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PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 24 juni 2010
klokke 13:45 uur

door

Ana Paula Körbes

geboren te Porto Alegre (Brazilië)

in 1979

Promotiecommissie

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	Prof.dr. P.J.J. Hooykaas

Cover art and design: Tom Boff & Ana Körbes

Printed: Wöhrmann Print Service, Zutphen, the Netherlands

ISBN: 978-90-8570-571-0

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CHAPTER 1

General introduction

Ana Paula Körbes and Johan Memelink

Stress signaling in plants

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. To effectively avoid invasion by microbial pathogens and herbivorous insects, plants have evolved sophisticated mechanisms to provide several strategic layers of constitutive and induced defenses. Preformed physical and biochemical barriers constitute the first line of defense and fend off the majority of pathogens and insects. However, when a pathogen or herbivore overcomes or evades these constitutive defenses, recognition of pathogen-derived or insect-induced signal molecules by plant receptors leads to the activation of a concerted battery of defense responses designed to prevent further pathogen spread or plant damage.

Perception of stress signals often results in the biosynthesis of one or more of the major secondary signaling molecules jasmonates (JAs; Balbi and Devoto, 2008; Turner et al., 2002; Wasternack, 2007), ethylene (ET; Wang et al., 2002; Guo and Ecker, 2004) and salicylic acid (SA; Shah et al., 2003). Production of one or more of these hormones generates signal transduction networks that lead to a cascade of events responsible for the physiological adaptation of the plant to the external stress. In general, it can be stated that defense against pathogens with a biotrophic lifestyle is mediated by the SA signal transduction route, whereas responses to wounding and insect herbivory are mediated by JA and attack by necrotrophic pathogens triggers JA/ET-dependent responses (Dong, 1998; Glazebrook, 2005; Howe and Jander, 2008). Over the past decade, it has become increasingly clear that a plant's resistance to attack is not brought about by the isolated activation of parallel, linear hormonal pathways, but rather is the consequence of a complex regulatory network that connects the individual pathways, enabling each to assist or antagonize the others (Grant and Jones, 2009; Pieterse et al., 2009). The JAs, 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; Pieterse et al., 2009). These signaling pathways affect each other through extensive cross-talk occurring at different levels (Pieterse et al., 2009). Whereas SA works mainly antagonistically to jasmonates, ET can have either synergistic or antagonistic effects on certain subsets of genes regulated by jasmonates. Genes encoding proteins involved in defense against necrotrophic pathogens, such as the anti-microbial plant defensin PDF1.2, are synergistically induced by a combination of JA and ET, whereas genes encoding proteins involved in defense against herbivorous insects, such as the acid phosphatase VSP1, are strongly induced by JA alone and ET has a strong negative effect on the JA response. In addition other factors, such as growth conditions, tissue type and age, and other hormones such as abscisic acid, affect the response output to JAs and ET (Pauwels et al., 2008; De Vleesschauwer et al., 2010).

Jasmonate signal perception

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 (Balbi and Devoto, 2008; Turner et al., 2002; Wasternack, 2007). These signaling molecules affect a variety of plant processes including fruit ripening (Creelman and Mullet, 1997), production of viable pollen (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000), root elongation (Staswick et al., 1992), tendril coiling (Devoto and Turner, 2003), response to wounding (Zhang and Turner, 2008) and abiotic stresses, and defense against insects (McConn et al., 1997) and necrotrophic pathogens (Thomma et al., 1999). There is evidence that the jasmonates 12-oxo-phytodienoic acid (OPDA), JA, and methyl-jasmonic acid (MeJA) act as active signaling molecules (Wasternack, 2007). It now appears that a highly active jasmonate is (+)-7-*iso*-Jasmonoyl-L-Isoleucine (Fonseca et al., 2009), which is perceived by the receptor CORONATINE INSENSITIVE1 (COI1; Fonseca et al., 2009; Katsir et al., 2008; Yan et al., 2009). (+)-7-*iso*-JA-L-Ile (in short JA-Ile) is synthesized from (+)-7-*iso*-JA, which is produced by the octadecanoid pathway for jasmonate biosynthesis (Wasternack, 2007), by conjugation to Isoleucine by the enzyme JASMONATE RESISTANT1 (JAR1; Staswick and Tiryaki, 2004). Remarkably, many COI1-dependent, wound-responsive genes are expressed normally in a *jar1* mutant (Chung et al., 2008; Suza and Staswick, 2008). The *jar1* mutant still produces 10-25% of wild-type levels of JA-Ile, which may be sufficient to support gene expression. Alternatively other jasmonates might serve as signaling molecules. It remains to be determined whether COI1 serves as a receptor for other jasmonates besides JA-Ile.

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 protein factors that interact with them. In general, these *cis*-acting elements are concentrated in a relatively small promoter region of a few hundred nucleotides upstream of the transcriptional start site, although there are examples of regulatory sequences located at a distance of several thousands of nucleotides from the gene they control. Several *cis*-acting elements in various gene promoters that mediate jasmonate responsiveness have been identified. The most common jasmonate-responsive promoter sequences are the GCC motif and the G-box. In addition several other jasmonate-responsive promoter elements have been reported.

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 deletion of this JERE results in an inactive and unresponsive *STR* promoter derivative. A tetramer of the JERE fused to a minimal promoter confers MeJA-responsive gene expression on a reporter gene, showing that the JERE is an

autonomous MeJA-responsive sequence (Menke et al., 1999). Within this JERE a GCC-box-like sequence is present. In *Arabidopsis*, a GCC motif (GCCGCC) plays a role in conferring JA responsiveness to the *PDF1.2* promoter (Brown et al., 2003). The GCC motif 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 ET (Penninckx et al., 1998), which is likely caused by a convergent action of both signals on the GCC motif. However, not all GCC motifs confer JA- and ET-responsive gene expression, since the *STR* gene does not respond to ET (Memelink, unpublished results). This may be due to the sequence of the *STR* GCC motif (GACCGCC), which differs slightly from the consensus sequence.

The G-box (CACGTG) or G-box-like sequences (e.g. AACGTG) that are essential for the jasmonate response were found in the promoters of the potato *PIN2* gene (Kim et al., 1992), the soybean vegetative storage protein B gene (*VSPB*; Mason et al., 1993), the Arabidopsis *VSP1* gene (Guerineau et al., 2003), the tomato leucine aminopeptidase gene (*LAP*; Boter et al., 2004), the tobacco putrescine N-methyltransferase 1a gene (PMT1a; Xu and Timko, 2004) and the Octadecanoid-derivative Responsive *Catharanthus* AP2-domain gene (*ORCA3*; 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 (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 *NtPMT1a* promoter, the G-box is flanked by a GCC motif, and both sequences are essential for MeJA-responsive promoter activity (Xu and Timko, 2004). In the *ORCA3* promoter the G-box-like sequence is flanked by an A/T-rich sequence which is important for the expression level (Vom Endt et al., 2007).

Several additional JA-responsive promoter sequences have also been reported. 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 in tobacco (Kim et al., 1993, 1994) and of the barley lipoxygenase 1 gene promoter (*LOX1*; Rouster et al., 1997). A monomer or a tetramer of the *as-1* sequence from the Cauliflower Mosaic Virus (*CaMV*) *35S* promoter also conferred JA-responsive expression to a reporter gene in transgenic tobacco (Xiang et al., 1996). Two jasmonate-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 Long Terminal Repeat (LTR) promoter of the tobacco retrotransposon *Tto*1 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 jasmonate-responsive elements appear to exist. The best characterized elements are the G-box and closely related variants, which are commonly found in promoters that respond to jasmonates and are negatively affected by ET, and the GCC motif, which is commonly present in promoters that respond in a synergistic manner to JAs combined

with ET. It has been well established that the JAs-responsive activity of promoters containing the GCC motif (e.g. *PDF1.2*; Lorenzo et al., 2003) or the G-box (e.g. *VSP*; Benedetti et al., 1995) is dependent on COI1. For promoters containing other elements COI1 dependency has not been established. The *OPR1* gene for example, containing the JASE1/2 motifs in its promoter, has been shown to be wound-inducible in a *coi1* mutant background (Reymond et al., 2000), and is inducible by OPDA but not by JA in an *opr3* mutant background (Stintzi et al., 2001). Therefore it remains to be established whether so-called JA-responsive elements other than the GCC motif and the G-box confer responses to bioactive JAs via COI1.

Transcription factors and JA responses

JAZ repressors and COI1 control the activity of AtMYC2

To identify molecular components of jasmonate signal transduction, screenings for *Arabidopsis* mutants that are insensitive to (Me)JA or to coronatine (a bacterial toxin which is a structural and functional analogue of JA-Ile) or that show constitutive jasmonate responses have been performed (Lorenzo and Solano, 2005; Browse, 2009).

The *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 JAs (Feys et al., 1994), is defective in resistance to certain insects and pathogens and fails to express jasmonate-regulated genes (Turner et al., 2002). 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 (Del Pozo and Estelle, 2000). 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). Plants that are deficient in other components or regulators of SCF complexes, including AXR1, COP9 and SGT1b, also show impaired jasmonate responses (Lorenzo and Solano, 2005). The existence of a *COI1* function in species other than *Arabidopsis* was demonstrated in tomato (Li et al., 2004), soybean (Wang et al., 2005), *Nicotiana attenuata* (Paschold et al., 2007), tobacco (Shoji et al., 2008) and potato (Halim et al., 2009). COI1 is a component that is specific to the JA pathway, whereas SGT1b and AXR1 are shared by other signaling pathways. Mutations in *AXR1* or *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 jasmonate signaling mutants has been described by Lorenzo et al. (2004). Screening for mutants affected in JA-induced root growth inhibition in an *ethylene-insensitive*3 (*ein3*) background resulted in the identification of 5 loci called *JA-insensitive* (*JAI*) 1-5. The *JAI1* locus corresponds to the *AtMYC2* gene (Lorenzo et al., 2004), encoding a basic-

Helix-Loop-Helix (bHLH) transcription factor which regulates a subset of jasmonate-responsive genes involved in wounding responses and resistance against insects (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007). Recombinant AtMYC2 binds *in vitro* to the G-box and related sequences (De Pater et al., 1997; Chini et al., 2007; Dombrecht et al., 2007). The *JAI2* locus corresponds to the previously characterized *JAR1* gene (Staswick et al., 1992), encoding an enzyme that couples JA to amino acids with a preference for isoleucine (Staswick and Tiryaki, 2004). 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 12 members that are induced at the gene expression level by JAs are called Jasmonate ZIM domain (JAZ) proteins (Chini et al., 2007; Thines et al., 2007). They contain in addition to the highly conserved central ZIM domain a highly conserved C-terminal Jas domain and a less conserved N-terminal region. In the *jai3* mutant an aberrant protein is expressed with a deletion of the C-terminal region including the Jas domain. The wild-type JAI3 (or JAZ3) protein is rapidly degraded in response to JA in a COI1-dependent manner, whereas the jai3 mutant protein is stable. The JAI3/JAZ3 protein was shown to interact *in vitro* and in yeast with AtMYC2. Based on these findings it was postulated that JAI3 is a repressor of AtMYC2 which is rapidly degraded in response to JA thereby activating AtMYC2 (Figure 1; Chini et al., 2007). However it remains to be experimentally demonstrated that the full-length JAI3/JAZ3 protein is indeed a repressor of gene expression. There are many examples of full-length transcription factors which have opposite activities to certain deletion derivatives (e.g. Gill and Ptashne, 1988; Fan and Dong, 2002; Miao and Lam, 1995).

JAZ variants lacking effective Jas domains also occur naturally in *Arabidopsis*. For JAZ10.1, two more stable variants have been described which are translated from alternatively spliced mRNAs. JAZ10.3 misses a few amino acids at the C terminus, making it more stable in response to jasmonate (Chung and Howe, 2009), and therefore it has dominant-negative effects on jasmonate responses when overexpressed (Yan et al., 2007). The splice variant JAZ10.4 lacks the entire Jas domain, rendering it completely stable and turning it into a strong dominant-negative repressor when overexpressed (Chung and Howe, 2009).

In independent studies, members of the *JAZ* gene family in *Arabidopsis* were characterized as being predominant among genes induced in anthers after 30 min 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 region including the Jas domain is stable. Interestingly, these authors were able to detect interaction between JAZ1 and COI1 in a yeast two-hybrid assay in the presence of JA conjugated to Ile (JA-Ile) in the yeast growth medium or in an *in vitro* pull-down assay in the presence of JA-Ile (Thines et al., 2007). No interaction was detected in the presence of OPDA, JA, MeJA or JA conjugated to Trp or Phe, whereas JA-Leu was about 50-fold less effective in promoting interaction between COI1 and JAZ1 than JA-Ile. JA-Ile

and JA-Leu are products of the JAR1-mediated conjugation reaction (Staswick and Tiryaki, 2004). JA-Ile and coronatine were also shown to promote the interaction between JAZ3 and JAZ9 in a yeast two-hybrid assay, whereas JA or MeJA are ineffective (Melotto et al., 2008). The C-terminal regions containing the conserved Jas domain of tomato JAZ1 (Katsir et al., 2008) and *Arabidopsis* JAZ1, JAZ3, JAZ9 (Melotto et al., 2008) and JAZ10.1 (Chung and Howe, 2009) were shown to be necessary for binding to COI1 in a JA-Ile or coronatine dependent manner. In addition it was shown that the Jas domains of tomato JAZ1 (Katsir et al., 2008) and *Arabidopsis* JAZ1, JAZ3, and JAZ9 (Melotto et al., 2008) are sufficient for binding to COI1 in a JA-Ile or coronatine dependent manner.

Using tomato SlCOI1 and SlJAZ1, it was shown that the complex binds radiolabeled coronatine (Katsir et al., 2008). Binding can be displaced with unlabeled coronatine or JA-Ile. Combined with the coronatine-dependent interaction between COI1 and JAZ proteins in yeast, these experiments provide strong evidence that COI1 is the receptor for at least certain JAs including JA-Ile, as well as for the microbial JA-Ile mimic coronatine. They do not exclude, however, the possibility that proteins co-purified with COI1 from the plant extracts function as JA receptor. A recent report combining purified COI1 (expressed *in vitro* in insect cell lysates) with surface plasmon resonance and photoaffinity labelling technology provided more evidence that COI1 directly binds JA-Ile and coronatine (Yan et al., 2009).

The expression of the *JAZ* genes in *Arabidopsis* is induced by JA (Mandaokar et al., 2006; Chini et al., 2007; Thines et al., 2007; Yan et al., 2007) and is controlled by AtMYC2 (Chini et al., 2007). The model is therefore that AtMYC2 and JAZ proteins form a jasmonate-responsive oscillator, where JAZ proteins negatively regulate AtMYC2 activity at the protein level, JAs cause JAZ degradation and AtMYC2 activation, and AtMYC2 switches on the expression of JAZ repressors at the gene level (Figure 1). Homo- and heterodimerization of JAZ proteins likely play important roles in AtMYC2 gene repression and in the interaction with COI1 (Chini et al., 2009; Chung and Howe, 2009), although it remains to be formally proven that the complexes are dimers and not higher order complexes. Although there are some discrepancies in the two reports (Chini et al., 2009; Chung and Howe, 2009), it can be concluded that most JAZ proteins are able to form homo- and heterodimers. Specific amino acids in the TIFY motif are important for dimer formation mediated by the ZIM domain (Chung and Howe, 2009). Interestingly, the dominant-negative effect of the naturally occurring splice variant JAZ10.4, which is stable due to the absence of the Jas domain, depends on a functional ZIM domain (Chung and Howe, 2009), which implies that the functional repressing unit is a JAZ (hetero)dimer. It has been reported that expression of the jai3 (JAZ3ΔJas) protein stabilizes other full-length JAZ proteins *in trans* (Chini et al., 2007). This phenomenon can be explained by assuming that the jai3 protein heterodimerizes with other JAZ proteins and thereby stabilizes them, although the molecular mechanism for such stabilization remains to be determined.

The picture that emerges for jasmonate signal transduction is highly reminiscent of auxin signal transduction. In the absence of auxin, auxin-responsive gene expression is inhibited by the action of Auxin/Indole-3-Acetic Acid (Aux/IAA) repressors which bind to ARF (Auxin

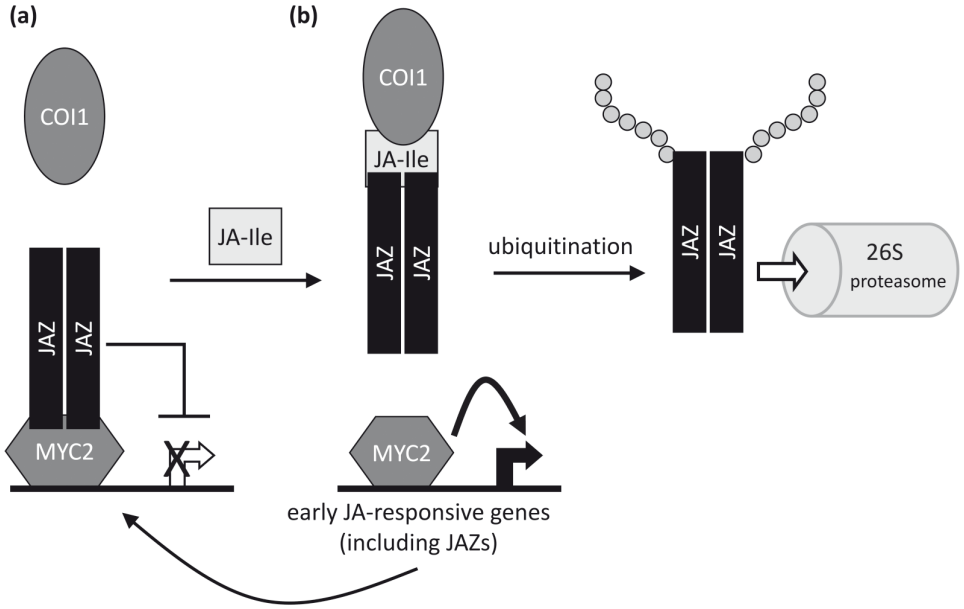

Figure 1. Model for regulation of jasmonate-responsive gene expression by AtMYC2 and JAZ proteins. Although depicted as a single protein, COI1 forms part of the putative E3 ubiquitin ligase SCF^{COI1}. **(a)** In the absence of JA-Ile, a (hetero)dimer of JAZ proteins interacts with AtMYC2 maintaining this transcription factor inactive. **(b)** JA-Ile promotes the interaction between JAZ and COI1. SCF^{COI1} causes the ubiquitination of JAZ resulting in degradation by the 26S proteasome. AtMYC2 is liberated and activates transcription of target genes, including the genes encoding JAZ proteins, resulting in a negative feedback loop.

Response factor) transcriptional activators. The F-box protein TRANSPORT INHIBITOR RESPONSE PROTEIN1 (TIR1) is the auxin receptor (Kepinski and Leyser, 2005; Dharmasiri et al., 2005). Auxin acts as the molecular glue between TIR1 and Aux/IAA proteins (Tan et al., 2007), resulting in their ubiquitination (Maraschin et al., 2009) and degradation (Guilfoyle, 2007). 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 (Gagne et al., 2002).

