluoride (PMSF). After centrifugation at 12000 rpm for 15 min at 4 °C, supernatants were transferred into clean tubes, frozen in liquid nitrogen, and stored at -80 °C. Protein concentrations were determined using Bio-Rad protein assay reagent with bovine serum albumin as the standard.

Immunoblot analysis

Protein extracts were separated on 10% (w/v) SDS-PAA gels and transferred to Protran nitrocellulose (Schleicher&Schuell) by semidry blotting. After blocking 1 hr in Tris-buffered saline-Tween (TBST; 20 mM Tris-HCl pH 7.6, 140 mM NaCl and 0.05% Tween 20) with 5 % non-fat dry milk at room temperature, the Western blots were incubated overnight with anti-HA peroxidase antibodies (1:2000, Roche) or with anti-GFP antibodies (1:5000) in TBST with 3 % bovine serum albumin. After 1 hr incubation at room temperature the blots were washed 4 x with TBST. After incubation with anti-GFP antibodies, blots were incubated for 1 hr with anti-rabbit IgG antibodies linked to peroxidase (1:10000) in TBST and 5% non-fat dry milk, followed by 4 washings. Finally, the blots were incubated in 6 ml luminol solution (250 µM sodium luminol (Sigma), 0.1 M Tris-HCl pH 8.6, 0.01% H₂O₂) mixed with 60 µl enhancer solution (67 µM p-hydroxy coumaric acid (Sigma) in DMSO) to visualize the proteins by enhanced chemiluminescence detection using X-ray films (Fuji, Tokyo, Japan).

Biological materials, growth conditions and treatments

Arabidopsis thaliana wild-type plants, *coi1-1* mutant plants, and all transgenic plants are in the genetic background of ecotype Col-0. Seeds were surface-sterilized by incubation for 1 minute in 70 % ethanol, 15 minutes in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for three hours (<http://plantpath.wisc.edu/~afb/vapster.html>). Surface-sterilized seeds were transferred to 250 ml Erlenmeyer flasks containing 50 ml MA medium (Masson and Paszkowski, 1992) or grown on plates containing MA medium supplemented with 0.6% w/v agar. Transgenic plants from T2 or T3 generations were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin for ORA59-overexpressing plants. Following stratification for 3 days at 4 °C, seeds were incubated at 21 °C in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days or otherwise as indicated. Seeds in liquid medium were placed on a shaker at 120 rpm. Transgenic plants carrying an XVE expression module containing the *ORA59-HA* gene were treated with 2 µM estradiol (Sigma-Aldrich) dissolved in DMSO (0.1% final concentration). Seedlings were treated for different time periods with 50 µM JA (Sigma-Aldrich) dissolved in DMSO at a final concentration of 0.1%. Control seedlings were treated with 0.2% DMSO.

Binary constructs and plant transformation

For the construction of the XVE-*ORA59-HA* lines, the *ORA59-HA* cassette was created by removing the stop codon and in frame fusion with a double HA tag. *ORA59-HA* was amplified by PCR with the primer set 5'-GGG GTA CCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CGG GAT CCT TAA GCG TAA

TCT GGA ACA TCG TAT GGG TAA CCA GCG TAA TCT GGA ACA TCG TAT GGG TAG AGC TCT TGA GAA CAT GAT CTC ATA AG-3', and was first cloned as a KpnI/BamHI fragment into pRT101. The expression cassette was transferred as a XhoI/XbaI fragment to pER8 (Zuo et al., 2000) digested with XhoI/SpeI. The binary vector pER8-ORA59-HA was introduced into *A. 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.

RNA extraction and Northern blot analyses

For each treatment, 15 to 20 10-days-old seedlings were transferred from plates with solidified MA medium to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 mL MA medium and incubated on a shaker at 120 rpm for 4 additional days. Seedlings were first treated for 10 min with 100 μ M cycloheximide (CHX) dissolved in DMSO (0.1% final concentration) and then JA (Sigma-Aldrich) dissolved in DMSO at a final concentration of 50 μ M or JA combined with 1 mM of the ethylene releaser ethephon (Sigma-Aldrich) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) were added for times as indicated. Total RNA was extracted from frozen tissues by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% ethanol, and resuspended in water. As described by Memelink et al. (1994), 10 μ g RNA samples were subjected to electrophoresis on 1.5% w/v agarose/1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). All probes were ^{32} P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed to Fuji X-ray films.

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Chapter 4

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

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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 *AOC1*, 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,

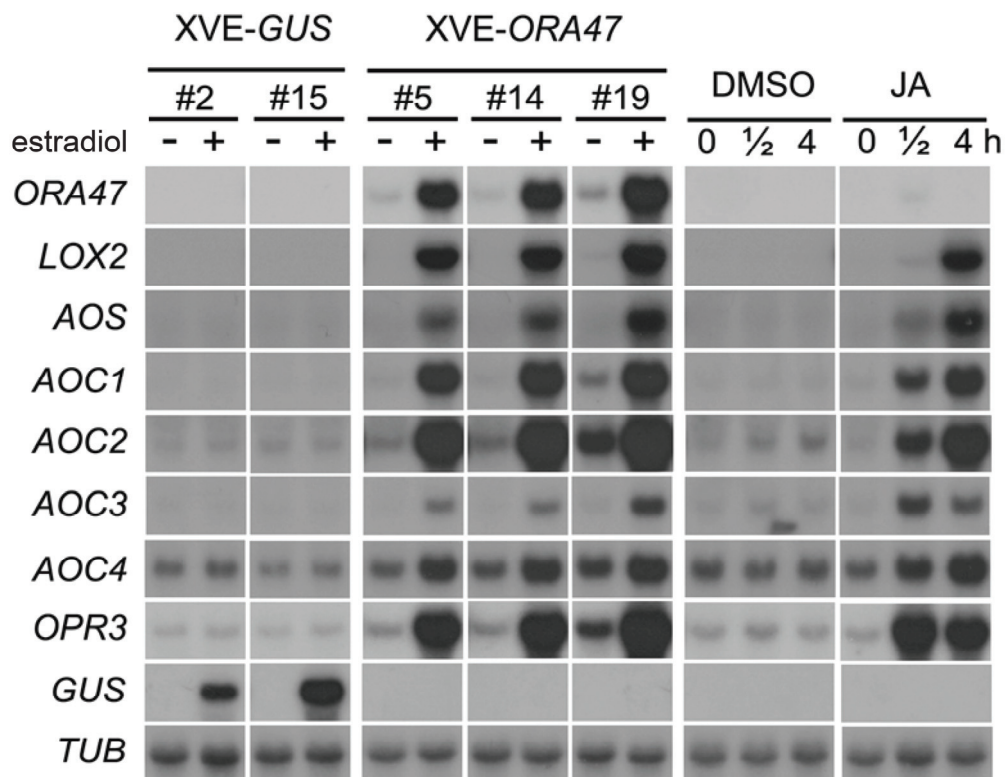


Figure 1. *ORA47* overexpression increases the expression of all four AOC gene family members. RNA gel blot analyses with two-weeks-old seedlings from three independent *XVE-ORA47* and two independent *XVE-GUS* lines treated for 24 hrs with 2 μ M estradiol (+) or with 0.1% v/v of the solvent DMSO (-).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

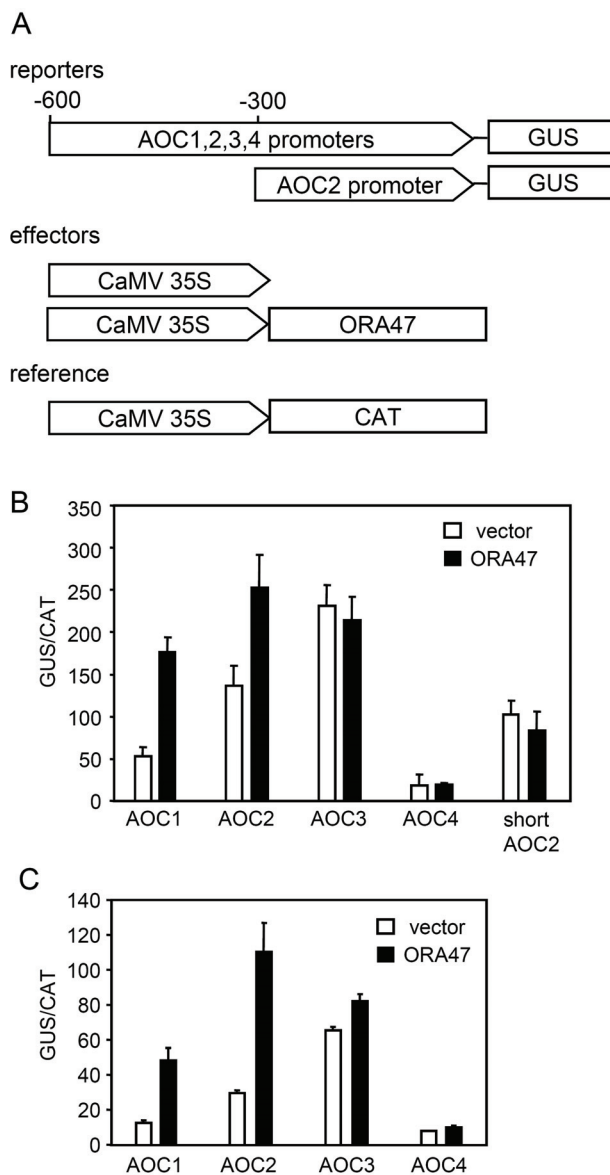


Figure 2. ORA47 trans-activates the *AOC1* and *AOC2* promoters. **(A)** Schematic representation of AOC promoter fragments fused to the *GUS* reporter gene used in transient trans-activation assays. Numbers indicate the position relative to the translation start site. Also depicted are the effector 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 $\pm$ SE (n=3).

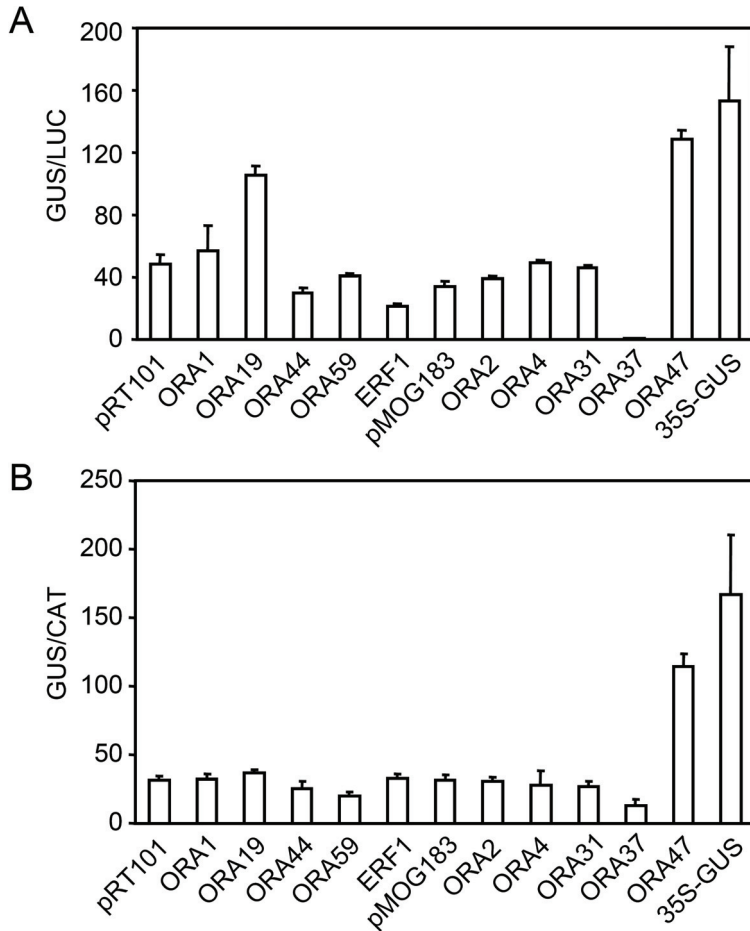


Figure 3. The *AOC2* promoter is trans-activated by ORA47 but not by other JA-responsive AP2/ERF-domain transcription factors. **(A)** Arabidopsis protoplasts or **(B)** *C. roseus* cells were co-transformed with a *GUS* reporter plasmid carrying the 600 bp *AOC2* promoter, and an effector plasmid expressing 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 $\pm$ 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

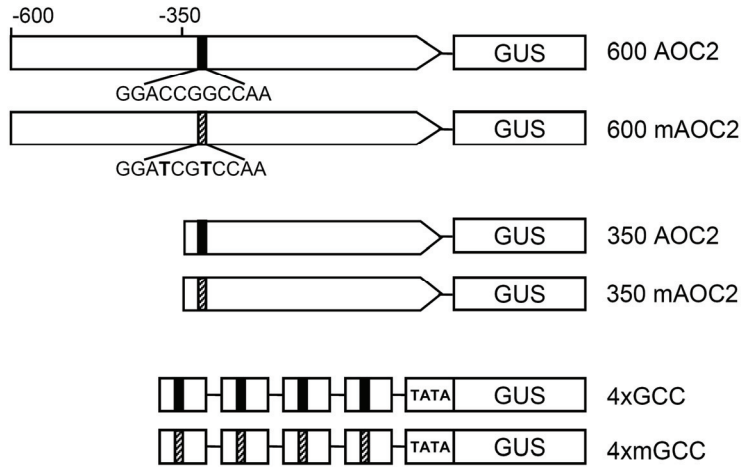
AOC2 promoter (Figure 4A), with two nucleotide substitutions in the GCC-2 box. In addition, we constructed artificial promoters consisting of 4 copies of the wild-type and mutated GCC-2 box fused head-to-tail to the TATA box from the CaMV 35S promoter.

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 4B). The EMSA also demonstrated that the two nucleotide substitutions in the GCC-2 box abolished *in vitro* binding of ORA47.

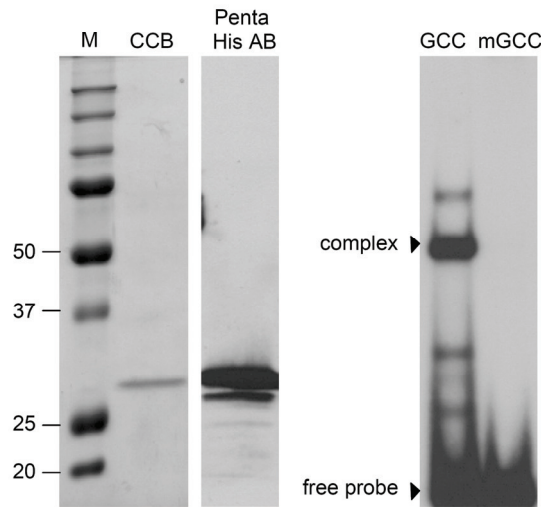
Next we tested whether the GCC-2 box was involved in trans-activation of the AOC2 promoter by ORA47. Arabidopsis protoplasts were co-transformed with one of the AOC2 promoter-*GUS* constructs shown in Figure 4A and an overexpression vector carrying the ORA47 ORF. ORA47 induced the activities of the 600AOC2 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.

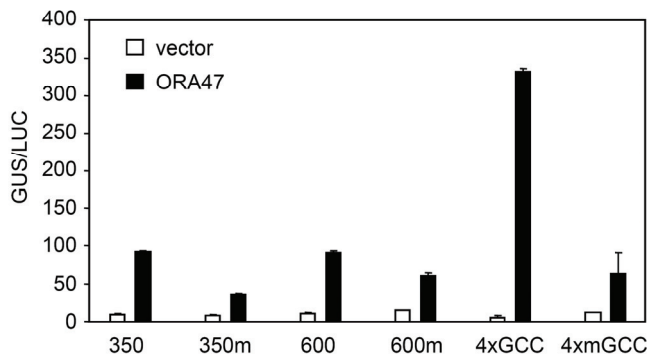
A



B



C



D

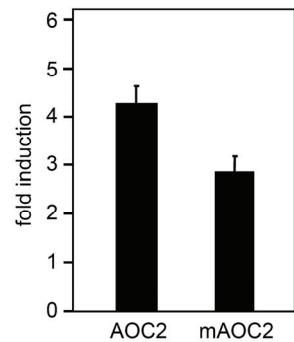


Figure 4. ORA47 protein interacts with a GCC-like box in the *AOC2* promoter. **(A)** Schematic diagram of reporter plasmids used in transient assays. *AOC2* promoter derivatives of 350 or 600 bp in length were fused to *GUS*. The position of the GCC-like box as well as its wild-type and mutated sequences 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 μ M JA for 24 hrs. Bars represent means $\pm$ 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 μ M 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 μ M 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

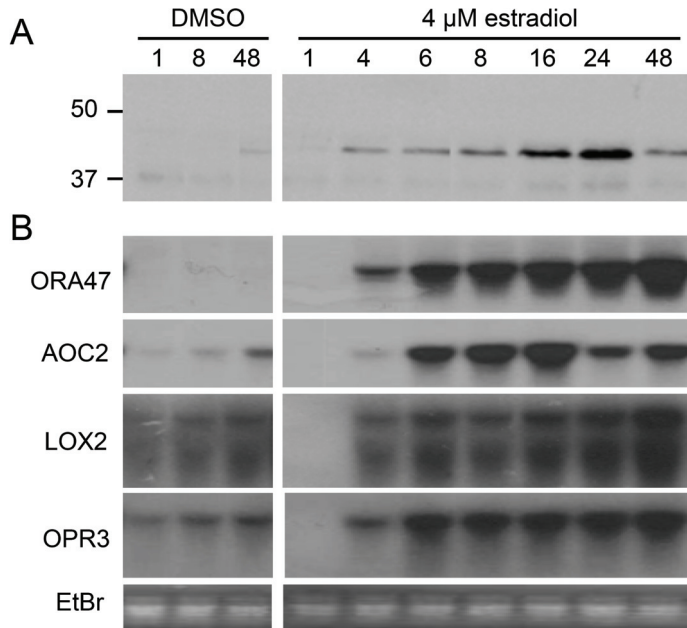


Figure 5. The ORA47-TAP protein is inducibly expressed and functional. Fourteen days old T2 seedlings from XVE-ORA47-TAP line #4 cultured in liquid medium were treated for varying times in hrs as indicated with 4 μ M estradiol or the solvent DMSO at a final concentration of 0.1% and protein and RNA was 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

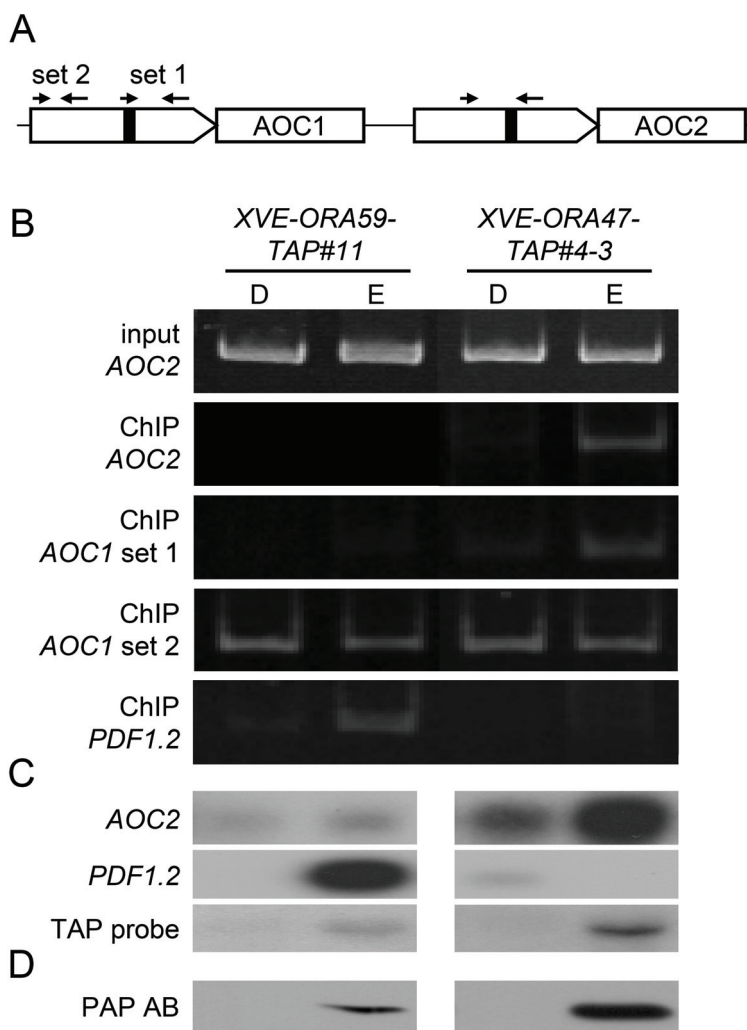


Figure 6. ORA47 binds to the AOC1 and AOC2 promoters in vivo. Fourteen days-old seedlings transformed with XVE-ORA47-TAP or XVE-ORA59-TAP were treated with 4 μ M estradiol (E) or DMSO (D) for 16 hrs. RNA and protein was extracted for Northern and Western blot analysis of transgene expression. Sonicated 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

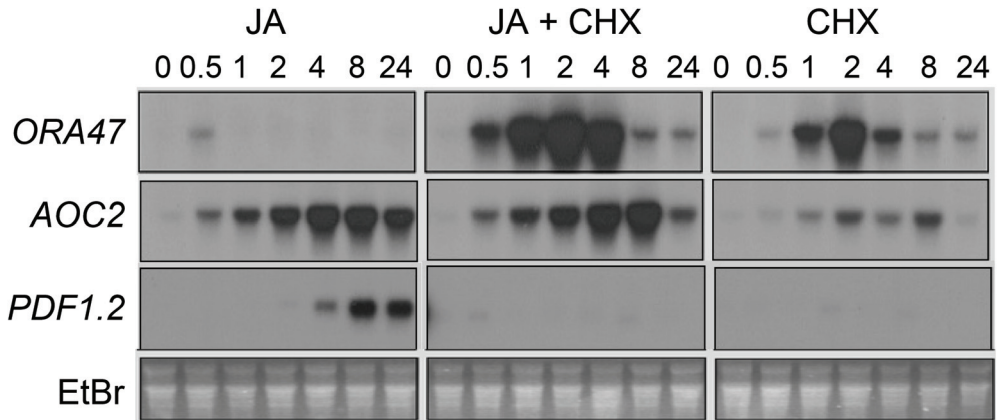


Figure 7. *AOC2* and *ORA47* are immediate-early JA-responsive genes. Fourteen days-old wild-type Arabidopsis seedlings were treated with 50 μ M JA, 100 μ M cycloheximide (CHX) or both for number of hrs as indicated. Blots were hybridized with probes corresponding to the complete open reading frames 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 GAAAAGAAAATTTTC 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 4°C, seeds were first germinated at 21°C 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 *AOC1*, 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 Sall 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 Sall 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 *Sall*/*XhoI* and cloned into pC1300intB-35SnosBK-TAP. The *ORA47-TAP* fusion was excised with *Sall*/*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 pMOG22ΔCAT (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*/*Sall* 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 NaHPO₄, 2 mM K₂HPO₄, pH 7.4, 1x Complete protease inhibitor cocktail (Roche) and 0.5% v/v Triton X100). After centrifugation at 15000xg for 10 min at 4°C, supernatants were transferred into 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 (BSA) 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% v/v H₂O₂) 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 *Sall* and *HindIII* and labeled by filling in the overhangs with the Klenow fragment of DNA polymerase I and α-³²P-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 °C 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 °C. 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 4×10^6 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 ³²P-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 °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).