AP2/ERF-domain transcription factors and jasmonate responses

In *Arabidopsis*, the AP2/ERF-domain transcription factor family comprises 122 members (Nakano et al., 2006). The expression of the AP2/ERF gene *ORA59* is induced by JA or ET, and is synergistically induced by both hormones (Pré et al., 2008). 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 *PDF1.2*. Plants overexpressing *ORA59*

were more resistant to infection by the necrotrophic fungus *Botrytis cinerea*.

Plants overexpressing ERF1, a closely related member of the AP2/ERF-domain family, were previously shown to have an elevated *PDF1.2* expression level (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 AP2/ERF-domain family, suggest that ORA59 and ERF1 have redundant functions in JA and ET signal transduction. However, an essential role for 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 (Pré et al., 2008). 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 (Figure 2). An evaluation of whether ERF1 has essential roles or whether it is a dispensable functionally redundant transcription factor awaits analysis of *ERF1* knock-out mutants.

The transcription factor AtERF2, encoded by a JA-inducible gene, has also been reported to control the expression of JA/ET-responsive genes including *PDF1.2* (Brown et al., 2003; McGrath et al., 2005). In addition, overexpression of the related transcription factor AtERF1 (which is also encoded by a JA-inducible gene, and which is different from ERF1) led to increased levels of *PDF1.2* expression (Pré et al., 2008). These observations apparently contradict the finding that loss-of-function of ORA59 by RNAi abolishes *PDF1.2* expression in response to JA, to combined JA/ET treatment or to infection with *B. cinerea* or *A. brassicicola* (Pré et al., 2008), indicating that no other AP2/ERF domain transcription factor or member of another class of transcriptional regulators was able to activate the expression of *PDF1.2* in response to these treatments. In experiments where transcription factors were inducibly expressed in transgenic plants or transiently expressed in protoplasts AtERF1 and AtERF2 failed to activate *PDF1.2* expression in contrast to ORA59 and ERF1 (Pré et al., 2008). One possible explanation for these observations is that overexpression of AtERF1 or AtERF2 causes a stress condition leading non-specific expression of defense genes including *PDF1.2*, whereas ORA59 and ERF1 are *bona fide* direct regulators of *PDF1.2*.

AtERF4 differs from the AP2/ERF-domain transcription factors encoded by JA-responsive genes described above 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 (Ohta et al., 2001). The *AtERF4* gene is induced by JA (McGrath et al., 2005; Yang et al., 2005), ET (Fujimoto et al., 2000; Yang et al., 2005), infection with *Fusarium oxysporum* (McGrath et al., 2005) or wounding (Cheong et al., 2002). Overexpression of *AtERF4* had no effect on the basal transcript level of several JA-responsive genes in untreated plants. However, upon JA and/or ET treatment, *AtERF4*-overexpressing plants showed significantly

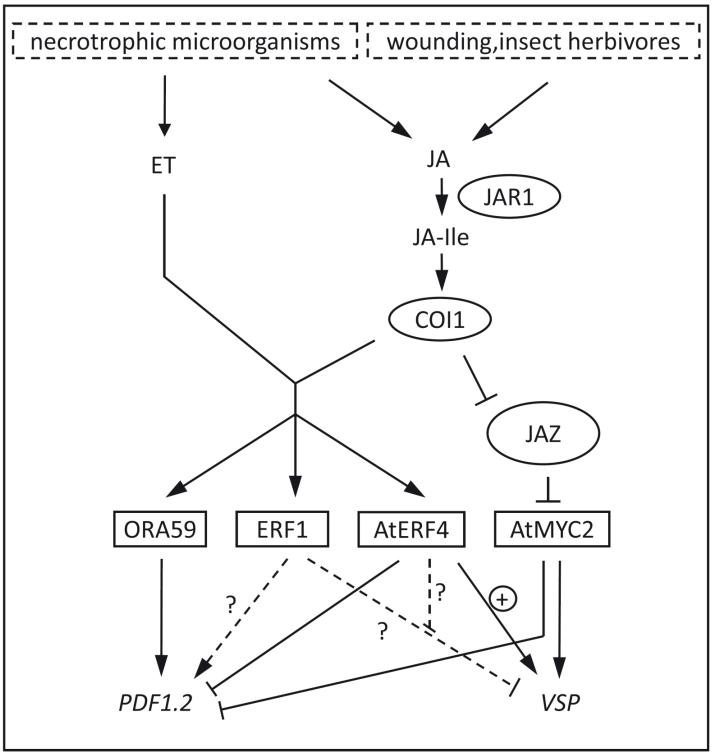

Figure 2. Model for the role of transcription factors in the stress-responsive network involving the JA and ET signaling pathways. Different types of biotic or abiotic stress, including wounding, attack by herbivorous insects and infection with necrotrophic pathogens, induce the synthesis of JA and related oxylipins. JAR1 converts JA into the biologically active JA-Ile. Some stress signals such as infection with necrotrophic pathogens simultaneously induce ET biosynthesis. JAs induce the expression of several genes encoding transcription factors, including ORA59, ERF1, AtERF4 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 bHLH-type transcription factor AtMYC2 positively regulates the expression of wound-responsive genes (e.g. *VSP*) and represses other genes, including *PDF1.2*. The JA and ET signals cooperate to induce the expression of genes encoding the AP2/ERF-domain transcription factors ORA59, ERF1 and AtERF4. ORA59 is the key regulator of JA/ET-responsive genes including *PDF1.2*, whereas the role of ERF1 in gene regulation remains unclear and awaits analysis of a knockout mutant (indicated by dashed lines and question marks). Conversely, AtERF4 represses the induction of JA/ET-responsive genes including *PDF1.2*. AtERF4 also enhances the JA-induced expression of AtMYC2 target genes including *VSP* (circled plus), possibly by repressing the negative effect of ET executed by ERF1 (dashed bar line and question mark).

lower induction of a subset of JA- and ET-responsive genes, including *PDF1.2,* compared to control plants (McGrath et al., 2005; Pré, 2006). On the other hand, plants in which *AtERF4* expression was silenced via T-DNA insertion (McGrath et al., 2005) or via RNAi (Pré, 2006)

showed increased *PDF1.2* transcript levels after JA- and/or ET-treatment compared to control plants, corroborating the complementary results obtained with *AtERF4*-overexpressing plants. This demonstrates that AtERF4 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 (Pré et al., 2008) and ERF1 (Lorenzo et al., 2003).

In addition, overexpression of the *AtERF4* 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 AtERF4 positively regulated the expression of these genes in response to JA treatment. It is not clear how the positive effect of *AtERF4* overexpression on JA signaling for this gene subset is operating at the molecular level, but assuming that AtERF4 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 AtMYC2 (Figure 2; Boter et al., 2004; 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 AtERF4 antagonizes the ERF1-mediated negative effect of ET on the expression of a subset of JA-responsive genes, including *VSP* genes (Figure 2). AtERF4 and AtMYC2 seem to positively regulate the same subset of JA-responsive genes. However, overexpression of *AtMYC2* is sufficient to activate *VSP2* expression in the absence of JAs (Lorenzo et al., 2004), which is not the case in *AtERF4*-overexpressing plants (Pré, 2006).

Therefore, JA and ET synergistically induce both activators (ORA59 and ERF1) and repressors (AtERF4) acting on the same set of genes. The functional importance of the simultaneous induction of both positive and negative regulators by JA and ET remains unclear. The balance between AP2/ERF-domain activators and repressors on common target promoters may provide a mechanism for switch-like transcriptional control. Additionally or alternatively, such a mechanism might be necessary to coordinate the response output to JAs and ET with other signals, such as growth conditions, tissue type and age, and other hormones (Pauwels et al., 2008).

Conclusions

Frequently occurring jasmonate-responsive promoter sequences are the GCC motif, which is commonly found in promoters activated synergistically by jasmonate and ethylene, and the G-box, which is commonly found in promoters activated by jasmonates and repressed by ethylene. Important transcription factors conferring jasmonate-responsive gene expression in

Arabidopsis are ORA59 (Pré et al., 2008) and AtMYC2 (Boter et al., 2004; Lorenzo et al., 2004), with other transcription factors acting as positive (e.g. ERF1, ANAC019/055) and negative (e.g. AtERF4) modulators of the gene expression response. ORA59 controls the expression of genes that are synergistically induced by jasmonates and ethylene, whereas AtMYC2 interacts *in vitro* with the G-box and related sequences (Zarei, 2007; Chini et al., 2007; Dombrecht et al., 2007), and controls genes activated by jasmonate alone.

The activity of AtMYC2 is postulated to be controlled by JAZ proteins, which are hypothesized to act as repressors (Chini et al., 2007). The bioactive jasmonate (+)-7-*iso*-JA-L-Ile promotes the interaction between JAZ proteins and the putative ubiquitin ligase complex SCF^{COI1} (Fonseca et al., 2009), presumably leading to ubiquitination of JAZ proteins and resulting in their degradation by the 26S proteasome. It has been proposed that different biologically active JAs could promote the binding between COI1 and specific JAZ family members, and that these family members could act as repressors of different downstream targets, presumably other transcription factors (Thines et al., 2007). However there is no evidence to support that notion, and in fact 10 of the 12 JAZ proteins were shown to interact with AtMYC2 (Chini et al., 2009). While this leaves open the possibility that JAZ4 and JAZ7 repress other transcription factors, the question remains whether and how other jasmonate-responsive transcription factors such as ORA59 are activated by jasmonates in a COI1-dependent manner. It is conceivable that JA-Ile or other biologically active JAs enhance binding between COI1 and hitherto unidentified repressors distinct from the JAZ proteins.

Thesis outline

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 specific subsets 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 involved in JA-responsive gene expression have been identified, including ORA59, ERF1, AtERF4, 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 the JA/ET-responsive transcription factor ORA59 in Arabidopsis.

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 were found to be 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 bound to the *PDF1.2* promoter *in vivo*. Interestingly, mutation of either GCC box was sufficient to completely abolish the expression of the *PDF1.2* promoter in response to JA alone or in combination with the ET-releasing agent ethephon in stably transformed plants.

Chapter 3 focuses on studies 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, JA-responsive 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 the characterization of the ORA59-interacting CCCH zinc finger protein ZFAR1 identified by yeast two-hybrid (Y2H) screening. Using Bimolecular Fluorescent Complementation (BiFC) it was shown that ORA59 and ZFAR1 interact in the cytoplasm of Arabidopsis cell suspension protoplasts. Consistent with this interaction ZFAR1 repressed ORA59 transcriptional activation of *PDF1.2* in protoplast trans-activation assays and had a negative effect on *Botrytis* resistance based on studies with plants with altered *ZFAR1* expression levels. Taken together the results indicate that ZFAR1 is a repressor protein that sequesters ORA59 in the cytoplasm.

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CHAPTER 2

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

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Abstract

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

Introduction

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

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

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

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

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

have been suggested to be positive regulators of *PDF1.2* gene expression (Brown et al., 2003; Lorenzo et al., 2003; Pré et al, 2008), a recent study showed that only ORA59 and ERF1 were able to function as transcriptional activators of *PDF1.2* gene expression, whereas AtERF2 and the related AtERF1 were not (Pré et al., 2008). Analysis of transgenic plants in which *ORA59* gene expression was silenced by RNAi, whereas the *ERF1* gene was normally expressed, showed that ORA59 is strictly required for *PDF1.2* gene expression in response to JA, JA/ET, and infection with necrotrophic fungi (Pré et al., 2008). Studies of the *PDF1.2* gene promoter (Brown et al., 2003; Manners et al., 1998) identified a GCC-box at positions -256 to -261 which is involved in the JA response. However interactions of the *PDF1.2* promoter with the relevant transcription factors ERF1 and especially ORA59 have not been reported, which prompted us to undertake the studies described in this chapter.

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

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

Results

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

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

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

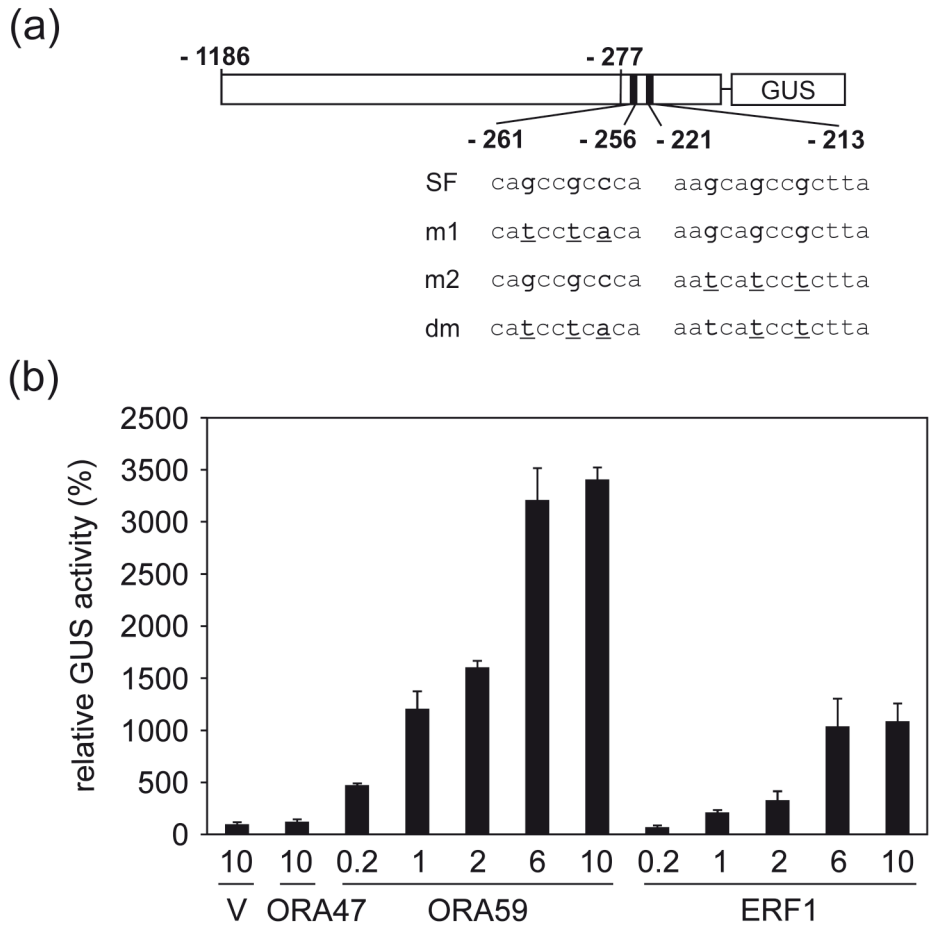

Figure 1. ORA59 and ERF1 trans-activate the *PDF1.2* promoter in a dose-dependent manner. **(a)** Constructs used in trans-activation assays. Reporter constructs consisted of the *GUS* gene driven by wild-type or mutated LF (long fragment) or SF (short fragment) *PDF1.2* promoter derivatives. Bold and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

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

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

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

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

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

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

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

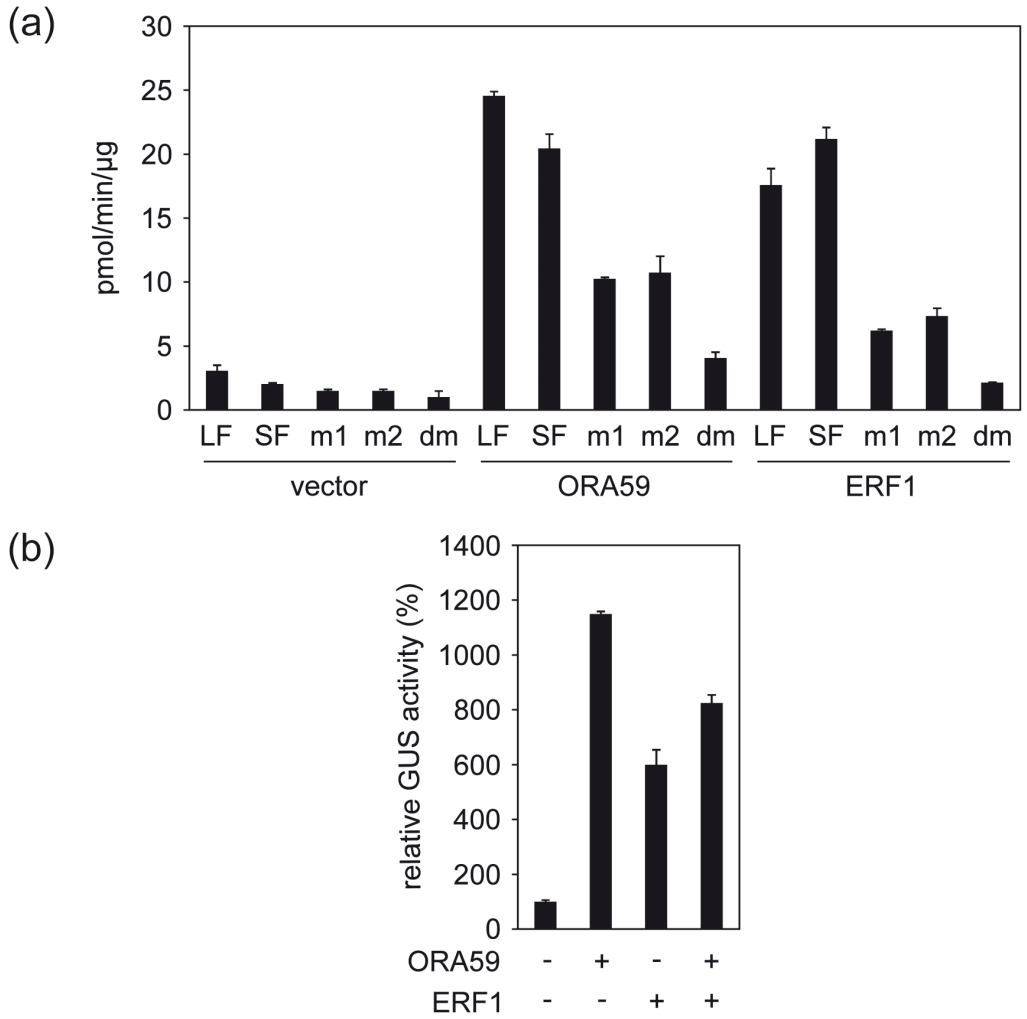

Figure 2. ORA59 and ERF1 trans-activate the *PDF1.2* promoter independently via two GCC boxes. **(a)** Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying different versions of the *PDF1.2* promoter shown in Figure 1A fused to *GUS* and overexpression vectors without or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

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

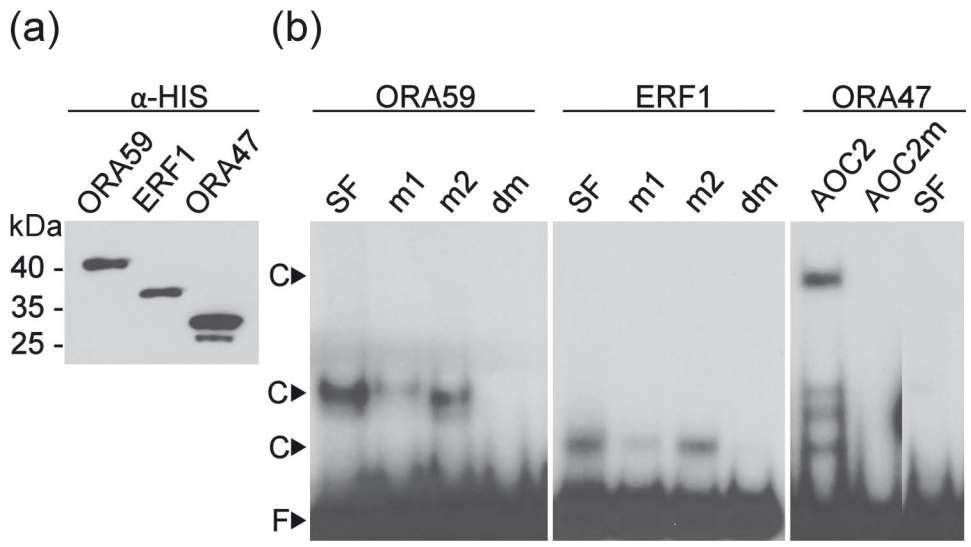

Figure 3. ORA59 and ERF1 bind to two GCC boxes in the *PDF1.2* promoter *in vitro*. **(a)** After SDS-PAGE recombinant proteins were detected with Penta-His HRP antibody conjugate following Western blotting. Protein size markers are indicated in k Dalton. **(b)** EMSAs were performed with recombinant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

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

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

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

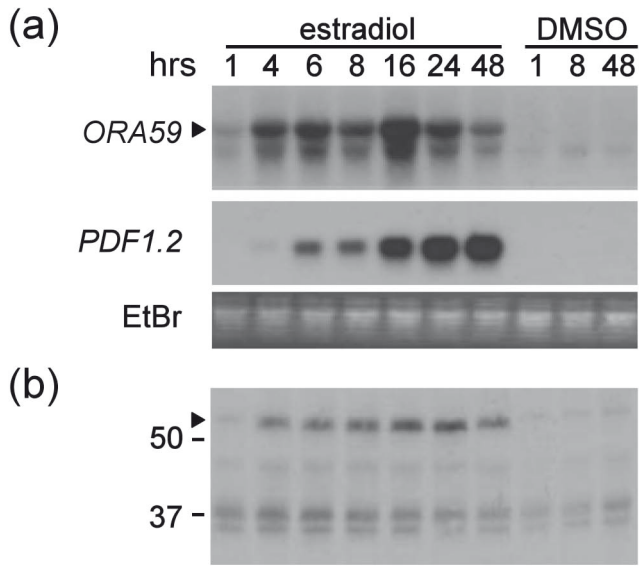

Figure 4. The ORA59-TAP protein is inducibly expressed and functional. Fifteen days-old T2 seedlings from XVE-ORA59-TAP line #4 cultured in liquid medium were treated for varying times in hours with 4 µM estradiol or the solvent DMSO at a final concentration of 0.1%. **(a)** Northern blot analysis. Gel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

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

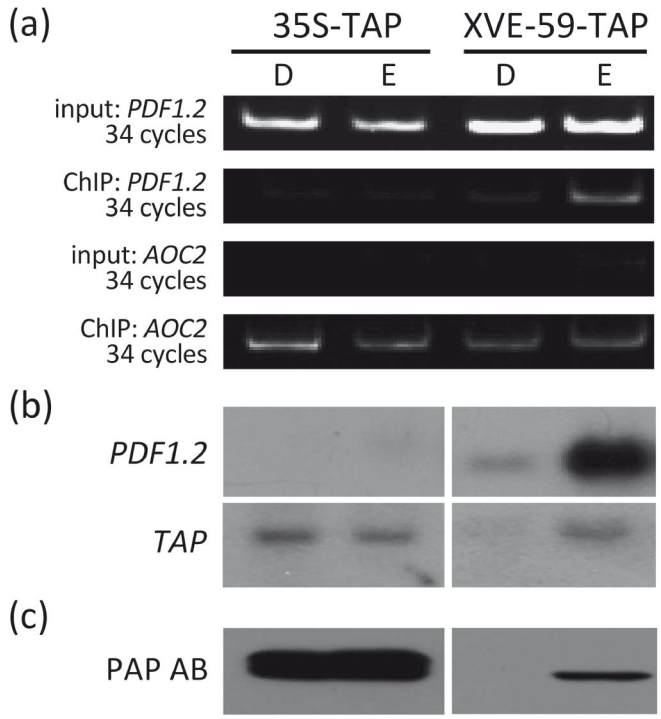

Figure 5. ORA59 binds to the *PDF1.2* promoter *in vivo*. Seedlings from XVE-ORA59-TAP line #4 and *35S*-TAP line #7 were treated with 4 µM estradiol (E) or 0.1% DMSO (D). RNA and protein was extracted for Northern and Western blot analysis of transgene expression. Sonicated chromatin prepared from the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

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

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

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

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

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

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

Discussion

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

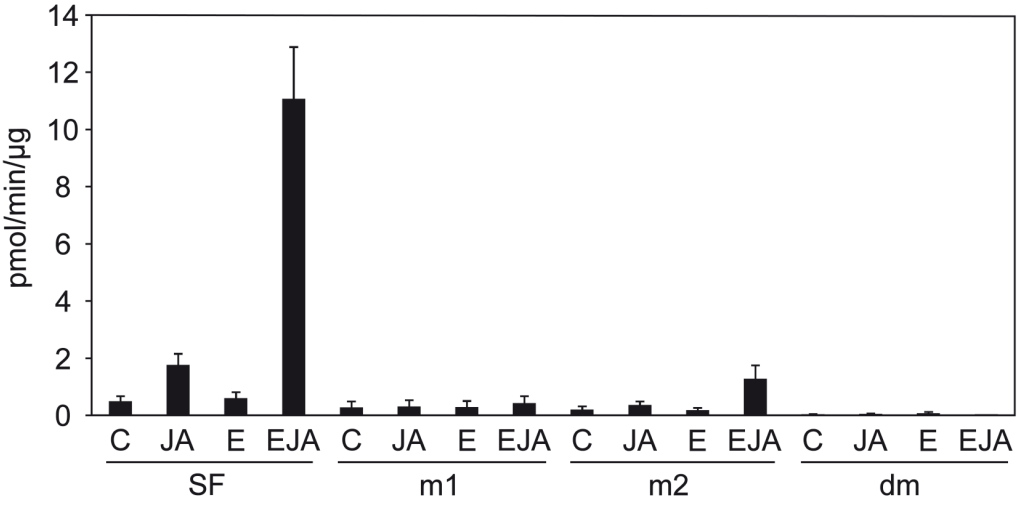

Figure 6. Both GCC boxes are essential for JA- and ethephon-responsive expression of *PDF1.2* promoter derivative SF in stably transformed Arabidopsis plants. Each bar represents average GUS activity values determined in pools of 10 T2 seedlings from 8 independent transformed lines for each construct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

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

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

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

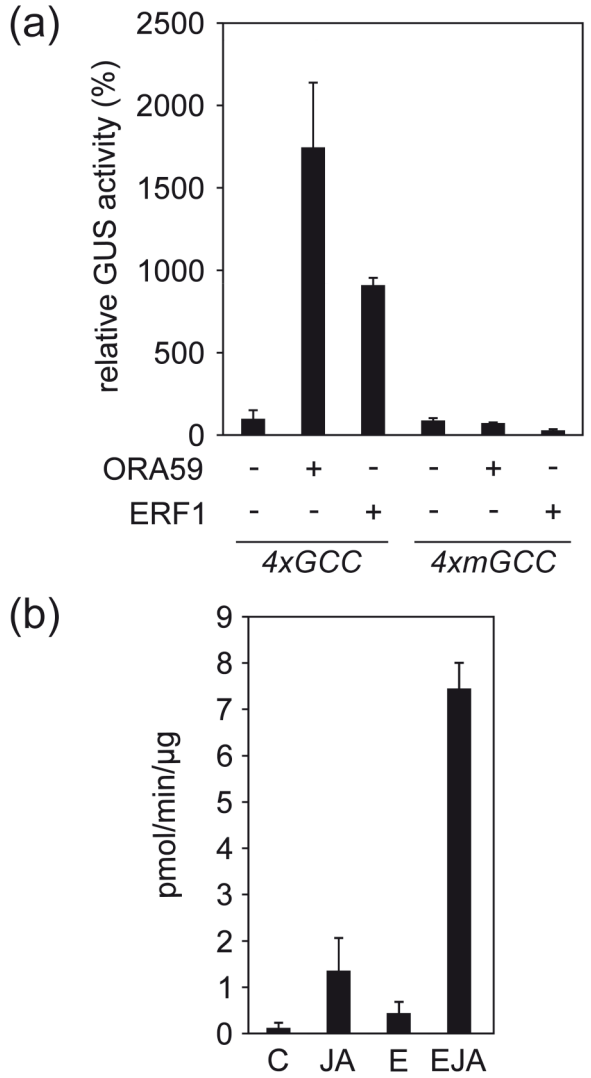

Figure 7. A GCC box tetramer is sufficient to confer JA- and ethephon-responsive expression in stably transformed Arabidopsis plants. **(a)** Arabidopsis protoplasts were co-transformed with 2 µg of wild-type or mutated 4xGCC-GUS and 6 µg of overexpression vectors containing *ORA59* or *ERF1* driven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

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

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

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

Materials and Methods

Growth conditions and treatments

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

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

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

Arabidopsis genomic DNA was used as template for the amplification of LF and SF fragments with forward primers 5'-CGG GAT CCA TGC AGC ATG CAT CGC CGC ATC-3' or 5' CGG GAT CCC CAT TCA GAT TAA CCA GCC GCC C-3', respectively, and the reverse primer 5'-GCG TCG ACG ATG ATT ATT ACT ATT TTG TTT TCA ATG-3'. Amplified products were digested with *Bam*HI and *Sal*I and cloned in plasmid GusXX (Pasquali et al., 1994). Mutations m1, m2 and dm were generated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and primers 5'-CCA TTC AGA TTA ACC ATC CTC ACC TGT GAA CGA TG-3' or 5'-CAT TAG CTA AAA GCC GAA TCA TCC TCT TAG GTT ACT TTA GAT ATC G-3', and their respective reverse complementary primers. For the construction of the *GCC box tetramers*, wild-type and mutant GCC-box monomers of the *PDF1.2* promoter were cloned by annealing the oligonucleotides 5'-GATC CTT AAC CAG CCG CCC ATG TGA-3' and 5'-GAT CTC ACA TGG GCG GCT GGT TAA G-3', and 5'-GATC CTT AAC CAT CCT CAC ATG TGA-3' and 5'-GAT CTC ACA TGT GAG GAT GGT TAA G-3', respectively, and ligating them into the plasmid pIC-20H (Marsh et al., 1984) digested with *Bam*HI and *Bgl*II. Monomers were then tetramerized in a head-to-tail configuration using the *Bam*HI and *Bgl*II sites. The tetramers were cloned as *Bam*HI/*Bgl*II fragments in the plasmid GusSH-47 (Pasquali et al., 1994) digested with *Bam*HI such that the orientation of the GCC-boxes relative to the downstream ORF was the same as in the *PDF1.2* promoter. The *ORA59* (At1g06160) open reading frame (ORF) was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-CGG GAT CCA TAT GGA ATA TCA AAC TAA CTT C-3' and 5'-CGG GAT CCT CAA GAA CAT GAT CTC ATA AG-3', digested with *Bam*HI and cloned in pRT101 (Töpfer et al., 1987). The *ERF1* ORF was PCR-amplified using the primer set 5'-GAA GAT CTT CAT CAC CAA GTC CCA CTA TTT TC-3' and 5'-GAA GAT CTC ATA TGG ACC CAT TTT TAA TTC AGT CC-3', digested with *Bgl*II and cloned in *Bam*HI-digested pRT101. The *ORA47* (At1g74930) ORF was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-GAA GAT CTC ATA TGG TGA AGC AAG CGA TGA AG-3' and 5'-GAA GAT CTT CAA AAA TCC CAA AGA ATC AAA G-3' and following digestion with *Bgl*II cloned in pIC-20R (Marsh et al., 1984). The *ORA47* insert was excised with *Bgl*II and inserted into pMOG183 (Mogen International, Leiden, The Netherlands) digested with *Bam*HI.

Binary constructs and plant transformation

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

ORA59 ORF lacking the stop codon (*ORA59*-Δstop) was amplified by PCR with the primer set 5'-ACG CGT CGA CAA AAT GGA ATA TCA AAC TAA CTT C-3' and 5' CCG CTC GAG CCT TGA GAA CAT GAT CTC ATA AG-3' and cloned in pGEM-T Easy (Promega). The *ORA59* ORF was excised from pGEM-T Easy with *Sal*I/*Xho*I and cloned into pC1300intB-35SnosBK-TAP. The *ORA59-TAP* fusion was excised with *Sal*I/*Spe*I from pC1300intB-35SnosBK-ORA59-TAP and introduced into the binary vector pER8 (Zuo et al., 2000) digested with *Xho*I/*Spe*I. The *PDF1.2* promoter derivatives SF, m1, m2 and dm fused to *GUS* and the tetrameric constructs *4xGCC:GUS* and *4xmGCC:GUS* were cloned into binary vector pMOG22λCAT (Pasquali et al., 1994; Menke et al., 1999) with *Xba*I/*Xho*I and *Xba*I/*Hin*dIII, respectively. The pMOG22λCAT binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993). Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MA medium containing 100 mg/L timentin and 20 mg/L hygromycin.

Protein production and immunoblot analysis

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

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

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

Electrophoretic mobility shift assays

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

Transient expression assays

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

Chromatin immunoprecipitation

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

RNA extraction and Northern blot analysis

Total RNA was isolated from tissue ground in liquid nitrogen by extraction with two volumes of phenol buffer (1:1 mixture of phenol containing 0.1% w/v 8-hydroxyquinoline and buffer containing 100 mM LiCl, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 100 mM Tris) and one volume of chloroform. After centrifugation, the aqueous phase was re-extracted with one volume of chloroform. RNA was precipitated overnight with LiCl at a final concentration of 2 M, washed twice with 70% ethanol, and resuspended in water. Northern blot analyses were performed as described (Memelink et al., 1994). Briefly, 10 µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose/1% v/v formaldehyde gels, and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). All probes were ^{32}P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washing were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed to Fuji X-ray films. The *PDF1.2* probe was PCR amplified from Arabidopsis genomic DNA using the primer set 5'-AAT GAG CTC TCA TGG CTA AGT TTG CTT CC-3' and 5'-AAT CCA TGG AAT ACA CAC GAT TTA GCA CC-3'. The TAP probe was excised from pBS1479 (Puig et al., 2001) with *Bam*HI.

Acknowledgements

A.C. was supported by a Marie Curie Intra-European fellowship within the European Community 5th Framework Programme (contract QLK5-CT-2002-51650). G.M. was supported by a Marie Curie Intra-European fellowship within the European Community 6th Framework Programme (contract MEIF-CT-2003-502102). A.Z. was supported by a grant from the Ministry of Science, Research and Technology, Iran (grant no. 7911580). A.C., G.M., A.Z. and A.P.K. were partially supported by a van der Leeuw grant from the Netherlands Organization for Scientific Research (NWO) awarded to J.M.

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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-domain transcription factor ORA59 acts as the integrator of the JA and ethylene signaling 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. 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 sequesters 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 including microbial pathogen attack and insect herbivory. Perception of stress signals results in the production of one or more of the secondary signaling molecules jasmonates (JAs), ethylene and salicylic acid (SA).

JAs are a group of related lipid-derived signaling molecules including the namesake compound jasmonic acid (JA) 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 a putative E3 ubiquitin ligase complex of the SCF type (Xie et al., 1998; 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; Chung et al., 2009). 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 resulting in expression of a set of genes involved in defense against wounding and herbivory.

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, Pré et al., 2008). 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; Pré et al., 2008) and confers resistance to the necrotrophic fungus *Botrytis cinerea* (Berrocal-Lobo et al., 2002; Pré et al., 2008). Analysis of plants in which *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 (Pré et al., 2008). Expression of *ORA59* (Atallah, 2005; Pré et al., 2008) and the subset of genes controlled by ORA59 including *PDF1.2* is also dependent on COI1 (Lorenzo et al., 2003; Pré et al., 2008). 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 superinduced by JA and ethephon consistent with previous reports (Penninckx et al., 1998; this thesis, Chapter 2). 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 JA/ethephon superinduction 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. ERF1, which is closely

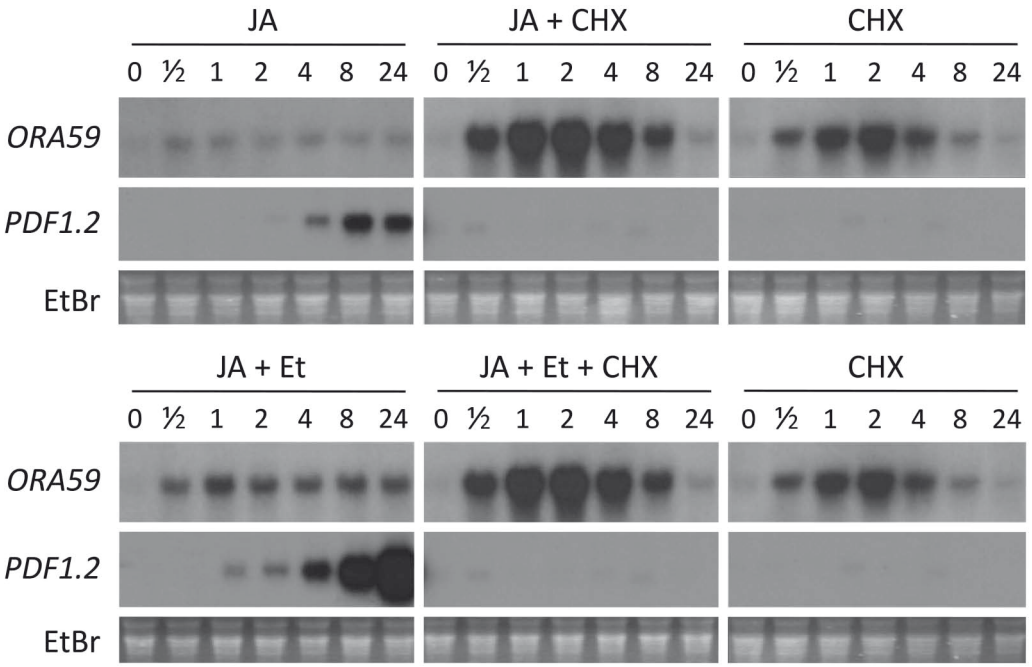

Figure 1. PDF1.2 is not an immediate-early JA-responsive gene. Fourteen-days old seedlings were treated with JA alone or in combination with the ethylene-releasing agent ethephon (Et) in the presence or absence of cycloheximide (CHX) for number of hrs as indicated. All panels hybridized with the same 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 or RNA loading.

related to ORA59 and can also switch on *PDF1.2* expression when overexpressed (Lorenzo et al., 2003; Pré et al., 2008), was also constitutively localized in the nucleus (Figure 2a).