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A jasmonate-responsive promoter element from *Catharanthus roseus* is active in *Arabidopsis thaliana* and is controlled by the transcription factor AtMYC2

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Abstract

Jasmonates are plant signaling molecules that play key roles in protection against certain pathogens and insects by switching on the expression of genes encoding defense proteins including enzymes involved in the biosynthesis of toxic secondary metabolites. In *Catharanthus roseus*, the AP2/ERF-domain transcription factor ORCA3 is involved in the jasmonate-responsive activation of terpenoid indole alkaloid biosynthetic genes. ORCA3 gene expression is itself induced by jasmonate. Its promoter contains an autonomous jasmonate-responsive element (JRE). Here we describe the jasmonate-responsive activity of the JRE from the ORCA3 promoter in Arabidopsis. We found that it interacts in vitro and in vivo with the basic helix-loop-helix transcription factor AtMYC2. Analysis of JRE-mediated reporter gene expression in an *atmyc2-1* mutant background showed that the activity was strictly dependent on AtMYC2.

Introduction

Jasmonic acid (JA) and its volatile methyl-ester (MeJA), both belonging to the group of jasmonate compounds, are fatty acid derivatives which are synthesized via the octadecanoid pathway (Turner et al., 2002; Wasternack et al., 2006). Jasmonates are involved in the regulation of a number of processes in plants, including certain developmental processes, senescence, and responses to wounding and insect and pathogen attack (Turner et al., 2002; Wasternack et al., 2006). JA is involved in plant protection by switching on the expression of genes encoding antimicrobial and anti-insect proteins including enzymes involved in the biosynthesis of toxic secondary metabolites (Gundlach, 1992; Memelink et al., 2001).

The AP2/ERF-domain transcription factor ORCA3 controls the JA-responsive expression of several genes involved in primary and secondary metabolism, leading to terpenoid indole alkaloid biosynthesis in the plant species *Catharanthus roseus* (van der Fits and Memelink, 2000; Memelink et al., 2001). The expression of the ORCA3 gene itself is rapidly induced by MeJA (van der Fits and Memelink, 2001). By loss- and gain-of-function experiments a 74 bp fragment called "D" was localized within the ORCA3 promoter, which functions as an autonomous jasmonate-responsive element (JRE) (Vom Endt et al., 2007). Block mutation analysis showed that the ORCA3 JRE is composed of two important sequences, a quantitative sequence responsible for a high level of expression, and a qualitative sequence that acts as an on/off switch in response to MeJA. The qualitative

sequence contains a G-box (CACGTG) with one mismatch, and is in fact a T/G hybrid box composed of T-box and G-box half-sites according to the terminology in Foster et al. (1994).

Using yeast one-hybrid screening, DNA-binding proteins belonging to the AT-hook class that interact with the quantitative sequence were isolated (Vom Endt et al., 2007). However those screenings failed to identify proteins that are able to bind specifically to the qualitative sequence. A similar T/G box in the promoter of the *leucine aminopeptidase* (*LAP*) gene from tomato (*Lycopersicon esculentum*) is necessary for its JA-responsive transcriptional activity (Boter et al., 2004). This T/G box interacts with two basic helix-loop-helix (bHLH) proteins called JAMYC2 and JAMYC10. The closest homologue in *Arabidopsis thaliana* is the bHLH protein AtMYC2 (also called RAP1 or rd22BP1). Knocking out AtMYC2 by T-DNA insertion abolishes the root shortening response to JA and affects JA-responsive gene expression (Boter et al., 2004; Lorenzo et al., 2004). AtMYC2 binds to G-box-like sequences (de Pater et al., 1997), including the G box and the T/G box (Chini et al., 2007). Based on its similarity to the *LAP* promoter element and its identity as a T/G box, the qualitative sequence in the *ORCA3* promoter is likely to bind a bHLH transcription factor with similarity to JAMYC2, JAMYC10 and AtMYC2.

The aim of this study was to investigate the activity of the JRE in Arabidopsis. On the one hand this might confirm that its activity is controlled by a bHLH transcription factor, which would be AtMYC2 in Arabidopsis. On the other hand, the JRE could serve as tool for the identification of additional regulatory proteins involved in JA-responsive gene expression by gain-of-function mutant analysis. In such an approach Arabidopsis transformants containing a selection gene under control of the JRE can be subjected to T-DNA activation tagging (Tani et al., 2004) to identify proteins that activate JRE-mediated gene expression when over-expressed.

Our results show that the JRE from the *ORCA3* promoter was JA-responsive in Arabidopsis, and that it interacted in vitro and in vivo with AtMYC2. Analysis of JRE-mediated reporter gene expression in an *atmyc2-1* mutant background showed that the activity was strictly dependent on AtMYC2.

Results

The JRE from the *ORCA3* promoter is JA-responsive in Arabidopsis

A tetramer of a fragment from the *ORCA3* promoter indicated as “D” was previously shown to function as an autonomous JRE when fused to the Cauliflower Mosaic Virus (CaMV) 35S minimal promoter and the *GUS* reporter gene (Vom Endt et al., 2007). This construct was

introduced in *Arabidopsis* to evaluate whether it has similar activity in this plant species. The T-DNA carries besides the *GUS* gene and the hygromycin resistance gene a *chloramphenicol acetyltransferase* (*CAT*) reporter gene driven by the CaMV 35S promoter, which is separated from the *GUS* gene by 5 kb of spacer DNA (Vom Endt et al., 2007). Besides the wild-type 4D tetramer, also the activity of the 4DM7 mutant, which is affected in the T/G box rendering it completely inactive in *Catharanthus*, was investigated to confirm that possible JRE activity in *Arabidopsis* also depends on the qualitative sequence. Seedlings from the T2 generation from 9 independent transformed lines were screened for JA-responsive expression of the *GUS* gene by histochemical staining with the substrate X-Gluc. For the wild-type D tetramer 3 lines were found to be active, whereas for the M7 mutant tetramer no GUS-positive seedlings were found. Subsequently, JA-responsive GUS activity was quantified in these three active 4D lines and compared to three randomly chosen 4DM7 lines. As shown in Figure 1A with the 4D lines strong increases in GUS activity were observed upon JA treatment, whereas in the 4DM7 lines only background levels were observed without any response to JA. The most active line was #3, which was used for further experiments.

Histochemical staining with the GUS substrate X-Gluc was performed of seedlings from 4D-*GUS*-47 line #3 to study whether the D tetramer showed tissue- or organ-specific expression. Representative examples of GUS staining are shown in Figure 1B. Without JA treatment seedlings never showed GUS staining in any cell type. Upon treatment with 100 μ M JA for 24 hrs weak GUS activity was observed in specific cell types. In leaves GUS activity was mainly localized in the hydathodes, with staining in other parts of the leaves in a fraction of the seedlings. In the roots staining was localized in the area where lateral roots emerged (Figure 1B) with weak staining in other root cell types in a fraction of the seedlings (data not shown). Upon treatment with 200 μ M JA for 24 hrs the majority of the seedlings showed the same tissue-specific GUS staining as after treatment with 100 μ M JA (not shown). A fraction of the seedlings showed intense GUS staining in extended areas of the leaves extending also in the petioles (Figure 1B). Root staining was similar for both treatments with different concentrations of JA.

The JRE from the *ORCA3* promoter responds specifically to JA independent of de novo protein synthesis

We asked the question whether the gene expression response conferred by the JRE is specific to JA, or whether other defense hormones also affect its activity. Fourteen-days-old T2 seedlings of 4D-*GUS*-47 #3 were treated with different hormones and expression of the

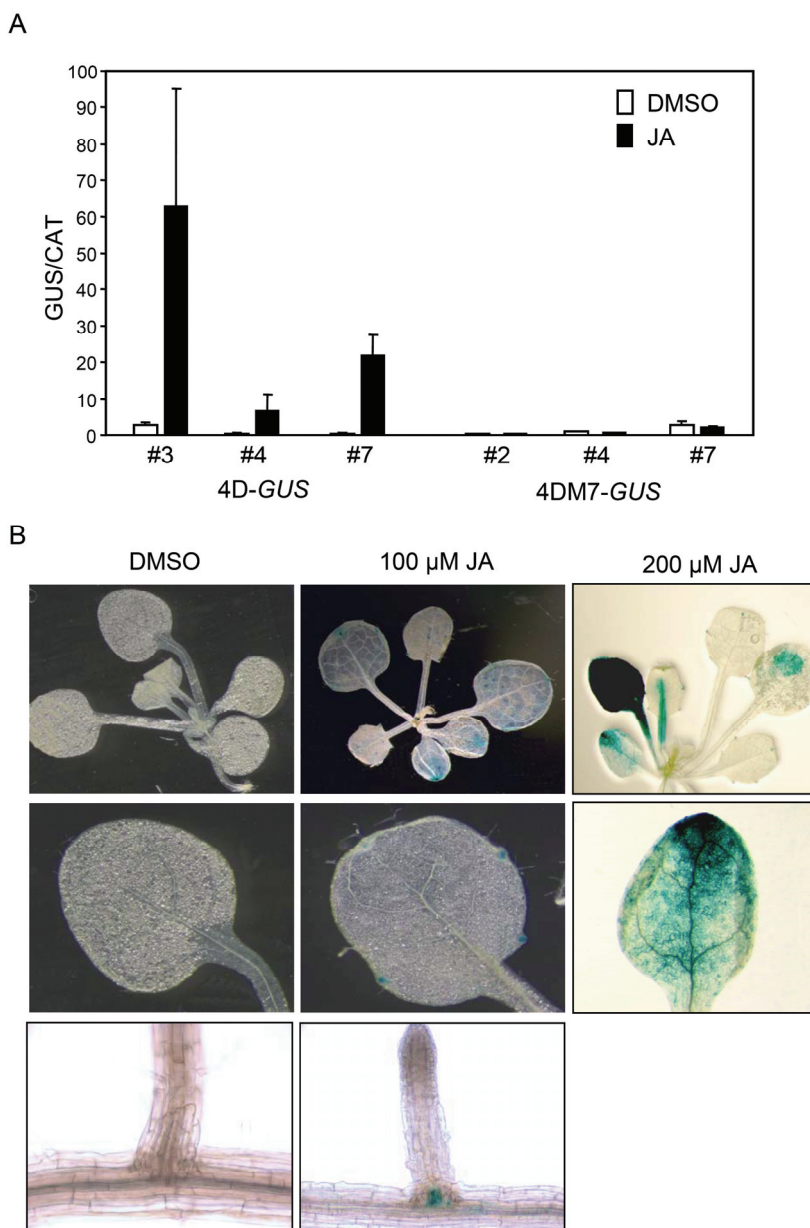


Figure 1. The JRE from the *ORCA3* promoter is JA-responsive in *Arabidopsis*. **(A)** Fourteen-days-old T2 seedlings from three independent transgenic lines (indicated by numbers) containing either 4D-*GUS*-47 (4 copies of the wild-type JRE) or 4DM7-*GUS*-47 (4 copies of the JRE mutant M7) were treated for 24 hrs with 50 μ M JA or as a control (C) with DMSO at a final concentration of 0.1% (v/v). GUS activities were related to chloramphenicol acetyltransferase activities encoded by the *CAT* gene controlled by the CaMV 35S promoter on the T-DNA. Absolute CAT activities were in a similar range for all transgenic lines (data not shown). GUS/CAT ratios are means $\pm$ SE determined in three pools of 10 seedlings. **(B)** Histochemical localization of GUS activity in fourteen-days-old seedlings treated with DMSO or JA at concentrations as indicated for 24 hrs.