The second remarkable observation was that when protoplasts transformed with either GFP-ORA59 (Figure 2a) or ORA59-GFP (Figure 2b) expression plasmids were treated with JA for 4 hrs, an increase in the proportion of cells showing nuclear localization of the GFP fusion protein was observed. A low frequency of nuclear localization was always observed in untreated or DMSO-treated protoplasts. Nuclear re-localization was a relatively slow process 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 demonstrate that the observed relocalization of ORA59 reflects a property of a functional

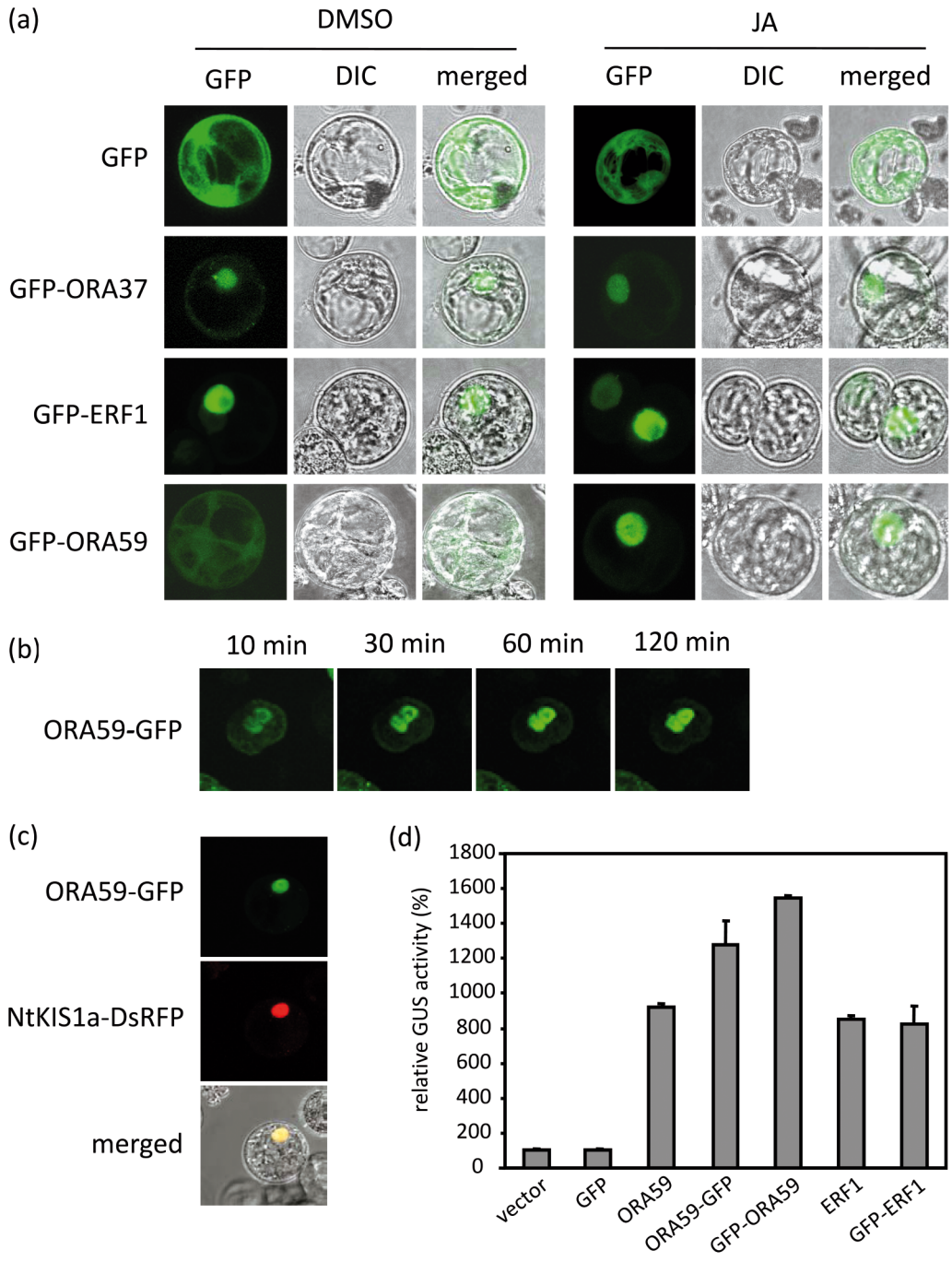

Figure 2. ORA59 accumulates in the nucleus of Arabidopsis protoplasts in response to JA. **(a)** GFP, GFP-ORA37, GFP-ERF1, GFP-ORA59 or ORA59-GFP constructs were transformed to Arabidopsis cell suspension protoplasts and examined by confocal laser scanning microscopy after treatment with 50 µM JA or 0.1% (v/v) DMSO as indicated for 4 hrs.

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 2d, the fusion proteins of ORA59 and ERF1 with GFP trans-activated the *PDF1.2* promoter to a similar level as the unfused ORA59 and ERF1 proteins. These results show that the GFP fusion proteins were functionally active as transcription factors.

ORA59 nuclear accumulation is also observed in *C. roseus* cells

To study whether this nuclear relocalization 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 *C. roseus* 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 protein 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.

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 wild-type leaf protoplasts and protoplasts derived from the mutant *coi1-1*. In *coi1-1* leaf protoplasts, the ORA59-GFP fusion protein relocalized to the nucleus in response to JA in a similar percentage of the cells as in wild-type protoplasts (Figures 4a and 4b), indicating that JA-induced nuclear accumulation of ORA59 did not require the COI1 protein.

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 µM 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 µM JA or 0.1% DMSO control for 1 hr. **(d)** GFP-transcription factor fusions are functional in transcriptional activation assays in Arabidopsis protoplasts. Arabidopsis protoplasts were co-transformed with the reporter construct *GUS* gene driven by a 277 bp *PDF1.2* promoter and one of the effector plasmids and the reference plasmid. The effector constructs consisted of an expression vector carrying the *CaMV 35S* promoter 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. Bars represent average GUS/LUC ratios from triplicate experiments ± SE expressed relative to the vector control set at 100%.

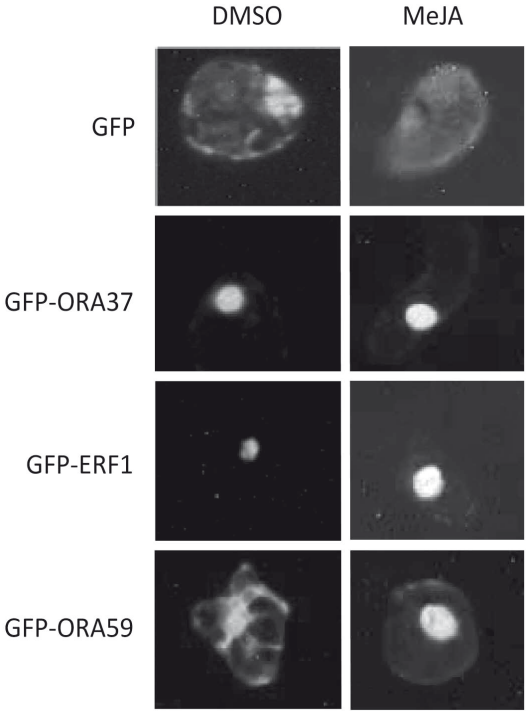

Figure 3. ORA59 accumulates in the nucleus of C. roseus cells in response to MeJA. GFP, GFP-ORA37, GFP-ERF1 and GFP-ORA59 expression plasmids were transformed to cell suspension cells of *C. roseus* and examined by confocal laser scanning microscopy after treatment with 100 µM methyl-jasmonic acid (MeJA)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

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/cytosolic distribution of a range of deletion derivatives. Domains with distinct features derived from *in silico* analysis of the ORA59 protein are shown in Figure 5a. The full-length ORA59 protein with GFP fused at its N-terminal end localized to the nucleus in about 45% of JA-treated protoplasts, whereas nuclear localization was observed in about 20% of untreated protoplasts (Figure 5b). ORA59 contains a putative bipartite nuclear localization signal (NLS) flanking the C-terminal end of the AP2 DNA-binding domain. Derivatives containing amino acids 48-139 or 81-139, both lacking the NLS, showed a dramatic reduction in nuclear localization, while derivatives 48-180 and 81-180, lacking both Ser-rich domains were virtually constitutively nuclear localized. Derivative 1-180 showed a qualitatively similar pattern of nuclear localization as full-length ORA59 but with 2-fold enhanced nuclear localization in cells without JA treatment (around 40% of the cells). Derivatives 48-244 and 81-244 did not show nuclear accumulation after JA treatment.

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 derivatives 1-180 and 81-244). In contrast there is a single domain responsible for JA-responsive nuclear localization since this function is only

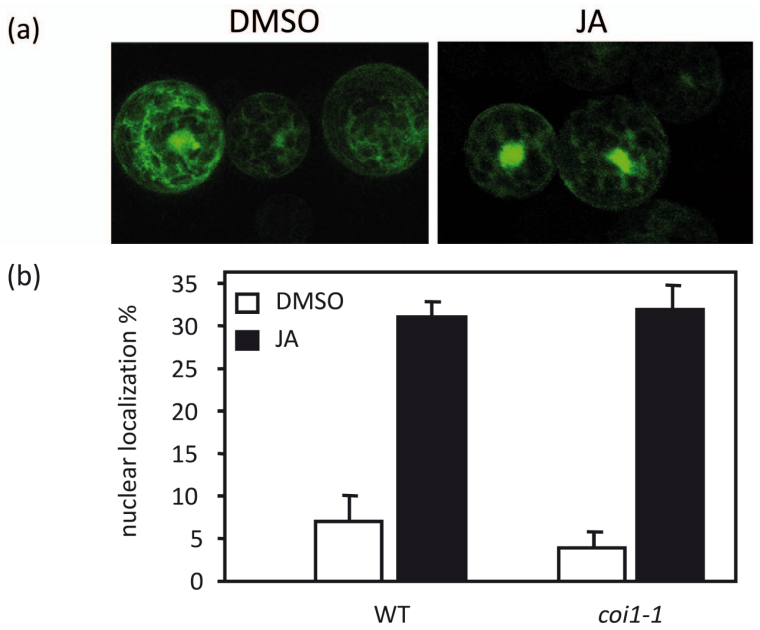

Figure 4. JA-responsive ORA59 nuclear localization is independent of COI1. **(a)** The ORA59-GFP expression plasmid was transformed to *coi1-1* leaf protoplasts and cells were examined by confocal laser scanning microscopy after treatment with 50 µM JA or 0.1 % DMSO for 2 hrs. **(b)** Nuclear localization 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.

present in full-length protein and in derivative 1-180. Based on the studied truncated proteins, both Ser-rich domains emerge as candidates for cytoplasmic retention, while the first Ser-rich domain could be involved in nuclear relocalization.

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 suggested 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 6a). Re-compartmentalization was clearly visible after 30 min of incubation in the presence of LMB and was nearly complete after 50 min (Figure

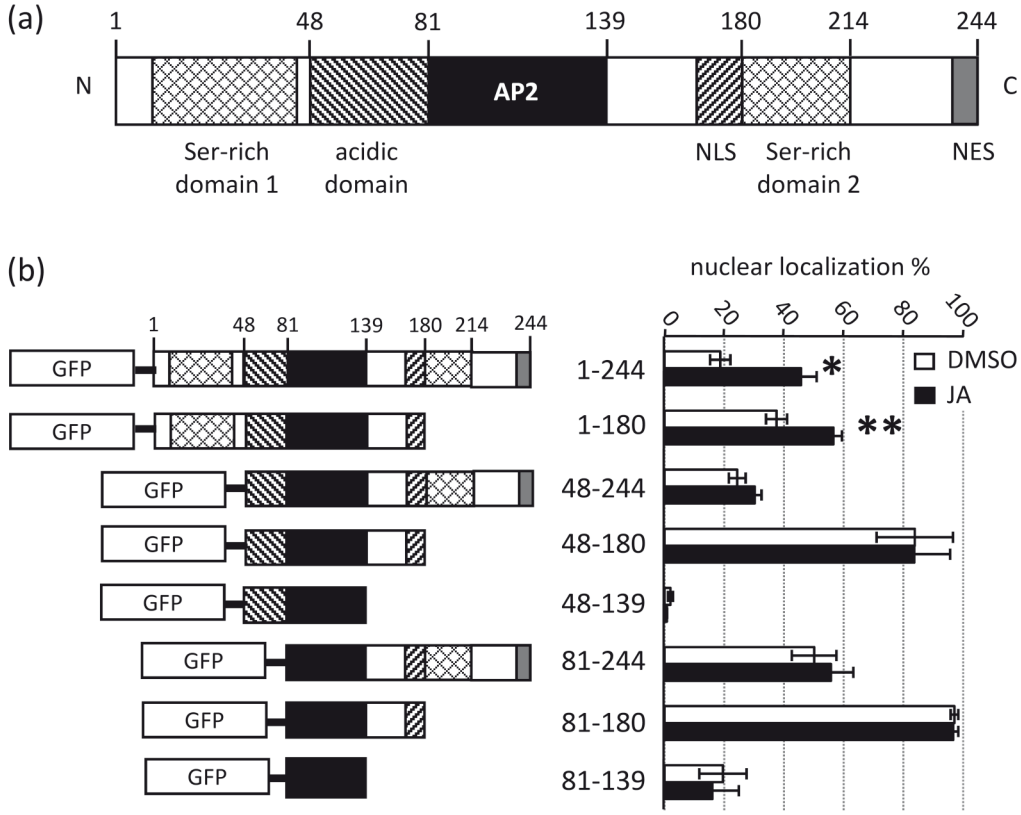

Figure 5. Mapping the ORA59 domain responsible for JA-responsive nuclear localization. **(a)** Schematic overview of domains in the ORA59 protein, which contains two Serine-rich domains (Ser-rich), an acidic domain, the AP2 domain, a putative bipartite nuclear localization signal (NLS) and a putative 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 µM 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 150 GFP-expressing protoplasts were analyzed. Bars represent average from triplicate experiments ± SE. Asterisks indicate significant differences between DMSO and JA treatments (T-test; *= 0.005; **= 0.014).

6b). This indicates that ORA59 shuttles between cytoplasm and nucleus at a slow rate.

ORA59 localization is determined by an active NLS and NES

Truncated proteins as used in the experiments in Figure 5 can exhibit conformational changes and thereby create experimental artefacts. To extend the observations of ORA59 localization

shown in Figures 5 and 6, GFP-fused ORA59 derivatives with point mutations in the NLS or specific deletions of the NLS or NES were studied. Since ORA59 contains a putative bipartite NLS, mutations were created in the first (m1), in the second (m2) or in both (m3) NLS parts (Figure 7a). Observation of Arabidopsis protoplasts expressing the mutant proteins showed that mutations in the first and second NLS parts dramatically reduced nuclear localization, whereas the double mutation resulted in exclusive cytosolic localization (Figures 7b and 7c). Deletion of the NLS (m4) had the same effect as the double mutation (Figures 7c). An interesting observation is that mutation m1 and also the double mutation m3 resulted in GFP signal mainly detected in the cytoplasm and often no signal at all in the nucleus, as shown in protoplasts co-transformed with a plasmid carrying a fusion between the nuclear tobacco protein NtKIS1a with the red fluorescent protein (DsRFP; Figure 7a - merged picture) (Jasinski et al., 2002). Mutation of the second NLS resulted in an even distribution of GFP signal in nucleus and cytoplasm, with a small proportion of cells with only nuclear signal. In agreement with the results obtained with the nuclear export inhibitor LMB (Figure 6), deletion of the NES (m5) prevented nuclear export and resulted in a high proportion of cells with nuclear localization of the GFP fusion protein (Figure 7b and 7c). The combination of double mutated NLS and lack of NES (m6) resulted in exclusive cytosolic distribution of the GFP fusion protein. These results confirm that indeed ORA59 shuttles between cytoplasm and nucleus due to the activity of a bipartite NLS and a NES.

ORA59 activation potential is determined by nuclear localization signals

Transcription factors work in the cell nucleus. Therefore, the fact that ORA59 shuttles between nucleus and cytoplasm prompted us to investigate whether ORA59 transcriptional activity was dependent on nuclear localization signals. ORA59 mutant proteins depicted in Figure 7a fused to GFP were tested for the ability to trans-activate the *PDF1.2* promoter in protoplasts. As shown in Figure 8a, ORA59 derivatives able to accumulate in the nucleus (m2 and m5) trans-activated the *PDF1.2* promoter. Surprisingly, the deletion derivative lacking the NES (m5) which showed increased nuclear accumulation activated the *PDF1.2* promoter to a 2-fold lower level than wild-type ORA59 protein. Trans-activation assays with unfused ORA59 derivatives gave similar results but with a less strong reduction of activation by m5 (Figure 8b). In conclusion, a functional NLS is necessary for nuclear localization and trans-activation, which is not surprising. What is surprising however is that removal of the NES resulted in a higher percentage of nuclear localization but in significantly lower trans-activation.

JA stabilizes 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

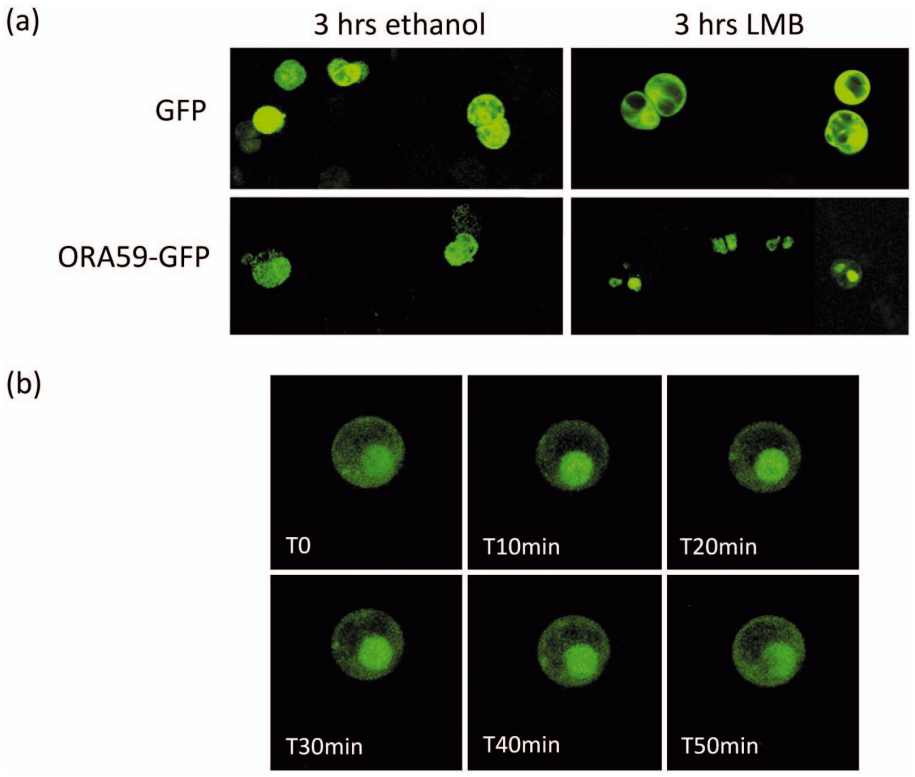

Figure 6. Inhibition of nuclear export leads to nuclear localization of ORA59. **(a)** Confocal laser scanning microscopy images of Arabidopsis cell suspension protoplasts expressing ORA59-GFP or GFP. Sixteen hrs after transformation with expression plasmids protoplasts were treated for 3 hrs with 2 µM 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 µM LMB.

two decades 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 9a). 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.