GUS gene in comparison with *AtMYC2* was analyzed by Northern blot hybridization. The expression of the *vegetative storage protein 1 (VSP1)* gene was also analyzed as a representative JA-responsive gene in Arabidopsis. As shown in Figure 2A, *GUS* mRNA accumulated only in response to JA, whereas ethylene, salicylic acid and abscisic acid (ABA) had no visible effect. Ethylene had a strong negative effect on the JA-responsive expression of the *GUS* gene. Consistent with previous reports (Lorenzo et al., 2004; Boter et al., 2004; Abe et al., 1997) *AtMYC2* expression was induced by JA and ABA with a negative effect of ethylene on the JA response. The expression of the *VSP1* gene was qualitatively very similar to *AtMYC2* expression with the difference that it responded slower to JA and ABA.

Gene expression analysis using the protein synthesis inhibitor cycloheximide (CHX) showed that in *Catharanthus* cells D-mediated JA-responsive expression does not require *de novo* protein synthesis, indicating that the JRE is a primary response element. To establish whether the JRE has similar activity in Arabidopsis, T2 seedlings from 4D-*GUS*-47 line #3 were treated with CHX or JA or both. Figure 2B shows that *GUS* expression was relatively weakly induced by JA, strongly induced by CHX and superinduced by the combination. This expression pattern is qualitatively very similar to the response in transgenic *Catharanthus* cells (Vom Endt et al., 2007). The expression of the *AtMYC2* gene was very similar to the *GUS* gene with the difference that it responded faster to JA. The *VSP1* gene showed a totally different expression pattern without a response to CHX alone and a strong negative effect of CHX on JA-responsive expression.

AtMYC2 binds to the G-box like sequence in the JRE in vitro

As a first experiment to test whether the activity of the JRE from the *ORCA3* promoter is controlled by AtMYC2, electrophoretic mobility shift assays were performed with recombinant AtMYC2 protein expressed in *Escherichia coli*. As shown in Fig.3B, AtMYC2 bound to the monomeric D fragment in vitro. Block mutations M1 to M5 (Figure 3A) did not affect complex formation whereas block mutations M6 and M7 completely abolished AtMYC2 binding.

AtMYC2 trans-activates the JRE via the G-box-like sequence in vivo

Next, the ability of AtMYC2 to trans-activate the *GUS* reporter gene via interaction with the JRE from the *ORCA3* promoter was tested. Arabidopsis protoplasts were co-transformed with combinations of *GUS* reporter genes controlled by tetramerized wild-type or mutant versions of the D fragment and an overexpression plasmid which was empty or expressed *AtMYC2* from the CaMV 35S promoter (Figure 4A). As shown in Figure 4B, AtMYC2 trans-activated the *GUS* reporter gene driven by the wild-type D tetramer 2-3 fold. Reporter constructs

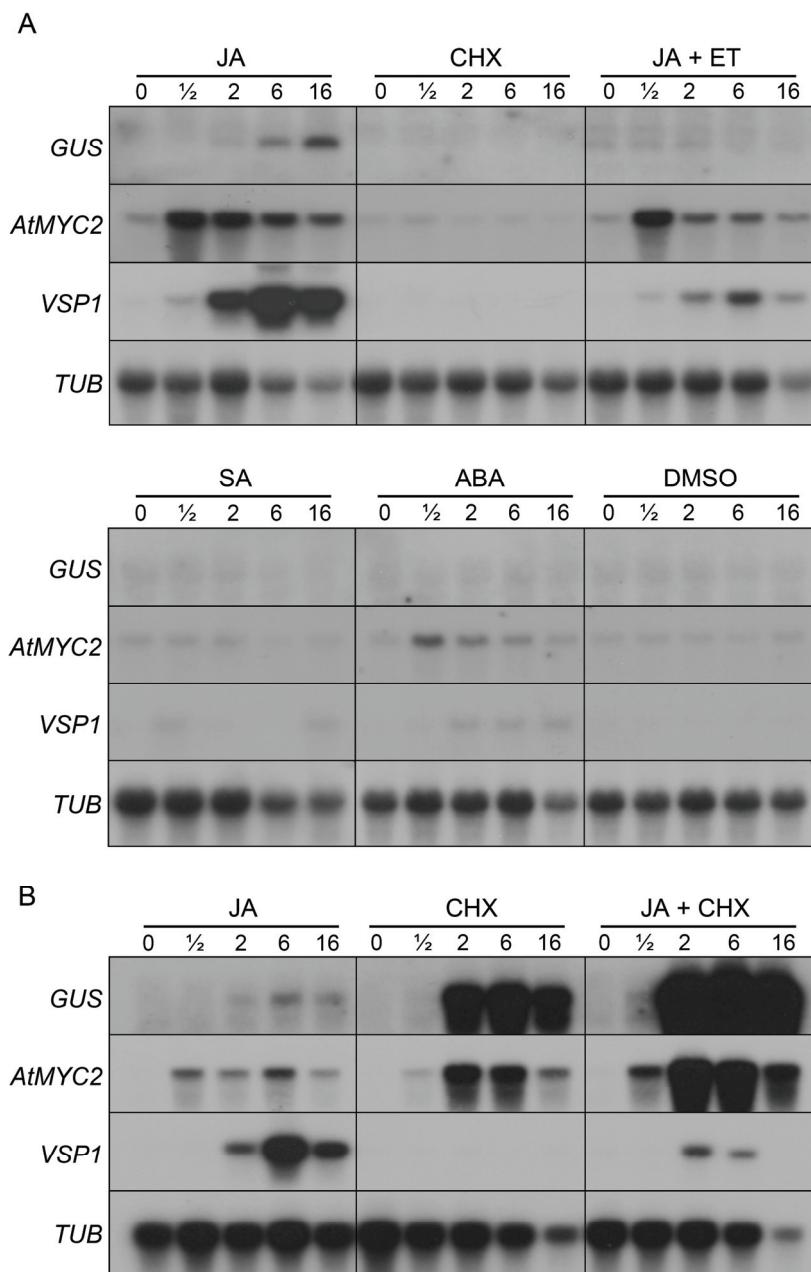


Figure 2. The JRE from the *ORCA3* promoter responds specifically to JA independent of de novo protein synthesis. **(A)** Fourteen-days-old seedlings of 4D-*GUS*-47 line #3 were treated for the indicated number of hours with 50 μ M JA, 1 mM ethephon (ET; an ethylene releaser), 1 mM salicylic acid (SA), 1 mM abscisic acid (ABA) or 0.1% of the solvent DMSO, or **(B)** with 50 μ M JA, 100 μ M cycloheximide (CHX) or both. RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading. In **(A)** all panels for each probe are from the same blot and exposed to film for the same time allowing direct comparison of expression levels.

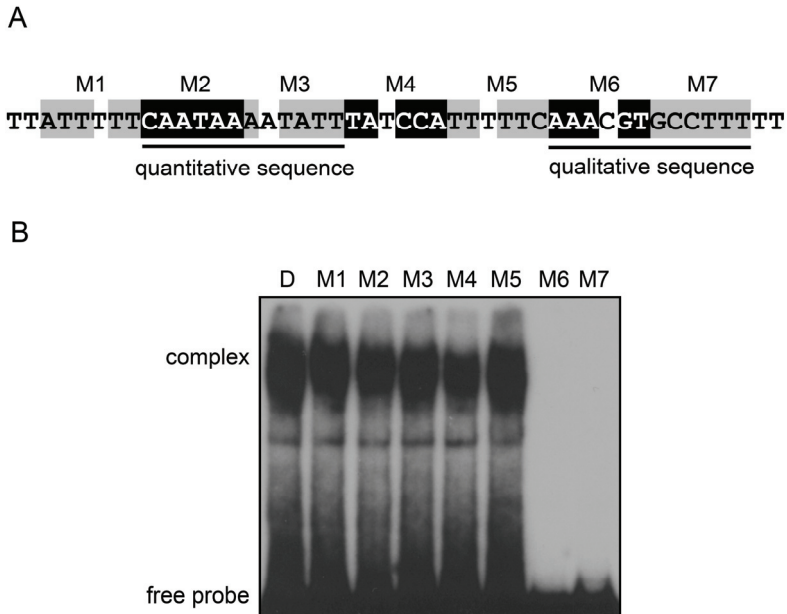


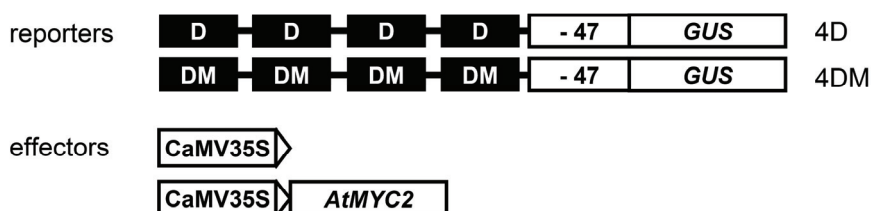
Figure 3. AtMYC2 binds to the G-box like sequence in the JRE in vitro. **(A)** Part of the wild-type sequence of the JRE is shown. Numbering of mutations is given above the sequence. In each mutant, boxed nt were changed into their complementary nt. The quantitative sequence responsible for maximum expression levels and the qualitative sequence forming the JA-responsive on/off switch are underlined. **(B)** In vitro binding of AtMYC2 to wild-type or mutated derivatives of fragment D as shown in (A).

carrying the DM1 to DM5 mutant tetramers were similarly trans-activated between 2 to 4 fold by AtMYC2. However, the mutant tetramers DM6 and DM7 did not confer trans-activation by AtMYC2. Thus there is a perfect correlation between the effects of mutations on in vitro binding of AtMYC2 to the D fragment, and the ability of AtMYC2 to trans-activate reporter gene expression in vivo. This indicates that AtMYC2 trans-activated the 4D-GUS-47 reporter construct in vivo via direct binding to the qualitative sequence containing the T/G box.