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

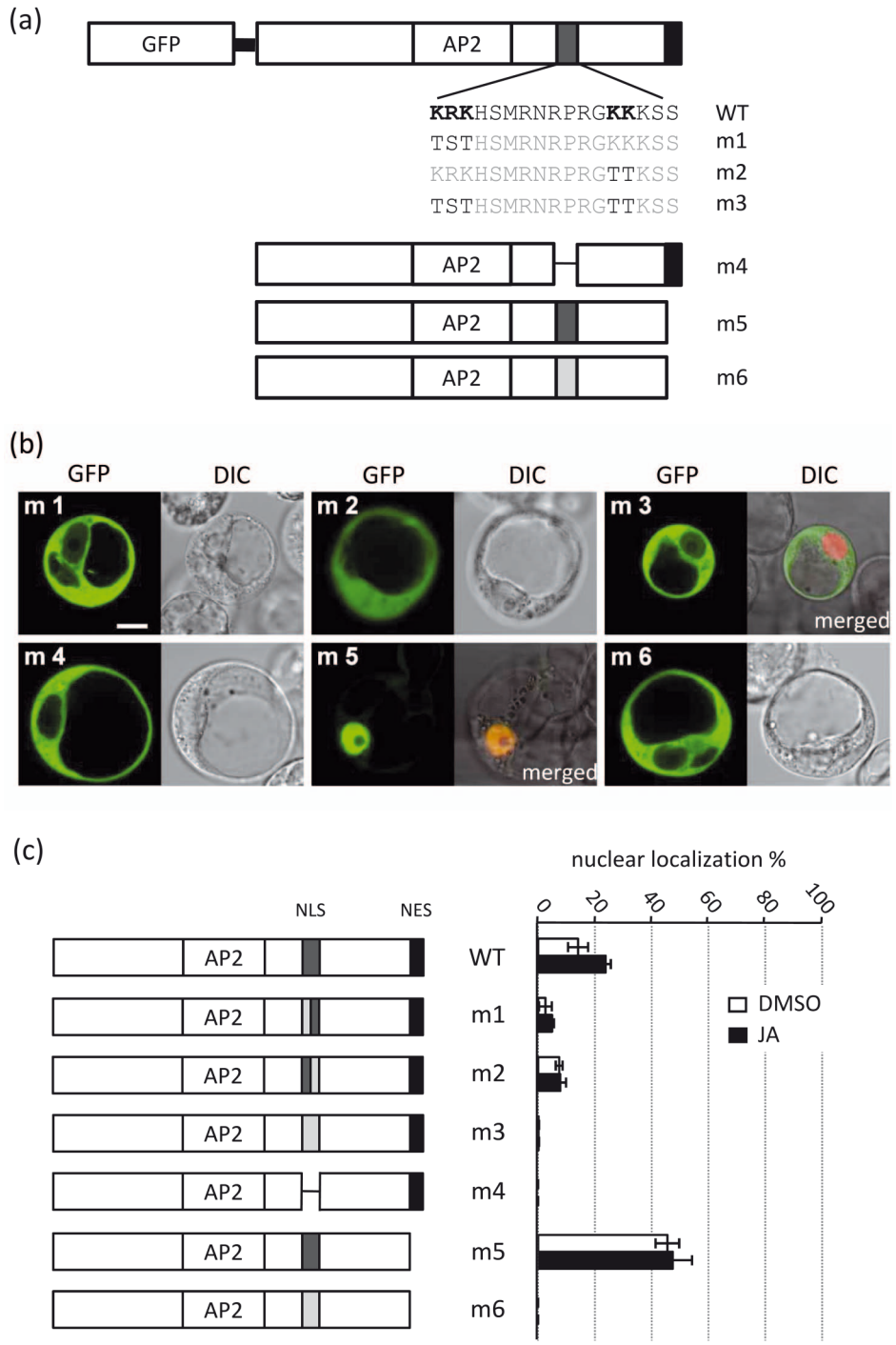

Figure 7. ORA59 localization is determined by an active NLS and NES. **(a)** Schematic overview of ORA59 derivatives with mutations in the putative bipartite nuclear localization

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 4, 8 and 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* (Figure 9b), demonstrating that the ORA59-HA fusion protein was functional as a transcriptional activator. As shown in Figure 9c, 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 9b). 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 transformed with *ORA59-GFP* expression plasmid were incubated in the dark for 16 hrs and then treated with MG132 or with the solvent DMSO for 2 hrs. Fluorescence microscopy showed that MG132, but not DMSO, increased nuclear and cytosolic abundance of ORA59-GFP in protoplasts (Figure 10a). Total protein was extracted from protoplasts co-transformed with *ORA59-GFP* and *GFP* expression plasmids and subjected to immunoblot analysis with anti-GFP antibodies. As shown in Figure 10c (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 10b 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,

signal (NLS) and nuclear export signal position (NES). Bold letter case indicates substituted amino acids. The dark gray box represents the wild-type NLS, whereas the light gray box represents the double mutated NLS. **(b)** GFP-ORA59 mutant constructs (m1-m6) were transformed to Arabidopsis cell suspension protoplasts and examined by confocal laser scanning microscopy. Pictures of representative cells are shown. Merged pictures indicate nucleus of protoplasts co-transformed with NtKIS1a-DsRed. Scale bar = 10 µm. **(c)** Sixteen hrs after transformation Arabidopsis cell suspension protoplasts transformed with the indicated ORA59 mutant derivative fused to GFP were treated for 2 hrs with 0.1% DMSO or 50 µM 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 150 GFP-expressing protoplasts were analyzed. Bars represent the average from triplicate experiments ± SE.

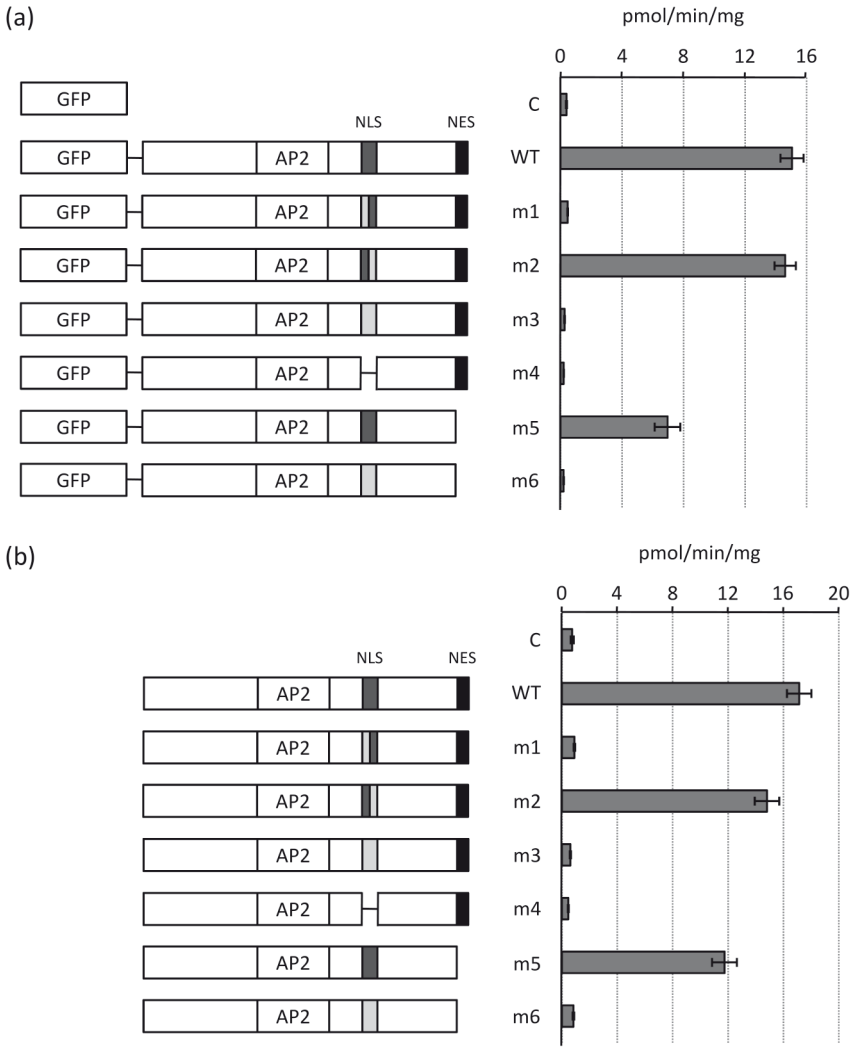

Figure 8. ORA59 activation potential is determined by nuclear localization signals. **(a)** Arabidopsis protoplasts were co-transformed with a *GUS* reporter gene driven by the 277 bp *PDF1.2* promoter and a *CaMV 35S* expression vector containing GFP fusions of wild-type (WT) or NLS/NES mutant versions of ORA59 or GFP alone, as indicated. **(b)** Same as (a) but with unfused proteins, as indicated. Empty vectors were used as control. Bars represent average GUS activity values from triplicate experiments corrected for protein concentrations ± SE.

indicating that there is not a single degron. Low amounts were detected of derivatives 1-180 and 81-244. Deletion derivatives 48-180, 48-139, 81-180 and 81-139 were almost as stable as GFP. This suggests that the Ser-rich domains are responsible for instability of ORA59. Next protein amounts were analyzed after treatment of transformed protoplasts with MG132 (Figure 10c). All

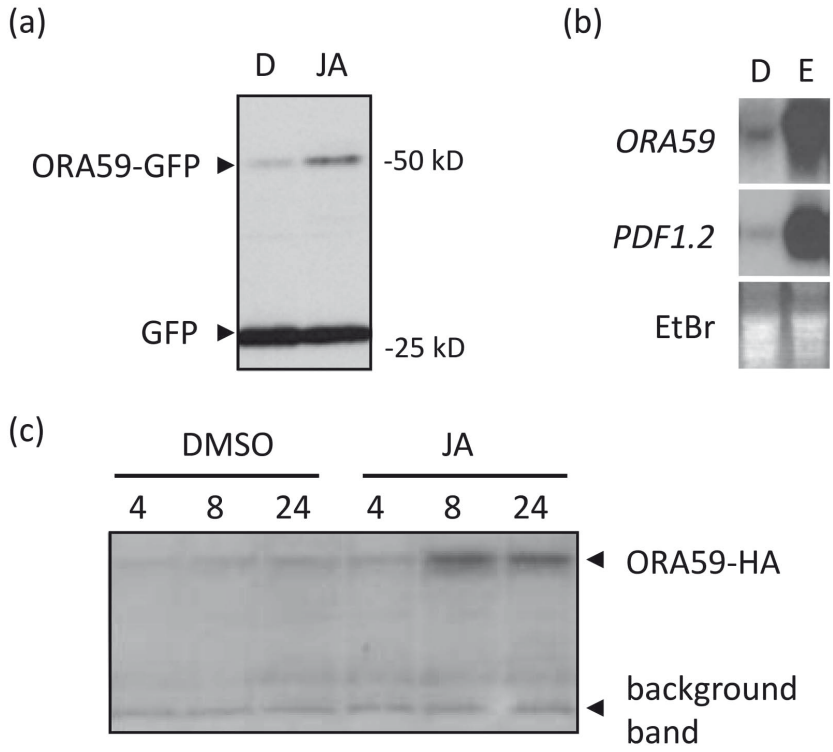

Figure 9. JA stabilizes the ORA59 protein. **(a)** Immunoblot analysis with anti-GFP antibodies of total protein extracts prepared from Arabidopsis cell suspension protoplasts co-expressing GFP and ORA59-GFP treated with 50 µM JA or 0.1% DMSO for 4 hrs. **(b)** Functional analysis of the XVE-ORA59-HA construct (line #18) treated with 2 µM estradiol (E) or 0.1% DMSO (D) for 16 hours. RNA blots were hybridized with the indicated probes. **(c)** Immunoblot analysis with anti-HA antibodies of total protein extracts from 14-days-old plants XVE-ORA59-HA (line #18) treated simultaneously with 2 µM estradiol and 50 µM JA or 0.1% DMSO. Intensities of the background band confirm equal protein loading.

deletion derivatives except GFP-81-139 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-48-139 that does not contain an NLS and did not accumulate in the nucleus (Figure 5b) 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

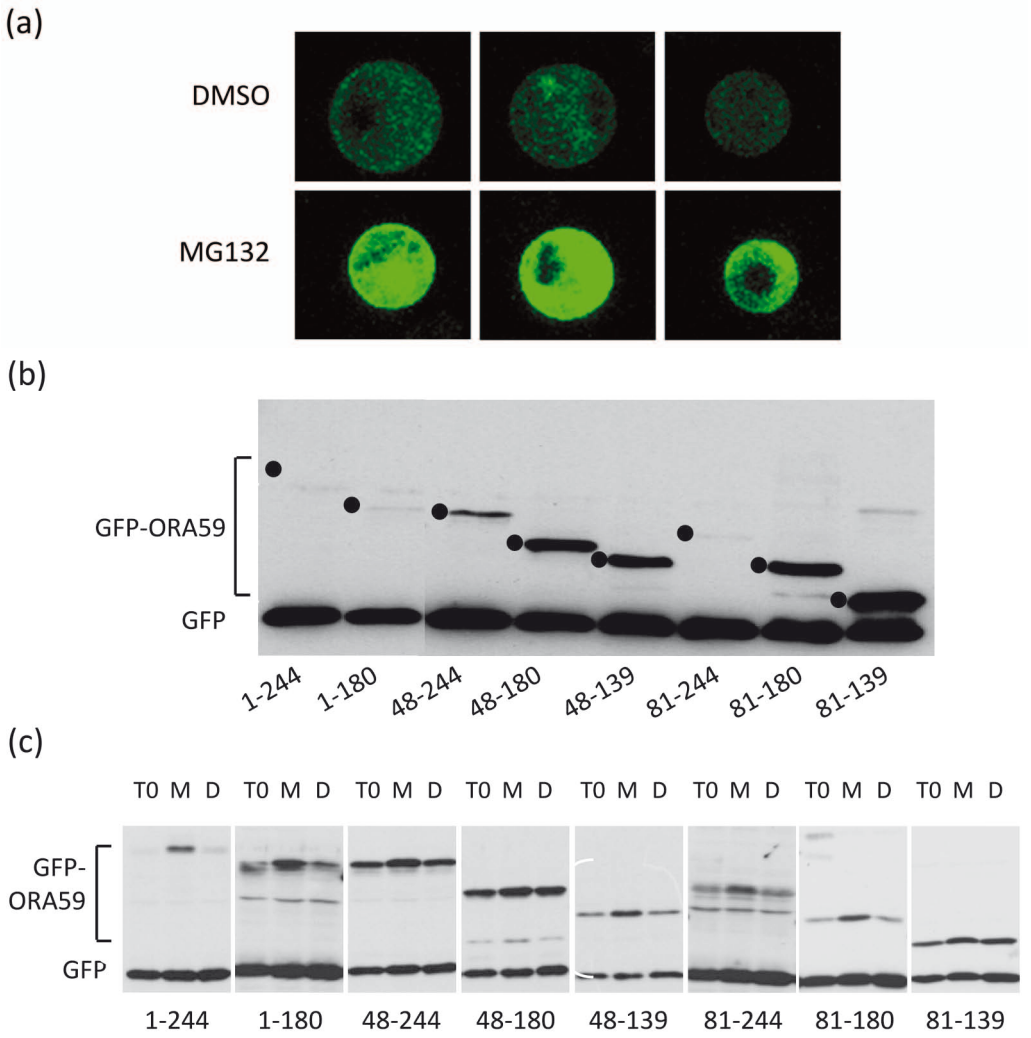

Figure 10. ORA59 is degraded by the 26S proteasome. **(a)** Confocal laser scanning microscopy images of Arabidopsis cell suspension protoplasts expressing ORA59-GFP. Sixteen hrs after transformation protoplasts were treated for 2 hrs with 50 µM MG132 or 0.1% DMSO. Pictures taken at identical confocal 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 µM of the 26S proteasome inhibitor MG132 (M). The upper band is the full-length 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

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 11, deletion derivatives lacking the putative NLS were inactive in the trans-activation assay, reconfirming the importance of a functional NLS. Derivative 81-180 containing the AP2 domain and the NLS had 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 derivatives 48-244 and 48-180 it appeared that the C-terminal Ser-rich region functions as an activation domain (Figure 11b). By comparing the activities of the GFP fusion derivatives 1-180 and 48-180 it appeared that the N-terminal Ser-rich region functions as an activation domain. The acidic region from positions 48-80 functioned only as an activation domain when deletion derivative 48-180 was not fused to GFP (compare to 81-180; Figure 11a).

In general activities of GFP fusion proteins were similar to those of the unfused deletion derivatives, with the differences that derivative 48-244 was relatively active as a GFP fusion, and that the activity of 48-180 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 11b), whereas the protein was undetectable by Western blotting (Figure 10b). Deletion derivative 48-180 on the other hand was almost inactive as a transcription activator when fused to GFP (Figure 11b), whereas the protein was as abundant as the co-expressed GFP (Figure 10b).

ORA59 is nuclear and cytosolic in transgenic plants

In order to assess ORA59 localization in Arabidopsis plants, transgenic lines overexpressing ORA59 fused C-terminally to GFP were generated. Independent lines with high overexpression of functional GFP-ORA59 were selected by measuring the expression levels of *ORA59* and its target gene *PDF1.2* (Figure 12a). Selected lines were also evaluated for levels of GFP-ORA59 protein accumulation by immunoblotting with anti-GFP antibodies, but it turned out to be undetectable. As shown in Figure 6, MG132 stabilized ORA59. Therefore seedlings were treated with MG132 or the solvent DMSO for 16 h prior to protein extraction. Among the lines tested, a low level of GFP-ORA59 protein could be detected only in line # 21 after MG132 treatment (Figure 12b). Lines # 3, 5 and 21 were further analyzed by confocal laser scanning microscopy, but GFP signal was difficult to detect. JA and MG132 improved the signal strength, but it remained difficult to detect GFP fluorescence. Remarkably, incubation of 4-7 days old seedlings in water for 24 h after

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.

germination in solid medium slightly improved the GFP signal. Under these conditions, ORA59-GFP was shown to be localized both in the nucleus and in the cytoplasm of root and leaf cells of line # 21 (Figure 12c).

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. Our results show that JA controls ORA59 at multiple levels, i.e. via transcriptional induction and by acting on 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. Comparison of *PDF1.2* trans-activation levels by derivatives 1-180, 48-244, 81-244 and NES deletion m5 indicate that the acidic domain is the N-terminal region partially important for activation, while the NES is responsible for the activation of the C-terminal 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 et al., 2008). ERF1 was also stabilized by MG132 (data not shown), but was 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, while the presence of both determined a stronger cytoplasmic retention.

ORA59 contains active NLS and NES sequences, resulting in protein shuttling. Removal of

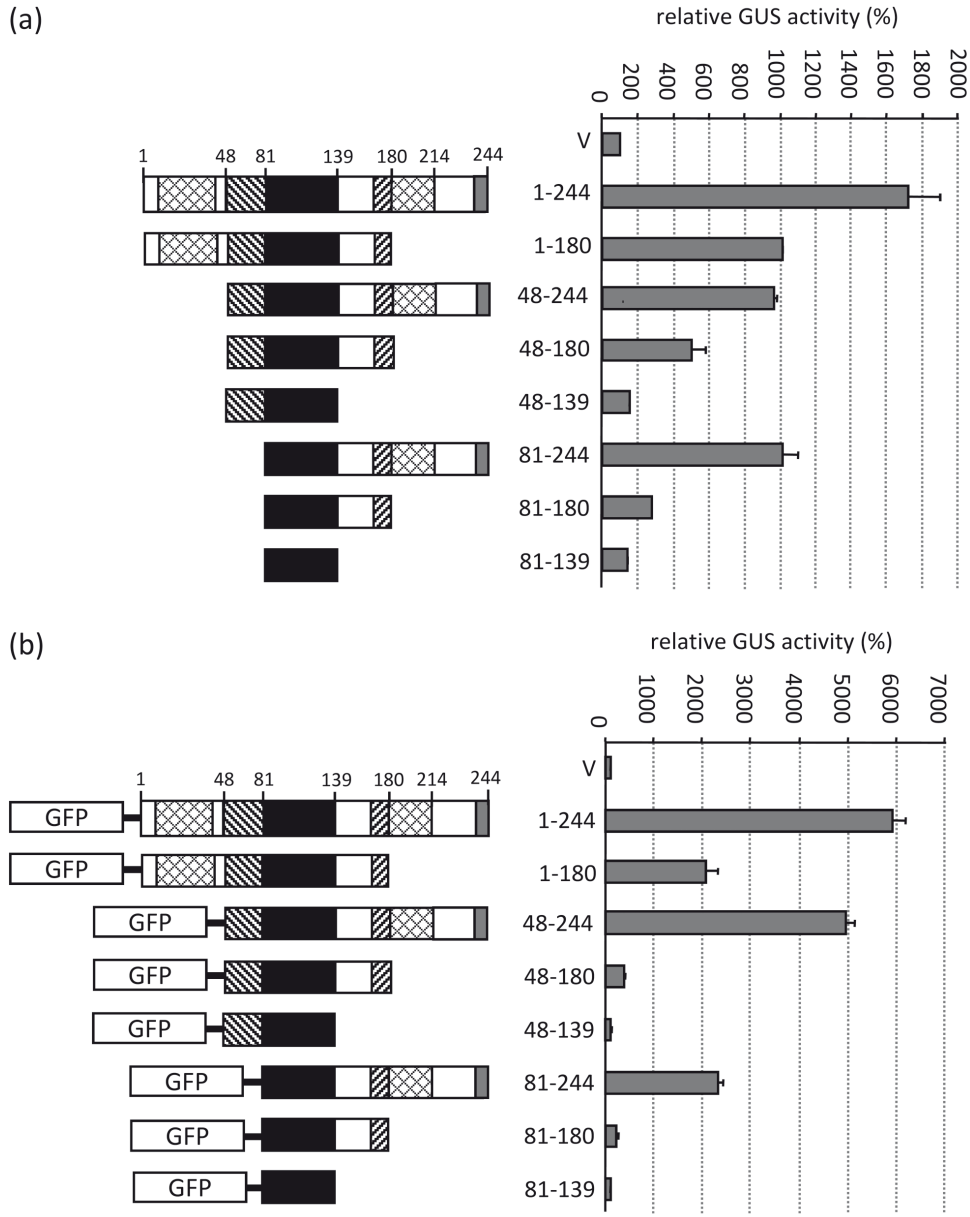

Figure 11. Functional mapping of the activation domain in ORA59. **(a)** Arabidopsis protoplasts were co-transformed with a *GUS* reporter gene driven by the 277 bp *PDF1.2* promoter and a *CaMV 35S* expression vector containing full-length (FL) ORA59 or one of the deletion derivatives as indicated. 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 ± SE expressed relative to the vector control set at 100%. **(b)** Same as in (a) but with fusions between ORA59 derivatives and GFP.

the NES resulted in nuclear localization in about 50% of the cells, whereas derivative 81-180, lacking in addition to the NES several other regions, showed nearly 100% nuclear localization. This indicates that one or more of these other regions are involved in cytoplasmic retention. Surprisingly removal of the NES, although resulting in a 2-fold increase in nuclear localization did not result in a concomitant increase in trans-activation of the *PDF1.2* promoter, but instead caused a 2-fold decrease. This suggests that active shuttling contributes to ORA59 activity, but how this comes about at the molecular level is unclear.

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 et al., 2008). COI1 is the jasmonate receptor which directly interacts with an Ile-conjugated form of jasmonic acid (Chung et al., 2009). Upon binding of JA-Ile COI1 interacts with the JAZ repressors 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 other biologically active jasmonates besides JA-Ile exist and do not bind COI1 and that ORA59 relocalization is COI1-independent indicates that at least one other 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.

The nucleocytoplasmic shuttling of ORA59 and its (in)stability shows striking similarities with the well studied regulation of the tumor supressor p53 in mammals. In normal cells under non-stressed conditions, p53 is a short-lived transcription factor which shuttles between the nucleus and the cytoplasm (Woods and Vousden, 2001) and is maintained in a latent form. Nuclear p53 levels are tightly regulated by the critical interacting protein Mdm2 or Hdm2 in human cells, an E3 ubiquitin ligase that promotes p53 nuclear export and cytoplasmic turnover. Studies suggest the presence of a cytoplasmic sequestration domain (CSD) in the C-terminus of p53 that inhibits association of importin with the C-terminal NLS1 (Liang et al., 1998; Liang et al., 1999), thereby preventing p53 nuclear import. In response to cellular stresses, p53 becomes transiently stabilized and translocates to the nucleus, where it forms a tetrameric complex which masks the C-terminal NES, thus trapping p53 in the nucleus (Stommel et al., 1999). An additional regulation is mediated by phosphorylation of the N-terminal NES in p53, which blocks p53 nuclear export (Zhang and Xiong, 2001).

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

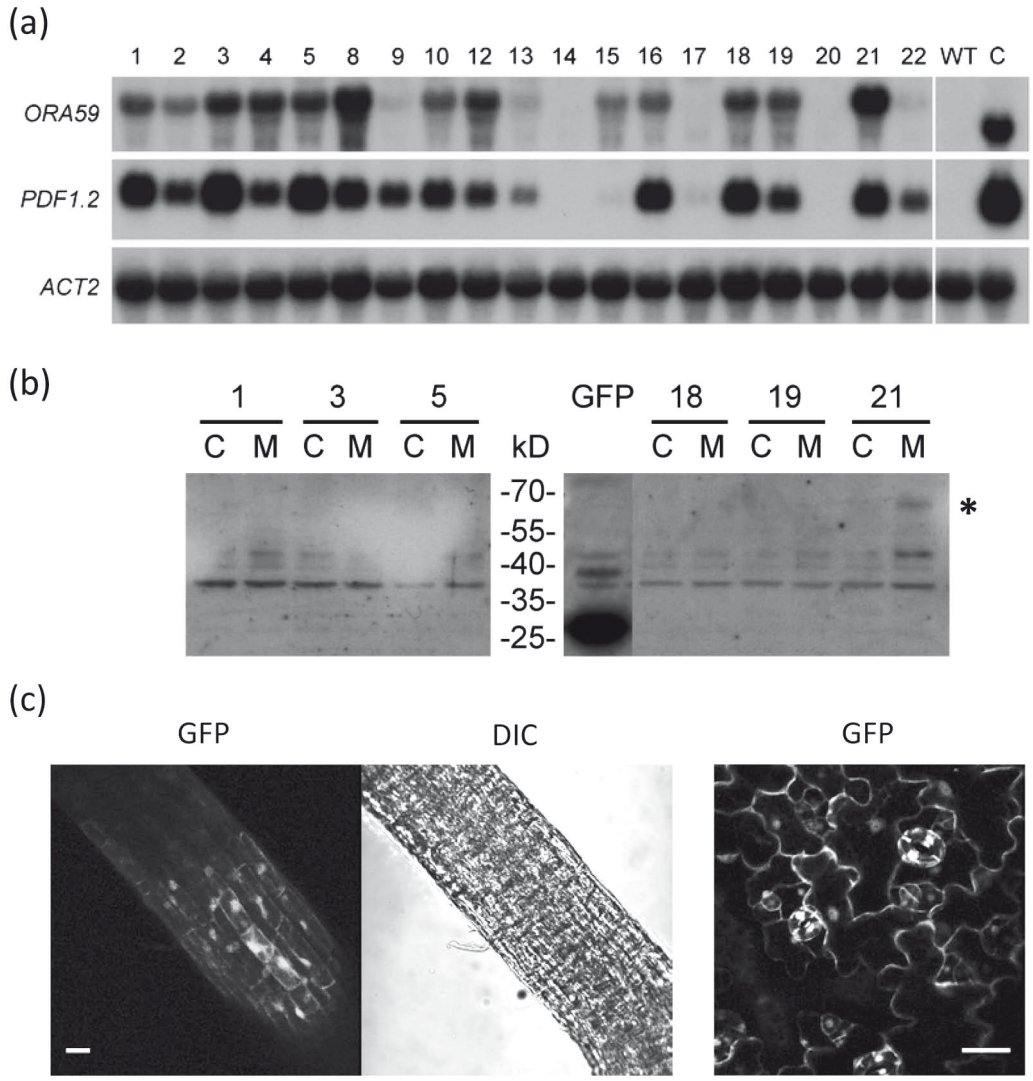

Figure 12. ORA59 is nuclear and cytosolic in transgenic plants. **(a)** Northern blot analysis. Fourteen-days-old seedlings from several *35S:ORA59-GFP* transgenic lines were screened by RNA blot hybridization with the indicated probes. A transgenic line (#2) expressing unfused ORA59 from the *35S* promoter was used as control. The ACTIN2 probe was used to verify equal loading. **(b)** Western blot analysis. Fourteen-days old seedlings were treated with 50 µM MG132 (M) or 0.1% DSMO (C) for 16 hours. Protein samples were separated by SDS-PAGE followed by Western blotting and immuno-probing with anti-GFP antibody. The asterisk indicates the position of the ORA59-GFP fusion protein. Positions of protein size markers are indicated in k Dalton. **(c)** Confocal laser scanning microscopy images of five-days-old *35S:ORA59-GFP* Arabidopsis seedlings of line #21, after 24 h incubation in water. Representative pictures of root and leaf tissues are shown. Scale bar = 25 µm.

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.

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

	act. PDF1.2 unfused[a]	act. PDF1.2 nGFP[a]	stability[b]	stabilized by MG132[c]	% nuclear DMSO	% nuclear JA
1-244	+	+	-	+	19	46
1-180	+	+	-	±	38	57
48-244	+	+	±	±	24	30
48-180	±	-	+	±	84	84
48-139	-	-	+	±	2	1
81-244	+	+	-	±	50	56
81-180	-	-	+	±	97	97
81-139	-	-	+	-	20	16

[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.

Materials and Methods

Constructs 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 *Bam*HI and *Eco*RI/*Spe*I respectively and cloned into pTH2^{BN} digested with *Bam*HI/*Bgl*II and *Eco*RI/*Spe*I 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 *Bgl*II and cloned into pBluescript SK+ digested with *Bam*HI, then *ERF1* was excised from pBluescript SK+-ERF1 with *Eco*RI/*Spe*I and cloned into pTH2^{BN} digested with *Eco*RI/*Spe*I to generate GFP-ERF1. ORA59 deletion derivatives 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 1-180, 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 48-244, 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 48-180, 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 48-139, 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 81-244, 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 81-180, 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 81-139. All ORA59 deletion derivatives were cloned in pGEM-T Easy (Promega, Madison, WI) and then inserts were excised with *Bam*HI and *Eco*RI and cloned in pRT101 (Töpfer et al., 1987). For N-terminal GFP fusions ORA59 deletion derivatives were excised from pGEM-T Easy with *Eco*RI and *Spe*I 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 *Sal*I/*Xho*I and cloned into pBluescript SK+ digested with *Sal*I. Then *ORA59-ΔSTOP* was excised from pBluescript SK+ with *Sal*I/*Eco*RI and cloned into pTH2^{SN} (another derivative of pTH2; Kuijt et al., 2004). *ORA59-ΔSTOP* was excised from pTH2^{SN} with *Sal*I/*Nco*I 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. Mutations of ORA59-NLS were generated according to the QuickChange Site-Directed Mutagenesis protocol (Stratagene) using pBluescript SK+-ORA59 as template and primers 5'-GTG ATA GCC TTG ACG TCG ACA CAC TCC ATG AGA-3' and 5'-TCT CAT GGA GTG TGT CGA CGT CAA GGC TAT CAC-3' for m1; 5'-GAA ACC GTC CTA GAG GAA CGA CGA CCT CGA GTT CTT CTT CGA CGT TG-3' and 5'- CAA CGT CGA AGA AGA ACT CGA GGT CGT CGT TCC TCT AGG ACG GTT TC-3' for m2 and m3 (using m1 in pGEM-T Easy as template); Deletions m1 and m2 were PCR amplified with 5'- CGG AAT TCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CG GGA TCC TCA AGA ACA TGA TCT CAT AAG-3' and ligated in pGEM-T-Easy. The NLS was deleted by excision of an *Nde*I/*Nco*I m3 fragment from pGEM-T-Easy, subsequently digested with *Sal*I and *Xho*I. Digested fragment was ligated in pGEM-T-Easy digested with *Nde*I and *Nco*I. Deletion of the NES was generated with primer set 5'- CGG AAT TCA AAA TGG AAT ATC AAA CTA ACT TC-3' and 5'- GAC TAC TAG TTC AAA GAA CCA CAA GTG TTG TAT TAC-3' for m5 and m6 (using m3 as template). Deletions m1, m2 m3 and m4 were excised with *Eco*RI and *Bam*HI from pGEM-T Easy and cloned in pRT101 digested with *Eco*RI and *Bam*HI. Deletions m5 and m6 were digested with *Eco*RI from pGEM-T Easy and cloned in pRT101 digested with *Eco*RI, respectively. Deletions m1, m5 and m6 were excised with *Eco*RI and *Spe*I from pRT101 and cloned in pTH2^{BN} digested with *Eco*RI and *Spe*I. Deletion m2 was excised with *Bgl*II and m3 and m4 with *Eco*RI from pRT101 and cloned in pTH2^{BN} digested with *Bam*HI and *Eco*RI, respectively.

Protoplasts prepared from *Arabidopsis thaliana* cell suspension culture ecotype Col-0 (Axelos et al., 1992) 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 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 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 from triplicate transformations were related to LUC activities in the same samples or to total protein content, determined by Bradford, to correct for differences in transformation and protein extraction efficiencies.

Microscopy

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 on slides in a drop of water and examination of GFP fluorescence using an Axioplan upright microscope (Zeiss, Germany) equipped with a BIORAD MRC1024ES scanhead with a krypton/argon laser. ORA59-GFP seedlings were germinated in solid MA medium for 4 to 7 days-old and incubated 24h in water prior to microscopy analysis.

For visualization of GFP the excitation wavelength was 488 nm while the emitted fluorescence was collected after passage through a broad band pass filter (500-550 nm). The resulting signal was amplified, digitalized and the consistent picture reconstituted by Leica software.

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 Na_3VO_4, 5 µg/ml leupeptin, 5 µg/ml antipain, 5 mM DTT and 1 mM phenylmethylsulfonyl-fluoride (PMSF)). Fourteen-days old seedlings were frozen in liquid nitrogen and then ground in 100 µl of TAP protein extraction buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM EDTA pH 8.0, 0.1% Igepal, 10 mM 2-mercaptoethanol, 2 mM benzamidine, 1 mM PMSF, 20 mM NaF, protease inhibitor cocktail Complete Mini (Roche)). 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% H_2O_2) 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) dissolved in DMSO (0.1% final concentration). Seedlings were treated for different time periods with 50 µM JA (Sigma-Aldrich) or 50 µM MG132 dissolved in DMSO at a final concentration of 0.1%. Control seedlings were treated with 0.2% DMSO. 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.

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 *Kpn*I/*Bam*HI fragment into pRT101. The expression cassette was transferred as a *Xho*I/*Xba*I fragment to pER8 (Zuo et al., 2000) digested with *Xho*I/*Spe*I. For the generation of *35S:ORA59-GFP* plants the expression cassette was transferred from $pTH2^{BN}$ as a *Hind*III/*Eco*RI fragment to pCAMBIA1300 (acc No. AF234296) digested with *Xho*I/*Spe*I. Binary vectors were 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 µM cycloheximide (CHX) dissolved in DMSO (0.1% final concentration) and then JA (Sigma-Aldrich) dissolved in DMSO at a final concentration of 50 µM 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 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 ^{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.

Acknowledgements

A.C. was supported by a Marie Curie Intra-European fellowship within the European Community 5th Framework Programme (contract QLK5-CT-2002-51650). A.Z. was supported by a grant from the Ministry of Science, Research and Technology, Iran (grant no. 7911580). A.Z. and A.P.K. were partially supported by a van der Leeuw grant from the Netherlands Organization for Scientific Research (NWO) awarded to J.M.

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CHAPTER 4

The CCCH zinc finger protein ZFAR1 interacts with the JA/ET-responsive transcription factor ORA59 and influences basal resistance against *Botrytis cinerea*

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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 (ET). In defense against necrotrophic pathogens, JA and ET signaling pathways synergize to activate a specific set of defense genes, including *PDF1.2* and *HEL*. The AP2-domain transcription factor ORA59 acts as the integrator of the JA and ET signaling pathways in *Arabidopsis thaliana*. Previous experimental results suggested that JA and ET affect the activity of ORA59 at the protein level via unknown mechanisms. To study regulation of ORA59 activity, we set out to identify and functionally characterize ORA59-interacting proteins. The CCCH zinc finger protein ZFAR1 was identified as a putative interacting protein from a yeast two-hybrid screening. Transient expression assays in Arabidopsis cell suspension protoplasts showed that ZFAR1 interacted with ORA59 in the cytoplasm and that it interfered with JA-induced nuclear localization of ORA59. Moreover, ZFAR1 repressed ORA59 activity in protoplasts. Transgenic plants overexpressing ZFAR1 showed accelerated disease progression while a knockout mutant was less severely affected than wild-type plants by *Botrytis* infection. Our results indicate that ZFAR1 acts as a repressor of ORA59 to fine-tune basal resistance against necrotrophic pathogens.