JA-responsive activity of the JRE from the *ORCA3* promoter is strictly dependent on AtMYC2 in vivo

AtMYC2 could be just one of several bHLH proteins in Arabidopsis that can bind to the JRE and can act as an activator of gene expression when overexpressed. Therefore we wanted to establish whether the activity of the JRE from the *ORCA3* promoter in Arabidopsis really depended on AtMYC2. To introduce the 4D-GUS-47 construct in an *atmyc2* mutant background, line #3 was crossed with the mutant line SALK_040500 from the SALK collection (Alonso et al., 2003) obtained via the Nottingham Arabidopsis Stock Centre. This

A



B

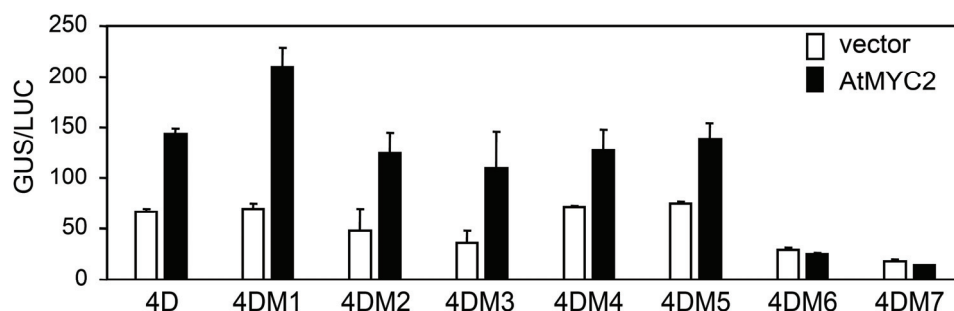


Figure 4. AtMYC2 transactivates the JRE via the G-box-like sequence in vivo. **(A)** Schematic representation of reporter and effector plasmids. Reporter plasmids contained 4 head-to-tail copies of the wild-type D element (containing the JRE) or one of the mutants shown in Fig. 3A fused to the CaMV 35S minimal promoter and the *GUS* gene. **(B)** Arabidopsis protoplasts were co-transformed with combinations of reporter and effector plasmids as indicated. A reference plasmid carrying the *Renilla LUC* gene fused to the CaMV 35S promoter was co-transformed in all experiments to correct for transformation and protein extraction efficiencies. Bars represent average GUS/LUC activity ratios $\pm$ SE of triplicate measurements. D, JRE containing element from *ORCA3* promoter; DM, corresponding element with mutated JRE.

atmyc2-1 (or *jin1-7*) mutant has a T-DNA insertion at nt 57 within the protein coding region of the *AtMYC2* gene which causes insensitivity in the JA root growth inhibition assay (Lorenzo et al., 2004) and affects JA-responsive gene expression (Boter et al., 2004).

The PCR analysis of wild-type and mutant *AtMYC2* alleles is shown in Figure 5A and confirmed that the two offspring lines selected from the cross by a first PCR screening were indeed homozygous for the T-DNA insertion. Analysis of GUS activity in the *atmyc2-1* mutant seedlings showed that the JA responsive activity was almost completely abolished compared to the activity in the parental wild-type line (Figure 6A). Analysis of the *GUS* mRNA level also showed that JA-responsive accumulation was strongly reduced in the *atmyc2-1* background (Figure 6B). With the *AtMYC2* probe the accumulation of a JA-responsive RNA was still detected, but consistent with a previous report (Boter et al., 2004) the size was slightly larger than the wild-type *AtMYC2* transcript, and it encodes a non-functional protein

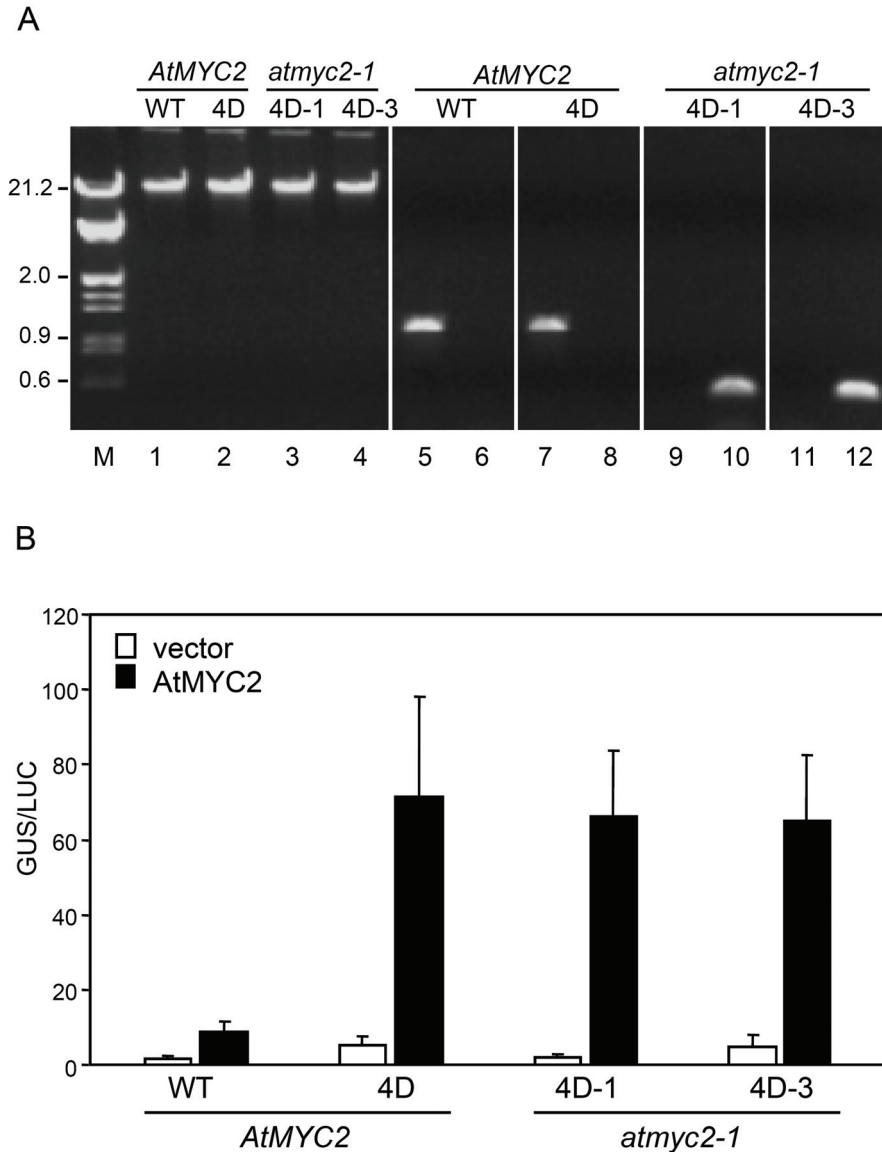


Figure 5. Molecular analysis demonstrating that the selected *atmyc2-1/4D-GUS-47* offspring is homozygous for the T-DNA insertion allele and contains a functional *GUS* reporter gene. **(A)** PCR detection of wild-type and T-DNA insertion alleles of *AtMYC2*. Lanes 1-4 contain samples of the genomic DNA used as templates for PCR. Lanes 5, 7, 9, and 11 contain PCR reactions performed with primers specific for the wild-type allele resulting in a fragment of 1037 bp. Lanes 6, 8, 10 and 12 contain PCR reactions performed with primers specific for the T-DNA insertion allele giving a fragment of 600 bp. Numbers 1 and 3 represent 2 different offspring selected from a cross between 4D-*GUS-47* line #3 and the *atmyc2-1* T-DNA knockout line. Lane M contains phage lambda DNA digested with *EcoRI*/*HindIII*. Fragment sizes in kb are indicated at the left. **(B)** The 4D-*GUS-47* gene in the *atmyc2-1* mutant is functional. Leaf protoplasts prepared from wild-type plants, or plants containing 4D-*GUS-47* in the wild-type or *atmyc2-1* background were transformed with the *AtMYC2* effector plasmid shown in Fig.4A and the 35S-LUC reference plasmid. GUS/LUC ratios are means $\pm$ SE from triplicate transformations.

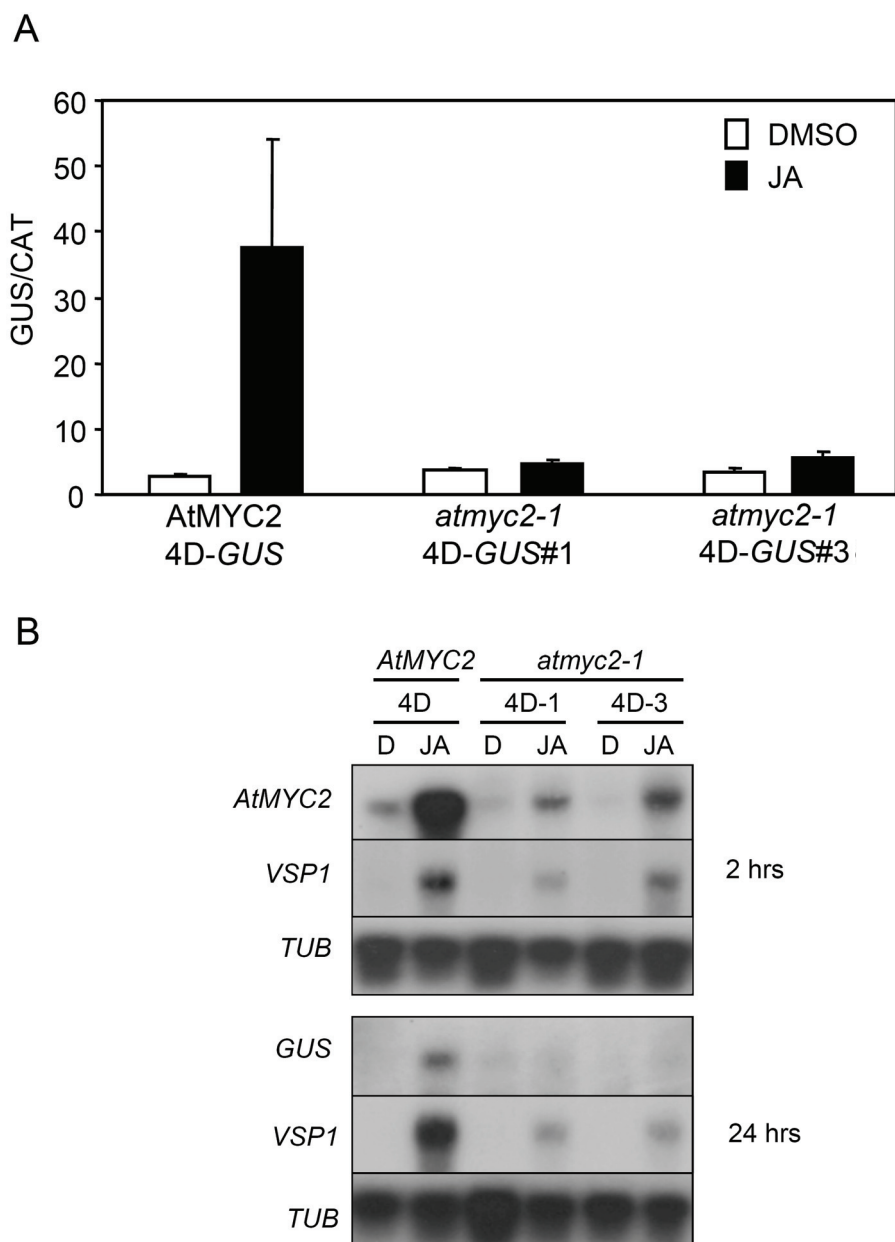


Figure 6. JA-responsive activity of the JRE from the *ORCA3* promoter is strictly dependent on AtMYC2 in vivo. **(A)** Fourteen days-old seedlings containing 4D-GUS-47 either in a wild-type or in the *atmyc2-1* mutant background were treated for 24 hrs with 50 μ M JA or with DMSO at a final concentration of 0.1% (v/v). GUS activities were related to CAT activities. Absolute CAT activities were in a similar range for all transgenic lines (data not shown). GUS/CAT ratios are means $\pm$ SE determined in three pools of 10 seedlings. **(B)** RNA gel blot analysis of 14 days-old seedlings treated with 50 μ M JA or 0.1% DMSO (D) for 2 or 24 hrs. with probes as indicated. All panels for each probe are from the same blot and exposed to film for the same time allowing direct comparison of expression levels.

containing only the first 17 amino acids of AtMYC2. JA-responsive *VSP1* expression was also reduced in the *atmyc2-1* background, although it appeared to be less affected than the *GUS* expression level.