Introduction

In natural environments plants are continuously exposed to many forms of biotic stress, including pathogen and herbivore attack, and abiotic stress, such as adverse light, water, temperature, nutrient or salt conditions. Their survival under such conditions is determined by the ability to perceive external signals and to build up highly adapted responses in a timely manner, mostly by switching on the expression of an appropriate set of genes.

The secondary signaling molecules jasmonates (JAs), ethylene (ET) and salicylic acid (SA) are the main endogenous molecules involved in regulating defense responses to biotic stresses in plants (reviewed by Pieterse et al., 2009). In general, it can be stated that JA- and JA/ET-dependent responses lead to cascades of events that are effective against herbivores and pathogens with a necrotrophic lifestyle, respectively, whereas SA-dependent defences are active against pathogens with a biotrophic lifestyle (Glazebrook, 2005). In addition, the JAs, ET and SA signal transduction pathways can act synergistically or antagonistically in a variety of responses (Kunkel and Brooks, 2002; Pieterse et al., 2009). To fully understand this intricate process of plant responses to environmental stresses, it is imperative to know the function of crucial genes and their regulation during different stress responses.

Several components of the JA signal perception and transduction pathway have been described (reviewed by Chung et al., 2009a; Memelink, 2009). A biologically active JA is perceived by the F-box protein Coronatine Insensitive1 (COI1), which forms part of a putative E3 ubiquitin ligase complex of the SCF type (Xu et al., 2002; Devoto et al., 2002). COI1 was demonstrated to directly bind JA-Isoleucine (JA-Ile) conjugate (Yan et al., 2009) and this promotes the binding of Jasmonate ZIM-motif (JAZ) proteins (Thines et al., 2007). Several members of this family were shown to also interact with the JA-responsive basic helix-loop-helix (bHLH) transcription factor AtMYC2 (Chini et al., 2007; Chung et al., 2009b). In response to JA-Ile JAZ repressor proteins are rapidly degraded (Chini et al., 2007; Thines et al., 2007), which is proposed to lead to derepression of AtMYC2 activity and leads to induction of several AtMYC2 target genes, such as *Vegetative Storage Protein 1* (*VSP1*).

Several members of the APETALA2/Ethylene-Response-Factor (AP2/ERF)-domain transcription factor family have also emerged as important players in JA-responsive gene expression (Memelink, 2009). The expression of the *Octadecanoid-Responsive Arabidopsis AP2/ERF 59 (ORA59)* gene is induced by JA or ET, and is synergistically induced by both hormones. Genome-wide microarray analysis showed that overexpression of *ORA59* resulted in increased expression of a large number of JA- and ET-responsive defense genes, including the *anti-microbial plant defensin 1.2* (*PDF1.2*). Moreover, plants overexpressing ORA59 or with silenced *expression of ORA59* via RNAi, were respectively more resistant or more susceptible to infection by the necrotrophic fungus *Botrytis cinerea* (Pré et al., 2008). Although JA and ET appear to regulate response genes via induction of ORA59 at the gene expression level, it is likely that the activity of ORA59 is also regulated at the protein level. ORA59-GFP fusion proteins overexpressed in protoplasts are mainly cytosolic but accumulate in the nucleus after JA treatment. In addition

ORA59 protein was found to be relatively unstable and its accumulation was induced by the proteasome inhibitor MG132 or by JA (Chapter 3). These observations indicate that ORA59 activity is also regulated at the protein level probably via interaction with regulatory proteins. Although expression of *ORA59* (Atallah, 2005; Pré et al., 2008) and of its target genes depends on COI1 (Lorenzo et al., 2003; Pré et al., 2008), ORA59 is not known to interact with members of the JAZ family of repressor proteins.

In order to further understand how ORA59 is regulated, we initiated a yeast screen to search for proteins interacting with this transcription factor. This resulted in the isolation of ZFAR1 as an ORA59-interacting protein. ZFAR1 contains putative protein interaction motifs in the form of 2 ankyrin repeats and 2 CCCH zinc finger motifs and belongs to a small group of highly related proteins. In Arabidopsis protoplasts ZFAR1 interacted with ORA59 in the cytoplasm, it interfered with JA-induced nuclear localization of ORA59, and it repressed transactivation of the *PDF1.2* promoter by ORA59. Transgenic plants overexpressing ZFAR1 showed accelerated disease progression while a knockout mutant was less severely affected than wild-type plants by *Botrytis* infection. Our results indicate that ZFAR1 acts as a repressor of ORA59 to modulate resistance against necrotrophic pathogens.

Results

Identification of a CCCH zinc-finger protein that interacts with ORA59

To identify proteins that interact with ORA59, yeast two-hybrid screenings were performed. Expression of full-length ORA59 fused to the GAL4 DNA-binding domain (BD) in the vector pAS2.1 auto-activated the expression of the Histidine selection gene in yeast strain PJ69-4A (Figure 1a-b). Auto-activation is a frequently occurring problem with transcription factor baits and can be circumvented by removal of the activation domain. A deletion derivative of ORA59 lacking 80 N-terminal amino acids (ORA59 81-244, Figure 1a) showed weak auto-activation that could be suppressed by the addition of 15-20 mM 3-AT in the medium (Figure 1b). With this bait 7.1 x 10^5 and 2.9 x 10^5 yeast transformants obtained with two Arabidopsis cDNA libraries generated from ecotype Col-0 seedlings treated with JA and ET or from untreated above-ground parts of mature ecotype *Landsberg erecta* plants in the vectors pAD-GAL4-2.1 or pACT2, respectively, were screened, resulting in 58 and 21 colonies that were able to grow on medium lacking histidine. Recovered prey plasmids were re-transformed and three plasmids from each library conferred growth on selective medium. From these candidate ORA59 interactors, only one cDNA sequence, retrieved from the library of untreated plants, was in frame with the GAL4 activation domain (Table 1). This plasmid contained a full-length cDNA encoding the protein corresponding to ZFAR1 (At2g40140) from Col-0. Sequence comparison between the two ecotypes revealed three nucleotide polymorphisms resulting in one amino acid difference.

ZFAR1 is a CCCH zinc finger protein of 597 amino acids which contains two tandem zinc finger

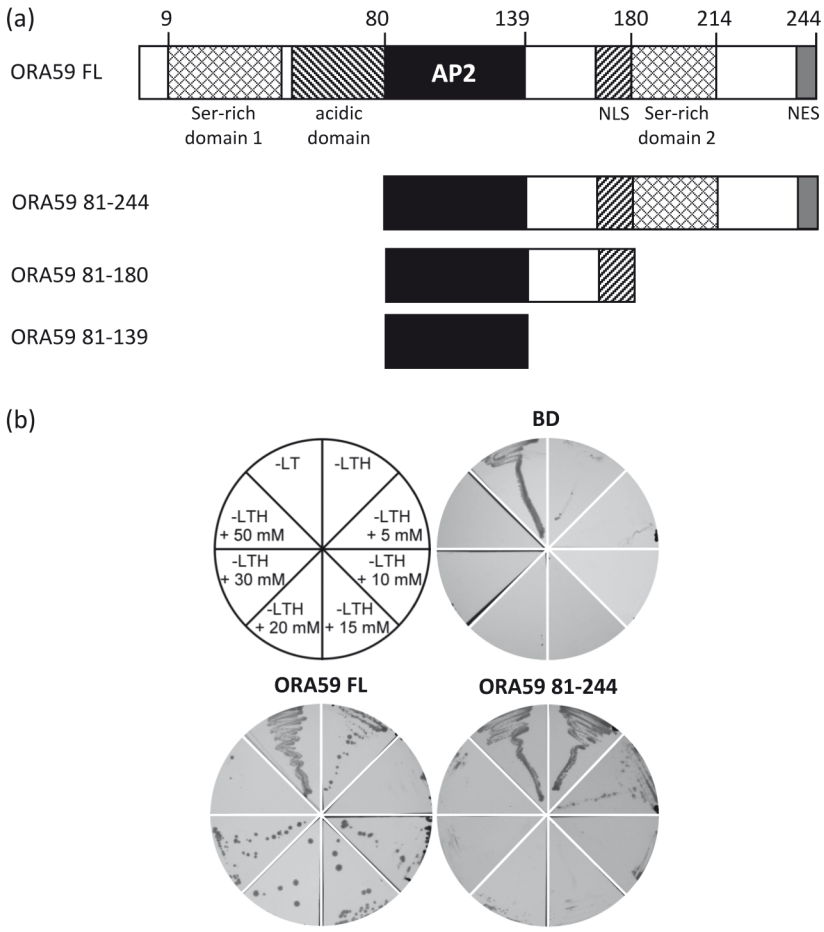

Figure 1. An N-terminal deletion derivative of ORA59 can be used in yeast two-hybrid screening. **(a)** Schematic overview of ORA59 full-length (FL) and ORA59 81-244 proteins. ORA59 contains two Serine-rich domains, an acidic domain, the AP2 domain, a bipartite nuclear localization signal (NLS) and a nuclear export signal (NES). **(b)** ORA59 exhibits strong auto-activation in yeast two-hybrid assay. Coding regions of ORA59 FL and 81-244 derivatives were fused to the GAL4 DNA-binding domain (BD-) and co-expressed in PJ69-4A yeast cells with the GAL4 activation domain expressed from the empty pACT2 vector (AD). Transformed yeast cells were re-streaked on minimal selective medium (SD/-Leu-Trp or -Leu-Trp-His) with increasing 3-AT concentrations (mM) and growth was monitored after 7 days. Binding domain (BD) and activation domain (AD) from the empty plasmids pAS2.1 and pACT2, respectively, were used as control.

(ZF) motifs in the middle of the protein and two ankyrin repeat domains in the N-terminal region (Figure 2a). Phylogenetic analysis of the CCCH zinc finger family of Arabidopsis (Wang et al., 2008) showed that ZFAR1 belongs to a subgroup which includes four other proteins with two CCCH-type zinc finger motifs (C-X_7-C-X_5-C-X_3-H and C-X_5-C-X_4-C-X_3-H) and two ankyrin repeat domains.

The highest amino acid identity is shared with At3g55980 (AtC3H47), subsequently called ZFAR2 (Figure 2a), with an overall identity of 70% (Figure 2b). The close similarity suggests that ORA59 could also interact with ZFAR2 and indeed Y2H assay confirmed interaction (Figure 2c). Yeast cells co-expressing ORA59 and ZFAR1 were able to sustain growth at 3-AT concentrations up to 50 mM on selective medium (Figure 2c), whereas co-expression of ORA59 and ZFAR2 conferred growth up to 20 mM. These interactions are considered significant, since background auto-activation of ORA59 derivative 81-244 conferred growth up to 10 mM 3-AT (Figure 2c).

Table 1. Yeast two-hybrid screening reveals one putative positive interaction. Alternative: Analysis of library prey plasmids conferring growth in the yeast two-hybrid assay with the ORA59 81-244 bait plasmid.

clone	library [1]	insert (bp)	AGI code	nBLAST hit	in frame[2]
3A	treated	921	At4g21600	endonuclease 5	no
5	treated	>1000	At2g24120	chloroplast RNA pol	antisense
11J	treated	825	At3g25760	AOC1	no
1Ua	untreated	673	At5g38410	rbcS-3B	no
2Ua	untreated	>1000	At4g12230	(thio)esterase/lipase	no
8Ua	untreated	2100	At2g40140	C3H zinc finger	yes

[1] Arabidopsis cDNA libraries generated from seedlings treated with JA and ET or from untreated above-ground parts of mature plants in the vectors pAD-GAL4-2.1 or pACT2, respectively.

[2] In frame with the GAL4 activation domain

ORA59 interacts with ZFAR1 and ZFAR2 *in planta*

To confirm the interaction of ORA59 with ZFAR1 *in planta*, a Bimolecular Fluorescence Complementation (BiFC) assay was employed. The N-terminal (YN) or C-terminal (YC) parts of the yellow fluorescent protein (YFP) were fused either N-terminally or C-terminally with ORA59 and ZFAR1. All YC constructs contained the influenza hemagglutinin (HA) epitope tag. The constructs were transiently co-expressed in Arabidopsis suspension cell protoplasts in all possible combinations of YN and YC fusion pairs with the unfused YFP parts as negative controls. Western blot analysis with anti-HA antibody showed that YC fusion proteins were correctly expressed in protoplasts and that ORA59 fusion proteins were less stable than ZFAR1, even after protein stabilization by JA or MG132 (Figure 3a). Reconstitution of a fluorescing YFP chromophore occurred only upon co-expression of both fusion proteins. The YFP signal was detected throughout the entire cell of Arabidopsis protoplasts co-transformed with YN-ORA59 and ZFAR1-YC (Figure 3b), ORA59-YC and YN-ZFAR1, or YC-ORA59 and ZFAR1-YN (not shown).

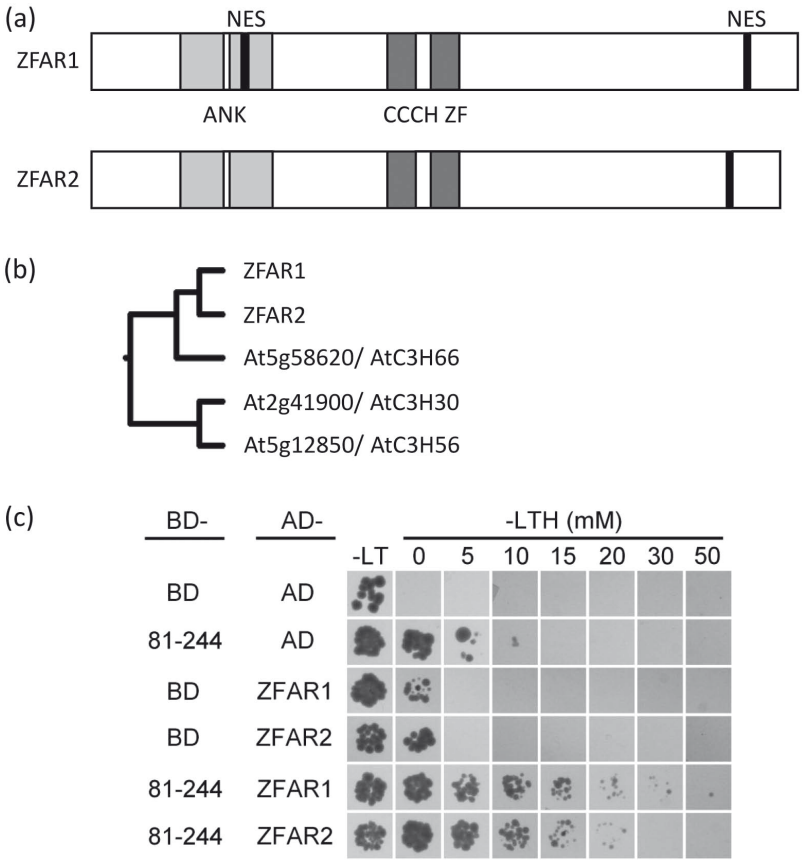

Figure 2. ORA59 interacts with ZFAR1 in yeast. **(a)** Schematic overview of ZFAR1 and ZFAR2 proteins, which contain two ankyrin-repeat domains, two CCCH3 zinc finger motifs and putative nuclear export signals (NES). **(b)** ZFAR1 shares high amino acid identity with ZFAR2. Phylogenetic tree of CCCH zinc finger proteins with two tandem ankyrin repeats from *Arabidopsis*. The unrooted neighbor-joining tree was constructed using full-length amino acid sequences aligned by ClustalW. **(c)** ORA59 interacts with ZFAR1 and ZFAR2 in yeast. Yeast cells expressing ORA59 81-244 fused to GAL4BD and ZFAR1 or ZFAR2 fused to GAL4AD were spotted on minimal selective medium (SD/-Leu-Trp or -Leu-Trp-His) with increasing 3-AT concentrations (mM) and growth was monitored after 7 days. Yeast transformed with the empty plasmids pAS2.1 and pACT2, expressing the binding domain (BD) and activation domain (AD) of GAL4, respectively, were used as control.

Cells transfected with single plasmids and any combination of empty YFP vectors produced no or only background fluorescence. No signal could be detected when the co-transfected proteins were either N-terminal or C-terminal YFP fusions (data not shown). These results demonstrate that ORA59 can also interact with ZFAR1 in plant cells. This interaction happens in the cytoplasm, in contrast to the clear nuclear interaction of AtMYC2 with JAZ1 (Figure 3b).

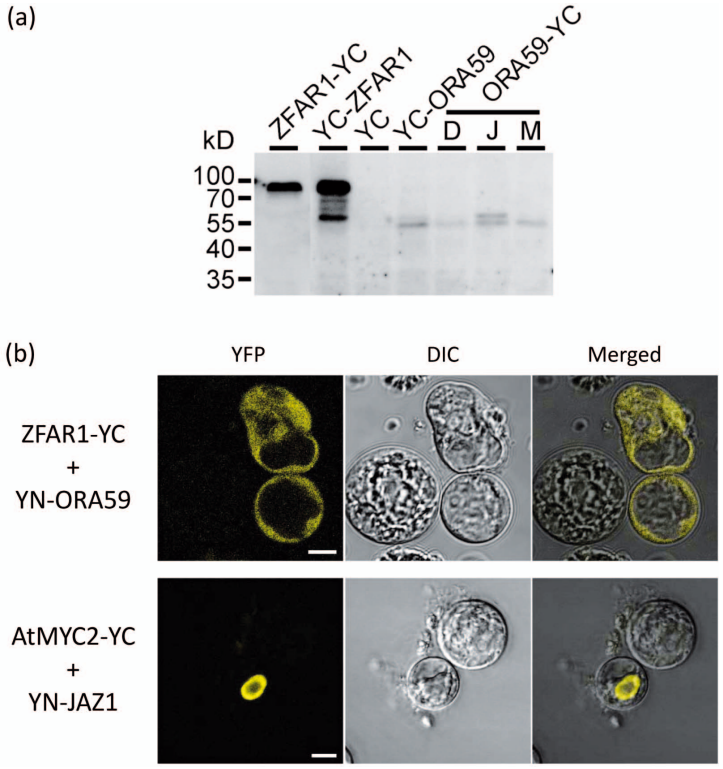

Figure 3. ORA59 interacts with ZFAR1 *in planta*. **(a)** BiFC protein fusions are correctly expressed. Western blot of proteins extracted from untreated or treated with 0.1% DMSO (D), 50 µM JA (JA) or 50 µM MG132 (M) protoplasts co-transformed with the indicated constructs. Protein samples of 30 µg were separated by SDS-PAGE and HA epitope present in YFP C-terminal (YC) fusions were immunodetected with anti-HA (α-HA) antibodies. Asterisks mark the positions of expected protein sizes. **(b)** YFP fluorescence and bright field images of Arabidopsis cell suspension protoplasts co- transformed with constructs encoding the indicated fusion proteins with YFP at the C-terminus (YC) or the N-terminus (YN). Scale bar = 10 µm.