Since the presence of the *GUS* reporter gene in the *atmyc2-1* mutant seedlings was deduced only from the fact that the seedlings were hygromycin-resistant, we wanted to prove that indeed the mutants contain a functional 4D-*GUS*-47 gene. Therefore leaf protoplasts prepared from plants carrying the reporter gene in the wild-type and mutant backgrounds were transformed with the *AtMYC2* overexpression plasmid and the *Renilla reniformis luciferase* reference gene. GUS activity analysis showed that introduction of the *AtMYC2* overexpression plasmid caused a strong induction of GUS activity in the selected mutant lines which was similar to the GUS activity level in the wild-type background (Figure 5B). This demonstrates that the *GUS* gene is functional and is able to respond to AtMYC2, which together with the other results shows that the activity of the JRE from the *ORCA3* promoter in *Arabidopsis* depended strictly on AtMYC2.

Discussion

Here we have demonstrated that the JRE from the *ORCA3* promoter is functional in *Arabidopsis*. In addition we have shown via various approaches that the JA-responsive activity of the JRE is controlled by the bHLH transcription factor AtMYC2. The most convincing evidence is the observation that the JRE is inactive in the *atmyc2-1* T-DNA knockout mutant.

The expression of the *VSP1* gene was not completely abolished in the *atmyc2-1* mutant. In addition, the expression of *VSP1* is strongly affected by the protein synthesis inhibitor cycloheximide. Both observations indicate that the promoter of *VSP1* does not directly interact with AtMYC2, although it was reported to contain a G-box-like sequence (Guerineau et al., 2003) that is a potential binding site for AtMYC2.

As in *Catharanthus* cells (Vom Endt et al., 2007), the JA-responsive activity of the JRE did not depend on de novo protein synthesis in *Arabidopsis*. This also is consistent with the notion that the JRE is directly interacting with AtMYC2. In addition, it shows that pre-existing AtMYC2 is activated in response to JA without requirement for newly synthesized proteins, which is consistent with the recent discovery of AtMYC2 activation by COI1- and proteasome-dependent degradation of members of the JAZ family of repressor proteins (Chini et al., 2007; Thines et al., 2007).

Our results show that AtMYC2 is a transcriptional activator when overexpressed in protoplasts in the absence of JA. One option is that an excess of AtMYC2 protein out-titrates the negative regulators from the JAZ family. Alternatively it is possible that the JA signal transduction pathway is activated due to protoplast preparation or to the transformation procedure.

Previously it was attempted to isolate the *Catharanthus* transcription factor interacting with the qualitative sequence in the JRE by yeast one-hybrid screening (Vom Endt et al., 2007). Although many JRE-binding transcription factors were isolated, none of them bound to the qualitative sequence. In that paper it was speculated that the *Catharanthus* protein is a bHLH transcription factor with similarity to AtMYC2. The results described here corroborate this hypothesis. Since bHLH proteins bind as homo- or heterodimers to their target sites in DNA, it was speculated by Vom Endt et al. (2007) that the *Catharanthus* bHLH transcription factor binds as an obligatory heterodimer and therefore escaped recovery in the yeast one-hybrid screening. AtMYC2 appears to bind as a homodimer when expressed in *E.coli* (de Pater et al., 1997; this report). The tomato proteins JAMYC2 and JAMYC10 were identified by yeast one-hybrid screening using the tomato *LAP* promoter as bait (Boter et al., 2004). It cannot be excluded that in planta the JAMYC proteins and AtMYC2 bind as heterodimers with a specific bHLH partner that strongly increases the DNA-binding affinity of the heterodimer compared to the homodimer. If AtMYC2 binds as a heterodimer in Arabidopsis, then its partner is not functionally limiting since in trans-activation experiments and in T-DNA knockout mutants AtMYC2 appears to be the only limiting factor (this report; Lorenzo et al., 2004; Boter et al., 2004).

The JRE is potentially useful as a tool for the isolation of new regulatory proteins involved in JA-responsive gene expression. It can for example be coupled to an antibiotic resistance gene. Transgenic plants or cells containing such a construct can then be super-transformed with an activation tagging T-DNA (Tani et al., 2004). In this way genes can be identified that activate the JRE when over-expressed. The advantage of a multimerized JRE over a natural JA-responsive promoter is that the presence of relatively few cis-elements reduces the likelihood that proteins that are not directly involved in the activity of JA-responsive sequences are activation-tagged. One concern with this JRE is that its activity is not very strong in Arabidopsis. In *Catharanthus* cells, the tetramerized JRE confers mRNA levels that are similar to those driven by the CaMV 35S promoter (Vom Endt et al., 2007), whereas in Arabidopsis relatively low mRNA levels were found even in the highest expressing transgenic line. This potential problem could be solved by screening more lines to find a higher expresser, or by using higher order multimers of the JRE.

Materials and methods

Biological materials, growth conditions and treatments

Arabidopsis thaliana wild-type plants, the *atmyc2-1* mutant and all transgenic plants were in the genetic background of ecotype Col-0. Seeds were surface-sterilized by incubation for 1 minute in 70% ethanol, 15 min in 25% commercial bleach (1.25% sodium hypochlorite), and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for three hours (<http://plantpath.wisc.edu/~afb/vapster.html>). Surface-sterilized seeds were transferred to plates containing MA medium supplemented with 0.6% agar (Masson and Paszkowski, 1992). Transgenic plants were selected on solid MA medium containing 20 mg/L hygromycin. Mutant *atmyc2-1* plants were selected on solid MA medium containing 25 mg/L kanamycin. Following stratification for 3 days at 4°C, seeds were incubated at 21°C in a growth chamber (16 h light/ 8 h dark, 2500 lux) for 10 days. For each treatment, 15 to 20 seedlings were then transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 mL MA medium and incubated on a shaker at 120 rpm for 4 additional days. Seedlings were treated for different time periods with 50 µM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO, 0.1% v/v final concentration), 1 mM of the ethylene releaser ethephon (Sigma-Aldrich) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration), a combination of JA and ethephon, 1 mM salicylic acid dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) or 100 µM abscisic acid dissolved in water. As a control, seedlings were treated with a combination of 0.1% DMSO and 0.5 mM sodium phosphate pH 7. Alternatively, seedlings were treated for 48 hrs with 100 µM JA dissolved in DMSO (0.2% v/v final concentration). As a control, seedlings were treated with 0.2% DMSO. For cycloheximide experiments, seedlings were first treated for 10 min with 100 µM CHX dissolved in DMSO (0.1% final concentration) and then JA dissolved in DMSO was added to 50 µM for times as indicated. The 4D-GUS-47 gene was introduced in the *atmyc2-1* mutant background by pollinating homozygous *atmyc2-1* mutant plants (T-DNA insertion line SALK_040500 from the SALK collection obtained from the Nottingham Arabidopsis Stock Centre) with pollen from 4D-GUS-47 #3 plants. Seeds were selected on solid MA medium containing 20 mg/L hygromycin and 25 mg/L kanamycin and plants were transferred to soil and were allowed to self-pollinate. Offspring was again selected on hygromycin and kanamycin and was allowed to self-pollinate and seed was collected. Seedlings from those seed batches were selected on antibiotics and screened for T-DNA insertions in both *AtMYC2* alleles using the primers 040500RP; 5'-TTT TCC TGT ACT CCT GAT CCG-3' and 040500LP; 5'-TGA ATT ATT AGC AAC GAC TCA CG-3', for detection of the wild-type allele, the primers 040500RP and LBb1; 5'-GCGTGGACCGCTTGCTGCAACT-3' (LBb1 primer is recommended by the SALK institute and 040500RP/040500LP were designed using the SALK iSECT tool online software) for detection of the T-DNA insertion allele. Genomic DNA was isolated from 20 mg of frozen leaves and buds. Frozen tissues were ground for 30 seconds in a microcentrifuge tube containing small metal beads using a Retsch Miller MM301 shaker. To the powder 400 µL of extraction buffer (0.3 M NaCl, 0.05 M Tris pH 7.5, 20 mM EDTA pH 8.0, 2% w/v sarkosyl, 0.5% w/v SDS, 5 M urea, 5% v/v Tris-saturated phenol) and 400 µL of phenol-chloroform-isoamylalcohol (25:24:1) were then added. After homogenization, tubes were centrifuged for 20 min at 6,000 rpm and the aqueous phase was transferred to a new tube. DNA was precipitated using 0.8 volume isopropanol and centrifuged for 5 min at 13,000 rpm. DNA pellets

were then washed with 70% ethanol and resuspended in 50 µL TE buffer containing 10 µg/mL RNase A. After incubation for 30 min at 37°C samples were ready for PCR analysis.

Plant transformation

Binary vectors containing tetramerized derivatives of the D fragment from the *ORCA3* promoter fused to the GUS gene were described previously (Vom Endt et al., 2007). *Agrobacterium*-mediated transformation of Arabidopsis plants was performed via 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.

RNA extraction and Northern blot analyses

Total RNA was extracted from frozen tissues by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% ethanol, and resuspended in water. As described by Memelink et al. (1994), 10 µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose/1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). All probes were ³²P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed to X-ray films (Fuji, Tokyo, Japan). DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5'-ATGACTGATTACCGGCTACAA-3' and 5'-CCGATTTTTGAAATCAAACCTTG-3' for *AtMYC2* (At1g32640); 5'-CGG GAT CCA TGA AAA TCC TCT CAC TTT-3' and 5'-CCC TCG AGT TAA GAA GGT ACG TAG TAG G-3' for *Vegetative Storage Protein 1* (*VSP1*, At5g24780); 5'-CGG AAT TCA TGA GAG AGA TCC TTC ATA TC-3' and 5'-CCC TCG AGT TAA GTC TCG TAC TCC TCT TC-3' for *TUB* (At5g44340).

Electrophoretic mobility shift assays

A PstI/NcoI fragment amplified on a *Rap-1* cDNA from ecotype C24 (Genbank acc.No. X99548, de Pater et al., 1997) with the primers 5'-CTG CAG GAT GAC TGA TTA CCG GCT ACA A-3' and 5'-CCA TGG TAC CGA TTT TTG AAA TCA AAC TTG-3' containing the complete *AtMYC2* open reading frame was cloned into pASK-IBA45 (IBA, Göttingen, Germany). Double Strep/His-tagged protein was expressed in *E. coli* strain Rosetta-gami B (DE3) pLysS (Novagen) and purified by sequential Ni-NTA agarose (Qiagen) and Strep-Tactin sepharose (IBA) chromatography. D wild-type and mutant fragments (Vom Endt et al., 2007) were isolated from pIC-20H with XbaI/XhoI and labeled by filling in the overhangs with the Klenow fragment of DNA polymerase I and α-³²P-dCTP. DNA-binding reactions contained 0.1 ng of end-labeled DNA fragment, 500 ng of poly(dAdT)-poly(dAdT), binding buffer (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% v/v glycerol) and protein extract in a 10 µL volume. Following addition of protein extract, reactions were incubated for 30 min at room temperature before loading on 5% w/v acrylamide/bisacrylamide (37:1)-0.5x Tris-Borate-EDTA gel under tension. After electrophoresis at 125 V for 1 hr, the gel was dried on Whatman DE81 paper and autoradiographed.

Transient expression assays

The *AtMYC2* gene was excised from the *Rap-1* cDNA in pBluescript SK (de Pater et al., 1997) with XmaI and cloned in the CaMV 35S cassette of the overexpression plasmid pRT101 (Töpfer et al., 1987). Protoplasts prepared from Arabidopsis cell suspension ecotype Col-0 were co-transformed with plasmids carrying the different D element derivatives fused to the *GUS* gene, the effector plasmid pRT101 carrying the *AtMYC2* gene 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 the different D element derivatives fused to the *GUS* gene with an empty pRT101 plasmid and p2rL7 were carried out. Protoplasts were transformed in triplicate using polyethylene glycol as described previously (Schirawski et al., 2000) with the three constructs in a ratio 2:2:6 (μg GUS/LUC/effector plasmid). Protoplasts were harvested 18 hours after transformation and frozen in liquid nitrogen. Alternatively, Arabidopsis leaf protoplasts were prepared and transformed as described (Sheen, 2002) using a ratio 2:6 (μg LUC/effector plasmid). GUS and LUC or CAT activity assays for each transformation were performed as described (van der Fits and Memelink, 1997; Dyer et al., 2000) with minor modifications. GUS activities were related to CAT or LUC activities to correct for differences in transformation and protein extraction efficiencies.

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Chapter 6

Summary

Jasmonic acid (JA) and related oxylipins, collectively known as jasmonates (JAs), are key regulators of plant development and plant responses to abiotic and biotic challenges (Turner et al., 2002; Farmer et al., 2003). More than four decades after the discovery of methyl jasmonic acid (MeJA) as the major lipid constituent of jasmine scent (Demole et al., 1962), the octadecanoid pathway for biosynthesis of JAs from linolenic acid is now well-established (Turner et al., 2002; Wasternack and Hause, 2002). The expression of all JA biosynthesis genes is induced by wounding or treatment with exogenous JA or MeJA (Turner et al., 2002), indicating that JA signaling is amplified by a positive feedback loop initiated by JA.

In contrast to JA biosynthesis, our current understanding of JA signal transduction steps lags behind (Lorenzo and Solano, 2005). Efforts to elucidate the JA signaling pathway have defined two steps. The first comprises components and regulators of the E3 ubiquitin ligase complex SCF^{coi1} (Lorenzo and Solano, 2005). The second step is defined by transcription factors including AtMYC2, octadecanoid derivative-responsive AP2/ERF-domain transcription factors (ORAs) and ERF1, which regulate the expression of specific sets of JA-responsive genes (Lorenzo and Solano, 2005; Pré, 2006). In the case of AtMYC2, these two steps are linked via members of the JAZ family of repressors (Chini et al., 2007; Thines et al., 2007).

The studies described in this thesis are mainly focused on the transcription factors comprising the second part of JA signaling. The interaction of JA-responsive transcription factors from *Arabidopsis* with their target genes had not been studied in much detail, which prompted the initiation of the studies described in this thesis. In addition, part of the studies was focused on possible activating effects of JA signaling on the activity of the transcription factor ORA59 at the protein level.

In *Arabidopsis*, the ERF transcription factor family comprises 122 proteins. In a family-wide screening, Atallah (2005) previously characterized a set of 14 genes which were rapidly induced by JA treatment in young seedlings. Later, Pré (2006) uncovered the function of several ORAs including ORA59, ORA47 and ORA37 by modulating their expression levels and studying the effect on gene expression and plant phenotype.

In certain defense responses, JA and ethylene signaling pathways synergize to activate a specific set of defense genes including *plant defensin1.2* (*PDF1.2*). The transcription factors ORA59 and ERF1 have been suggested to act as integrators of JA and ethylene signaling pathways in *Arabidopsis* to control this gene subset (Lorenzo et al., 2003; Pré, 2006). Overexpression of ORA59 as well as of ERF1 activates the expression of several defense-related genes including *PDF1.2* and causes increased resistance against the necrotrophic fungus *Botrytis cinerae* (Berrocal-Lobo et al., 2002; Pré, 2006). Analysis of

plants where *ORA59* expression is knocked out by RNAi shows that the JA- and ethylene-responsive expression of defense genes including *PDF1.2* is not controlled by ERF1 as previously reported (Lorenzo et al., 2003), but instead by *ORA59* (Pré, 2006). Therefore, the *ORA59* acts as the integrator of the JA and ethylene signalling pathways and is the key regulator of JA- and ethylene-responsive *PDF1.2* expression.

In **Chapter 2** studies are described which aimed at dissecting the interaction of *ORA59* and the related transcription factor ERF1 with the *PDF1.2* promoter. Two GCC boxes in the *PDF1.2* promoter are important for trans-activation by *ORA59* and ERF1 in transient assays in protoplasts and for in vitro binding. No synergistic effect between ERF1 and *ORA59* in trans-activation of the *PDF1.2* promoter was observed, indicating that each transcription factor acts independently on the *PDF1.2* promoter. Use of the chromatin immunoprecipitation (ChIP) technique showed that *ORA59* binds to the *PDF1.2* promoter in vivo. Interestingly, mutation of a single GCC box at positions -256 to -261 previously reported by others (Brown et al., 2003) to be important for JA-responsive expression completely abolished the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ethylene-releasing agent ethephon, suggesting that in vivo two functional GCC boxes are required for a switch-like effect of these defense hormones on *PDF1.2* gene expression.

The aim of the studies reported in **Chapter 3** was to determine whether JA has an activating effect on *ORA59* at the protein level. The results show that JA caused stabilization as well as nuclear localization of *ORA59*. Domain mapping of *ORA59* showed that stabilization and nuclear localization were conferred by more than one single domain. Interestingly, nuclear localization of *ORA59* did not require a functional COI1 protein. It is postulated that there is a jasmonate receptor distinct from COI1, an F-box protein that targets *ORA59* for degradation, and a repressor protein that retains *ORA59* in the cytoplasm.

ORA47 regulates a completely different subset of JA-responsive genes than *ORA59* does. Inducible overexpression of *ORA47* leads to elevated expression levels of a large number of genes encoding enzymes involved in JA biosynthesis, including AOC2, AOS and LOX2 (Pré, 2006). Oxylin measurements in plants overexpressing *ORA47* revealed strong alterations in the oxylin profile. These results suggest that *ORA47* is responsible for the regulation of the auto-stimulatory loop in JA biosynthesis.

The specific aims of the studies described in **Chapter 4** were to determine whether *ORA47* affects the expression of all four members of the small gene family encoding AOC, and whether the AOC genes are direct targets of *ORA47*. The results showed 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 the results showed that ORA47 interacted with the AOC1 promoter in vivo, and that ORA47 can trans-activate the AOC1 promoter in a transient assay. Mutation of the GCC-like box in the context of the 600 bp AOC2 promoter led to a 2.5-fold reduction in the induction level by JA, but did not abolish JA-responsive promoter activity. This indicates that the GCC-like box is important for JA-responsive expression, but that additional JA-responsive sequences are present in the 600 bp AOC2 promoter.

In *Catharanthus roseus* the AP2/ERF domain transcription factor ORCA3 controls the MeJA-responsive expression of several genes involved in terpenoid indole alkaloid biosynthesis (Memelink et al., 2001). *ORCA3* gene expression is itself induced by MeJA. A 74 bp fragment called “D” from the *ORCA3* promoter functions as an autonomous jasmonate-responsive element (JRE; Vom Endt et al., 2007). The JRE is composed of two important sequences, a quantitative sequence responsible for a high level of expression, and a qualitative sequence that acts as an on/off switch in response to MeJA. The qualitative sequence consists of a G-box with one mismatch (AACGTG). Based on the ability of members from the basic helix-loop-helix (bHLH) family of transcription factors from tomato to bind to such a G-box-like sequence in a JA-responsive promoter (Boter et al., 2004), the qualitative sequence from the *ORCA3* JRE was predicted to interact with a bHLH protein from *Catharanthus*.

The aim of the studies described in **Chapter 5** was to investigate whether the JRE is active in Arabidopsis, and if so, whether its activity depends on the bHLH transcription factor AtMYC2. The results show that the JRE element from the *ORCA3* promoter is functional in Arabidopsis. The JRE interacts in vitro and in vivo in a specific manner with AtMYC2. Analysis of JRE-mediated reporter gene expression in an *atmyc2-1* mutant background showed that the activity was strictly dependent on AtMYC2. As in *Catharanthus* cells (Vom Endt et al., 2007), the JA-responsive activity of the JRE did not depend on de novo protein synthesis in Arabidopsis. This also is consistent with the notion that the JRE is directly interacting with AtMYC2. In addition, it shows that pre-existing AtMYC2 is activated in response to JA without requirement for newly synthesized proteins, which is consistent with the recently discovered mechanism of AtMYC2 activation by COI1- and proteasome-dependent degradation of members of the JAZ family of repressor proteins (Chini et al., 2007; Thines et al., 2007).

The aim of the studies described in this thesis was the functional analysis of JA-responsive transcription factors in Arabidopsis with an emphasis on the interaction with the promoters of their target genes.

In short, the following new results were obtained. The promoter of the *PDF1.2* gene contains at least two functionally equivalent GCC boxes instead of one as reported earlier (Brown et al., 2003). The JA biosynthesis genes *AOC1* and *AOC2* are direct target genes of ORA47, corroborating the notion that ORA47 controls JA biosynthesis via regulation of the biosynthesis genes (Pré, 2006). JA controls ORA59 activity by promoting the nuclear localization and stabilization of the protein. JA-responsive nuclear localization is independent of the jasmonate receptor COI1, indicating the existence of an alternative jasmonate receptor. The JRE from the *ORCA3* promoter is active in Arabidopsis and its activity is controlled by the bHLH transcription factor AtMYC2, suggesting that a related bHLH protein controls *ORCA3* gene expression in *Catharanthus*.

Interesting follow-up questions concern the mechanisms of ORA59 stabilization and nuclear localization and the identity of the alternative jasmonate receptor. In addition, it will be interesting to identify the bHLH protein(s) from *Catharanthus* responsible for JA-responsive *ORCA3* expression, and to study their regulation and their impact on terpenoid indole alkaloid biosynthesis.

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Samenvatting

Jasmonzuur (JA) en verwante oxylipines, gezamenlijk aangeduid als jasmonaten (JA's), zijn sleutelregulatoren van plantenontwikkeling en van reacties van planten op abiotische en biotische stressprikkelers (Turner et al., 2002; Farmer et al., 2003). Meer dan vier decennia na de ontdekking van methyl-jasmonzuur (MeJA) als het belangrijkste lipide bestanddeel van jasmijngeur (Demole et al., 1962) is de octadecanoïedroute die leidt tot de biosynthese van JA's van linoleenzuur grotendeels opgehelderd (Turner et al., 2002; Wasternack en Hause, 2003). De expressie van alle jasmonaatbiosynthesegenen wordt geïnduceerd door verwonding of door behandeling met JA of MeJA (Turner et al., 2002), wat aangeeft dat JA signalering versterkt wordt door een positieve terugkoppelingslus aangezwengeld door JA.

In tegenstelling tot het beeld van JA biosynthese loopt ons inzicht in JA signaaltransductie wat achter (Lorenzo en Solano, 2005). Onderzoeksinspanningen om de JA signaaltransductieroute op te helderen hebben twee verschillende stappen gedefinieerd. De eerste stap omvat componenten en regulatoren van het E3 ubiquitine ligase complex SCF^{COI1} (Lorenzo en Solano, 2005), waarbij COI1 een F-box eiwit is dat zowel dienst doet als jasmonaatreceptor (Thines et al., 2007) als dat het eiwitten herkent ter markering met ubiquitine voor afbraak door het 26S proteasoom (Chini et al., 2007; Thines et al., 2007). Dat laatste is de normale functie van F-box eiwitten in eukaryote organismen, terwijl de receptorfunctie tot nu toe specifiek is voor planten. De tweede stap in JA signalering omhelst transcriptiefactoren zoals AtMYC2, octadecanoïed-responsieve AP2/ERF-domein transcriptiefactoren (ORA's) en ERF1, welke de expressie van specifieke sets van JA-responsieve genen reguleren (Lorenzo en Solano, 2005; Pré, 2006). In het geval van AtMYC2 zijn deze twee stappen met elkaar verbonden via leden van de JAZ repressorfamilie welke binden aan AtMYC2 en afgebroken worden via herkenning door COI1 in aanwezigheid van een biologisch actief jasmonaat (Chini et al., 2007; Thines et al., 2007).

De studies beschreven in dit proefschrift waren voornamelijk gericht op de transcriptiefactoren die deel uitmaken van de tweede stap in JA signalering. De interactie van JA-responsieve transcriptiefactoren van *Arabidopsis* met hun doelwitgenen was nog niet in detail bestudeerd, hetgeen de aanleiding vormde tot enkele van de studies beschreven in dit proefschrift. Daarnaast was een deel van het onderzoek gericht op het aantonen van mogelijke activerende effecten van JA signalering op de activiteit van de transcriptiefactor ORA59 op eiwitniveau.

In de zandraket (*Arabidopsis thaliana*) omvat de AP2/ERF-domein transcriptiefactorfamilie 122 leden. In een familiebrede screening heeft Atallah (2005) in het verleden een set van 14 zogenaamde ORA genen gevonden die snel worden aangeschakeld door JA in jonge zaailingen. Wat later heeft Pré (2006) de functie opgehelderd van

verschillende van deze ORA's, zoals ORA59 en ORA47, door hun expressieniveaus te moduleren en het effect daarvan op genexpressie en het aanzien van de planten te bestuderen.

In sommige verdedigingsresponsen werkt JA signaaltransductie samen met de signaleringsroute van ethyleen, een ander hormoon betrokken bij verdediging, teneinde een set van genen die ook het gen *PDF1.2* (plant defensin 1.2) omvat aan te schakelen. De transcriptiefactoren ORA59 (Pré, 2006) en ERF1 (Lorenzo et al., 2003) zijn naar voren geschoven als integrators van JA en ethyleen signalering in Arabidopsis. Overexpressie van ORA59 of ERF1 schakelt de expressie van *PDF1.2* en andere genen behorende bij deze genenset aan en veroorzaakt verhoogde resistentie tegen de necrotrofe schimmel *Botrytis cinerea* (Berrocal-Lobo et al., 2002; Pré, 2006). Analyse van planten waarin de expressie van ORA59 is uitgeschakeld door RNA interferentie (RNAi) toonde aan dat de expressie van deze genenset inclusief *PDF1.2* in respons op JA en ethyleen niet afhankelijk is van ERF1 zoals eerder gesuggereerd (Lorenzo et al., 2003), maar van ORA59 (Pré, 2006). De conclusie is daarom dat ORA59 de integrator is van JA en ethyleen signalering en de sleutelregulator is van JA- en ethyleen-responsieve expressie van het *PDF1.2* gen.

Hoofdstuk 2 beschrijft studies die gericht waren op de opheldering van de interactie van ORA59 en de gerelateerde transcriptiefactor ERF1 met de *PDF1.2* promotor. Twee GCC boxen in de *PDF1.2* promotor bleken belangrijk te zijn voor zowel de trans-activering door ORA59 of ERF1 in kortstondige expressietesten in Arabidopsis protoplasten als voor binding met deze eiwitten in vitro. Er kon geen samenwerking vastgesteld worden tussen ORA59 en ERF1, wat aangeeft dat deze transcriptiefactoren onafhankelijk van elkaar hun invloed uitoefenen op de *PDF1.2* promotor. Toepassing van de chromatine immunoprecipitatie techniek (ChIP) toonde aan dat ORA59 ook in vivo bindt aan de *PDF1.2* promotor. Gezien de gelijkwaardigheid van de twee GCC boxen in de hiervoor genoemde experimenten kwam de constatering dat mutatie van één enkele GCC box gelegen tussen posities -256 en -261 de respons van de *PDF1.2* promotor op JA alleen of in combinatie met ethyleen volledig plat legde als een verrassing. Deze bevinding is wel in overeenstemming met data beschreven in het artikel van Brown et al. (2003). Een verklaring zou kunnen zijn dat in vivo twee functionele GCC boxen nodig zijn voor een alles-of-niets respons van het *PDF1.2* gen op deze hormoonsignalen.

Het doel van de studies beschreven in **Hoofdstuk 3** was om vast te stellen of JA een activerend effect heeft op ORA59 op eiwitniveau. De resultaten toonden aan dat JA zowel stabilisatie als nucleaire lokalisatie van ORA59 teweegbracht. Bij het in kaart brengen van functionele domeinen in ORA59 bleek dat voor stabilisatie en nucleaire lokalisatie meerdere niet overlappende delen van het eiwit nodig waren. Een interessante waarneming

was het feit dat nucleaire lokalisatie van ORA59 niet afhankelijk was van COI1. Gebaseerd op deze resultaten is in **Hoofdstuk 3** gepostuleerd dat er nog een andere jasmonaatreceptor is naast COI1, dat er een F-box eiwit is dat ORA59 herkent ter markering voor afbraak, en dat er een repressoreiwit is dat ORA59 in het cytoplasma vast houdt in afwezigheid van JA.

ORA47 reguleert een heel andere subset van JA-responsieve genen dan ORA59. Induceerbare overexpressie van ORA47 leidt tot verhoogde expressieniveaus van genen die coderen voor enzymen betrokken bij jasmonaatbiosynthese, zoals AOC2, AOS, en LOX2 (Pré, 2006). Oxylipine metingen in planten die ORA47 tot overexpressie brengen onthulden sterke veranderingen in het oxylipine profiel. Deze resultaten geven aan dat ORA47 verantwoordelijk is voor de zelfstimulerende lus in JA biosynthese.

De specifieke doelstellingen van het onderzoek beschreven in **Hoofdstuk 4** waren om vast te stellen of ORA47 de expressie van alle vier leden van de AOC genfamilie controleert, en of de AOC genen directe doelwitten zijn van ORA47. De resultaten toonden aan dat de expressie van alle vier AOC genen werd aangeschakeld door overexpressie van ORA47. Een GCC-achtige box in de AOC2 promoter vertoonde specifieke interactie met ORA47 in vitro en in vivo, en deze GCC-achtige box was belangrijk voor ORA47-afhankelijke activiteit van de AOC2 promoter. Daarnaast werd aangetoond dat ORA47 bond aan de AOC1 promoter in vivo, en dat ORA47 de AOC1 promoter kan trans-activeren in een kortstondige expressietest in protoplasten. Mutatie van de GCC-achtige box in de context van een AOC2 promoterafgeleide van 600 bp lang resulteerde in een 2.5-voudige verlaging van de inductie door JA, maar niet in volledige inactivering. Dit geeft aan dat de GCC-achtige box belangrijk is voor de JA-responsieve activiteit van de AOC2 promoter, maar dat de 600 bp lange promoter ook nog additionele JA-responsieve sequenties bevat.

In de roze maagdenpalm (*Catharanthus roseus*) controleert de AP2/ERF-domein transcriptiefactor ORCA3 de MeJA-responsieve expressie van een aantal genen betrokken bij de biosynthese van terpenoïde indoolalkaloïden (Memelink et al., 2001). De expressie van het ORCA3 gen zelf wordt ook aangeschakeld door JA. Het zogeheten D fragment van 74 bp lang afkomstig uit de ORCA3 promoter functioneert als een autonoom (Me)JA-responsief element (JRE; Vom Endt et al., 2007). Deze JRE bevat twee belangrijke sequenties, namelijk een kwantitatieve sequentie die noodzakelijk is voor een hoog niveau van expressie, en een kwalitatieve sequentie die werkt als een MeJA-responsieve aan/uit schakelaar. De kwalitatieve sequentie is een zogenaamde G-box met één afwijking (AACGTG). Gebaseerd op het feit dat sommige leden van de basische helix-lus-helix (bHLH) familie van transcriptiefactoren van toemaat aan een G-box-achtige sequentie van een JA-responsieve promoter kunnen binden (Boter et al., 2004) is voorspeld dat de kwalitatieve sequentie van de ORCA3 JRE interactie vertoont met een bHLH eiwit van *Catharanthus*.

Het doel van de studies beschreven in **Hoofdstuk 5** was om te bepalen of de JRE actief is in Arabidopsis, en zo ja, of deze activiteit afhankelijk is van de bHLH transcriptiefactor AtMYC2. De JRE bleek inderdaad functioneel te zijn in Arabidopsis. Tevens werd vastgesteld dat de JRE in vitro en in vivo specifieke interactie vertoonde met AtMYC2. Meting van de activiteit van een reporter gen onder controle van de JRE in een *atmyc2-1* mutant toonde aan dat de activiteit volledig afhankelijk was van AtMYC2. Net als in Catharanthuscellen (Vom Endt et al., 2007) was de JA-responsieve activiteit van de JRE niet afhankelijk van de novo eiwitsynthese. Dit is consistent met de waarneming dat de JRE directe interactie vertoont met AtMYC2. Het toont tevens aan dat reeds aanwezig AtMYC2 wordt geactiveerd door JA signalering zonder dat nieuw aangemaakte eiwitten nodig zijn, wat klopt met de recente ontdekking dat AtMYC2 wordt geactiveerd door JA-, COI1-en proteasoom-afhankelijke afbraak van leden van de JAZ repressorfamilie (Chini et al., 2007; Thines et al., 2007).

Het doel van het onderzoek beschreven in dit proefschrift was de functionele analyse van JA-responsieve transcriptiefactoren in Arabidopsis met nadruk op hun interactie met de promotors van hun doelwitgenen.

In het kort zijn de volgende nieuwe inzichten verkregen. De promotor van het *PDF1.2* gen bevat minstens twee functioneel gelijkwaardige GCC boxen in plaats van één zoals eerder gerapporteerd (Brown et al., 2003). De JA biosynthesegenen *AOC1* en *AOC2* zijn directe doelwitgenen van ORA47, hetgeen een verdere ondersteuning vormt voor de gedachte dat ORA47 de biosynthese van JA bevordert door directe regulatie van de biosynthesegenen (Pré, 2006). JA beïnvloedt de activiteit van ORA59 door stabilisatie en nucleaire lokalisatie van dit eiwit. JA-responsieve lokalisatie in de kern is niet afhankelijk van de jasmonaatreceptor COI1, wat aangeeft dat er nog een andere jasmonaatreceptor bestaat. Het jasmonaat-responsieve element van de *ORCA3* promotor is actief in Arabidopsis, en deze activiteit wordt gecontroleerd door de bHLH transcriptiefactor AtMYC2, wat suggereert dat een verwant bHLH eiwit de expressie van het *ORCA3* gen in Catharanthus reguleert.

Interessante vragen voor volgend onderzoek betreffen de mechanismen van stabilisatie en nucleaire lokalisatie van ORA59 en de identiteit van de alternatieve jasmonaatreceptor. Ook zou het interessant zijn om (het) bHLH eiwit(ten) in Catharanthus te identificeren verantwoordelijk voor JA-responsieve *ORCA3* expressie, en om de regulatie en de impact van dit/deze eiwit(ten) op terpenoïde indoolalkaloïdbiosynthese te bestuderen.

Curriculum Vitae

Adel Zarei was born on the 23rd of July, 1966 in Babol, Iran. He attended high school at “Dabirestan Taleghani” in Babol. In 1986, he entered the Faculty of Agricultural Sciences, and in 1990 he finished his bachelor studies in Horticultural Sciences at the Chamran University. From September 1991 until 1993 he stayed at Tarbiat-Modaress University and received his master degree from the department of Horticultural Sciences. In 1994 he was appointed as a member of college of Agriculture, University of Mazandaran as instructor and co-researcher active in the department of Plant Pathology for 8 years. In 2003 he received an award from the ministry of Science, Research and Technology of Iran to start his PhD research under supervision of Prof. dr. Johan Memelink at the Institute of Biology, Leiden University, The Netherlands.