ZFAR1 CCCH zinc fingers interact with the C-terminal part of ORA59

Some members from the plant CCCH zinc finger family were shown to bind RNA and were suggested to be involved in RNA processing (Li et al., 2001; Addepalli and Hunt, 2007; Addepalli and Hunt, 2008; Pomeranz et al., 2010a), whereas several other members are thought to be involved in DNA binding (Wang et al., 2008; Kim et al., 2008; Pomeranz et al., 2010a). To determine whether ZFAR1 and ZFAR2 can be putative transcription factors, full-length proteins were fused to the GAL4 DNA-binding domain in pAS2.1. In addition, three ZFAR1 deletion derivatives were also constructed in pAS2.1 (Figure 4a). Expression of BD-ZFAR1 and BD-ZFAR2 in yeast cells resulted in weak, but detectable, transcription activation of the Histidine selection

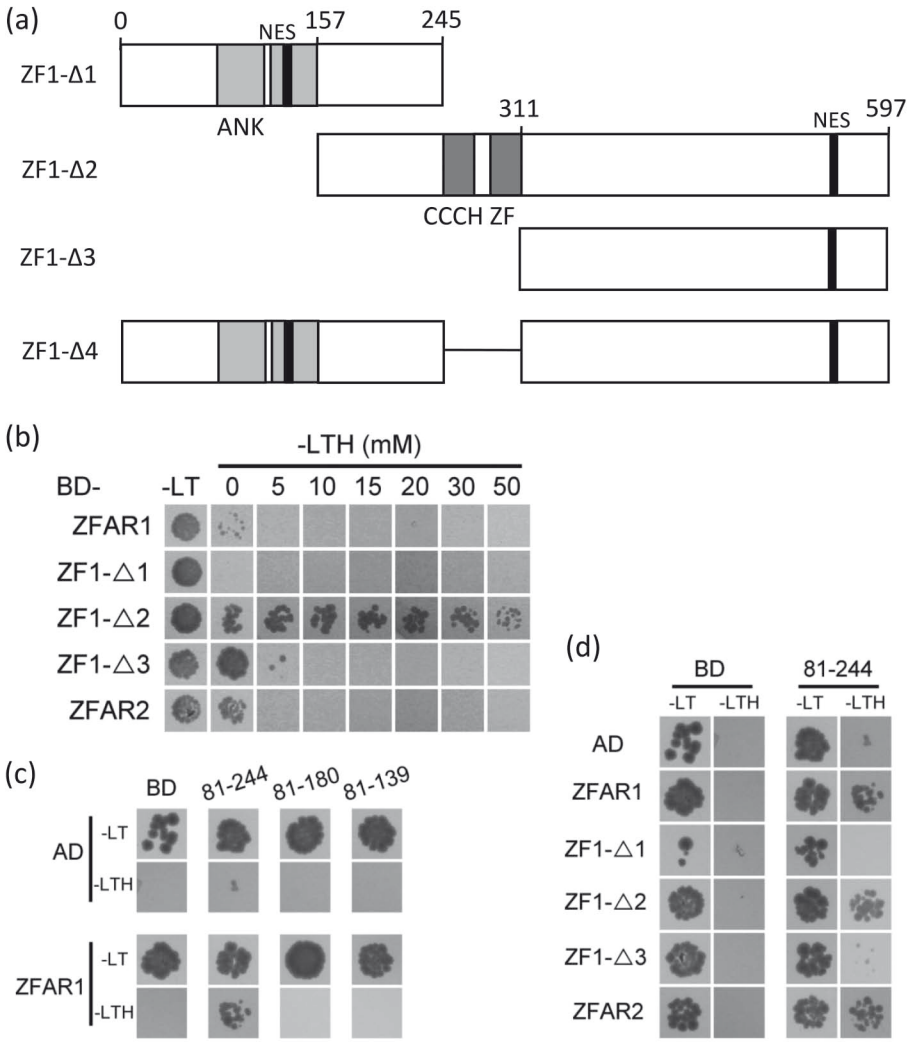

Figure 4. The zinc finger motifs of ZFAR1 promote transcriptional activation in yeast and interaction with the C-terminal region of ORA59. **(a)** Schematic representation of truncated versions of ZFAR1. Numbers indicate amino acid positions. **(b)** ZFAR1 and ZFAR2 exhibit auto-activation in yeast two-hybrid assays. Yeast cells co-expressing GAL4BD fused to ZFAR1 derivatives or full-length ZFAR2 and GAL4AD were spotted on minimal selective medium (SD/-Leu-Trp or -Leu-Trp-His) with increasing 3-AT concentrations (mM) and growth was monitored after 7 days. **(c)** Yeast cells expressing ORA59 truncated versions 81-244, 81-180 or 81-139 fused to GAL4BD and ZFAR1 fused to GAL4AD. **(d)** Yeast cells expressing ORA59 truncated version 81-244 fused to GAL4BD and ZFAR1 derivatives or full-length ZFAR2 fused to GAL4AD. Yeast suspensions were spotted on minimal (-LT) or selective medium (-LTH) with 10 mM 3-AT and growth was monitored after 7 days. Yeast transformed with the empty plasmids pAS2.1 and pACT2, expressing the binding domain (BD) and activation domain (AD) of GAL4, respectively, were used as control.

gene, indicating that the ZFAR proteins contain a functional transcriptional activation domain (Figure 4b). To determine the position of the activation domain, ZFAR1 deletion derivatives were also tested for auto-activation in yeast. As shown in Figure 4b, ZF1-Δ1, which contains the ankyrin repeats and lacks the zinc finger domains, did not show auto-activation. On the contrary, ZF1-Δ2 exhibited a very strong auto-activation that cannot be suppressed by addition of 3-AT, whereas ZF1-Δ3 conferred moderate growth on selective medium. This result shows that the two zinc-finger motifs are mainly responsible for transcriptional activation in yeast, although a weaker activation domain is also present in the C-terminal region.

To determine the interaction domains of ORA59 and ZFAR1, ORA59 deletion derivatives (81-244, 81-180 and 81-139) were fused to the GAL4 DNA-binding domain of pAS2.1 and the ZFAR1 deletions shown in Figure 4a were fused to the GAL4 activation domain of pACT2. ORA59 deletion derivative 81-180 differs from 81-244 in the absence of 64 C-terminal amino acids, whereas 81-139 contains only the 59 amino acids corresponding to the AP2 domain (Figure 1a). Co-expression of BD-ORA59 81-244, 81-180 and 81-139 with AD-ZFAR1 in yeast cells indicated that the 64 C-terminal amino acids of ORA59 were necessary for the interaction with ZFAR1 (Figure 4c). In addition, co-expression of BD-ORA59 81-244 with AD-ZF1-Δ1, -Δ2 and -Δ3 revealed that the zinc finger motifs of ZFAR1 were essential for protein interaction (Figure 4d).

Subcellular localization of ZFAR1 and ZFAR2

Since transcription factors are commonly found as nuclear proteins, localization of ZFAR1 and ZFAR2 fused to the green fluorescent protein (GFP) was analyzed in Arabidopsis cell suspension protoplasts. ZFAR1 and ZFAR2 have no predicted nuclear localization signals (NLS). ZFAR1 contains N- and C-terminal putative NES whereas ZFAR2 contains one C-terminal putative NES (Wang et al., 2008). N- and C-terminal fusions of ZFAR1 with GFP were found both in the cytoplasm and in the nucleus of Arabidopsis protoplasts, similar to GFP alone (Figure 5a). The ZFAR1-GFP signal was homogeneously distributed in some cells, but it was most often observed in cytosolic spots or in a combination of both. Although the spots were smaller and were found less frequently than with ZFAR1, similar subcellular localization was observed for the ZFAR2-GFP fusion (Figure 5a). To study the influence of the ankyrin and the zinc finger motifs in ZFAR1 protein localization, ZFAR1 deletions depicted in Figure 3b were C-terminally fused to GFP. An additional ZFAR1 deletion, ZFAR1Δ4, which lacks only the ZF domain, was constructed. As shown in Figure 5b, deletion ZF-Δ1, containing the 245 N-terminal amino acids and the ankyrin motifs showed a homogeneous GFP distribution in the nucleus and cytoplasm and no spots could be detected. A similar distribution was found upon deletion of only the ankyrin motifs (ZF-Δ2), but occasionally small spots were found as well. Deletion of both ankyrin and ZF motifs (ZF-Δ3) promoted the accumulation of more cytosolic spots. Intriguingly, the deletion ZF-Δ4, lacking only the ZF, showed accumulation in bigger spots. These results indicate that the spotted cytoplasmic distribution is mainly determined by the C-terminal region of ZFAR1, while the presence of the

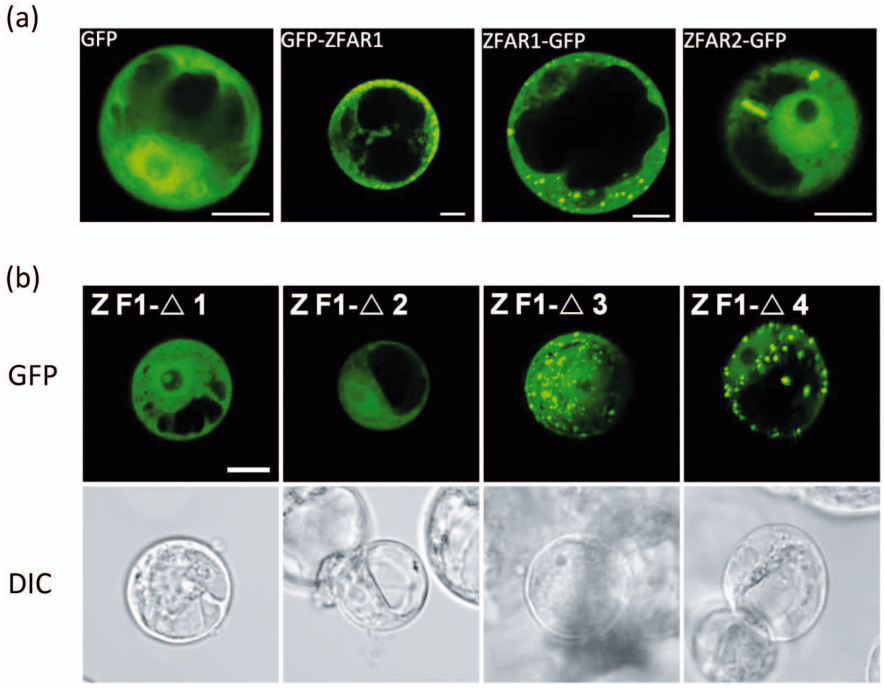

Figure 5. ZFAR1 and ZFAR2 proteins are localized both in the nucleus and in the cytoplasm. **(a)** GFP, GFP-ZFAR1, ZFAR1-GFP, GFP-ZFAR2. **(b)** GFP- ZFAR1 truncated versions shown in Figure 4a were transformed to Arabidopsis cell suspension protoplasts and examined by confocal laser scanning microscopy. Scale bar = 10 µm.

zinc finger determines an even cytosolic distribution.

ZFAR1 represses activation of *PDF1.2* promoter activity by ORA59

In order to elucidate the functional significance of the interaction between ZFAR1 and ORA59, transactivation assays were performed. Co-transformation of Arabidopsis protoplasts with a *PDF1.2* promoter - *GUS* reporter construct and an effector plasmid carrying *ORA59* fused to the *CaMV 35S* promoter resulted in strong activation of up to 30 fold (Figure 6a). ZFAR1 alone did not activate, but instead slightly repressed *PDF1.2* promoter activity. ZFAR1 caused a dose-dependent repression of ORA59 activity with a 4-fold reduction at a plasmid ratio of 2:6 µg of ORA59 : ZFAR1 (Figure 6a). To study the specificity of repression, the effects of ZFAR1 on ORA47, an unrelated AP2/ERF-domain transcription factor, were determined at 2:6 µg plasmid ratios. ORA47 trans-activated the promoter of the *allene oxide cyclase 2 (AOC2)* gene in the protoplast assay as previously reported (Zarei, 2007). In contrast to the effect on ORA59, ZFAR1 did not significantly affect transcriptional activation of the *AOC2* promoter by

ORA47 (Figure 6a). We tested whether ZFAR1 could interfere with JA-induced re-localization of ORA59 to the nucleus. Nuclear localization was studied by confocal laser scanning microscopy of Arabidopsis cell suspension protoplasts co-expressing ORA59 fused N-terminally to GFP and ZFAR1 after treatment with DMSO or JA. Interestingly ZFAR1 did not affect basal ORA59 nuclear localization, but it abolished JA-induced nuclear re-localization (Figure 6b). To establish whether the repression of *PDF1.2* promoter activation by ZFAR1 correlated with the observed changes in JA-responsive ORA59 re-localization, *PDF1.2* promoter trans-activation by ORA59 in protoplasts was performed with DMSO or JA treatments. JA slightly stimulated *PDF1.2* promoter activation by ORA59 (Figure 6c). As observed before (Figure 6a) ZFAR1 repressed basal trans-activation by ORA59 (Figure 6c), although it did not affect basal ORA59 localization (Figure 6b). In agreement with the negative effect on JA-responsive nuclear localization of ORA59 (Figure 6b), ZFAR1 abolished the stimulatory effect of JA on transactivation of the *PDF1.2* promoter by ORA59 (Figure 6c).

ZFAR1 has a modest influence on ORA59 target gene expression

Since gene expression patterns can provide important clues about gene function, we verified whether *ZFAR1* and *ZFAR2* are induced in wild-type Arabidopsis seedlings treated with JA, with the ET-releasing agent ethephon or with SA, applied alone or in pairwise combinations with JA. *ZFAR1* and *ZFAR2* were not responsive to JA or ethephon treatments. However, a modest induction was observed after SA treatment (Figure 7). Simultaneous treatment with JA did not affect the weak SA-induced gene expression.

To investigate whether *ZFAR1* regulates defense gene expression in plants, Arabidopsis constitutively overexpressing the *ZFAR1* gene were generated. Among several independent T2 lines tested, lines #2, #4, #5, #13 and notably #9 showed a high level of *ZFAR1* overexpression. Lines #2 and #9 were selected for further gene expression analysis (Figure 8a). These lines showed no morphological differences compared to wild-type plants. As shown by RNA blot analysis, *PDF1.2* expression in transgenic seedlings from *35S*:*ZFAR1* line #9 treated with JA, ethephon or a combination of both did not significantly differ from *PDF1.2* expression in the *35S*:*GUS* control line (Figure 8b). Since ORA59 regulates the expression of many stress-responsive genes (Pré et al., 2008), we also verified the expression of other target genes. Unlike *PDF1.2*, the expression of *Hevein-Like Protein* (*HEL*) is slightly weaker in the *35S*:*ZFAR1* than in the control lines. However more clear differences can be observed in the expression of *Anthocyanin 5-aromatic Acyltransferase* (*AN5-AT*) and *Chitinase* (*Chit*-At2g43580). Since *AN5-AT* is involved in secondary metabolism, other ORA59 target genes involved in primary or secondary metabolism might also be affected.

The results from the trans-activation assays together with the gene expression analysis of the *35S*:*ZFAR1* #9 line prompted us to test whether the absence of ZFAR1 would affect the expression of *ORA59* target genes. Since ZFAR1 and ZFAR2 might have overlapping roles, we

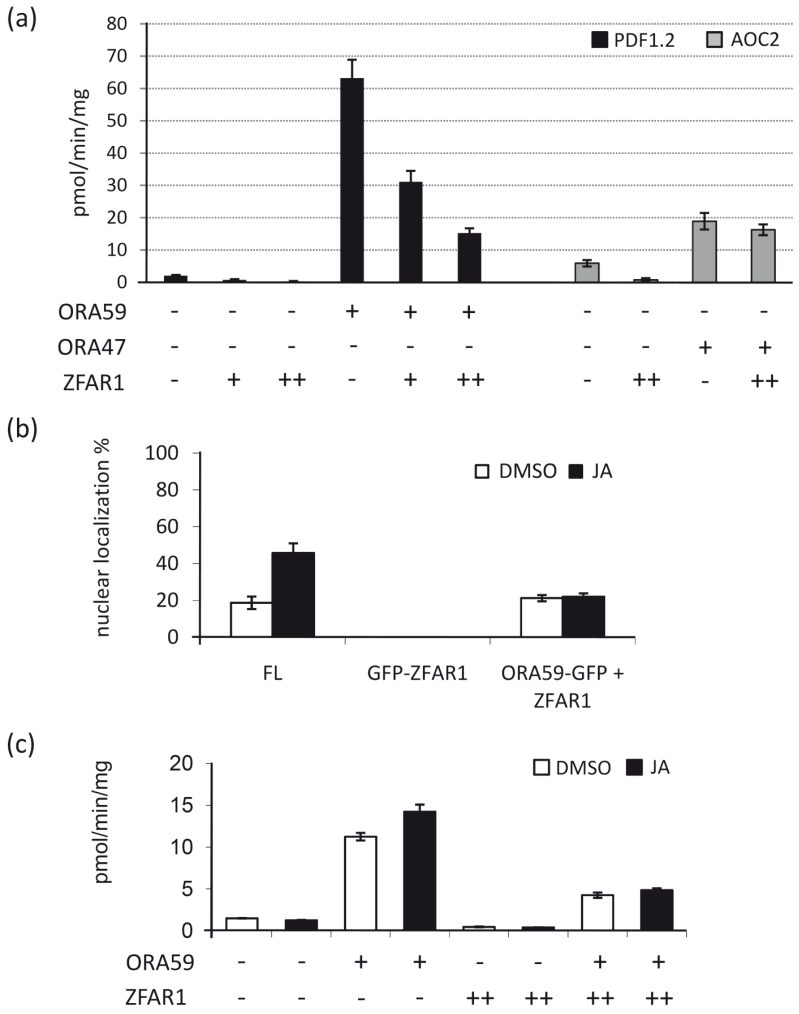

Figure 6. ZFAR1 represses ORA59 activity and JA-responsive nuclear localization. **(a)** ZFAR1 represses the activity of ORA59 but not of ORA47. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying *PDF1.2*:*GUS* or *AOC2*:*GUS* and overexpression vectors containing 35S:*ORA59* or *ORA47* and *ZFAR1* ORFs, as indicated. **(b)** ZFAR1 prevents JA-responsive ORA59 nuclear localization. Sixteen hrs after transformation, cell suspension protoplasts transformed with GFP-ORA59, GFP-ZFAR1 or co-transformed with GFP-ORA59 and ZFAR1 were treated for 2 hours with 0.1% DMSO or 50 µM JA. Values represent cells with nuclear localization as a percentage of the total number of GFP-expressing cells analyzed by confocal laser scanning microscopy. For each data point at least 150 GFP-expressing protoplasts were analyzed. Values represent means ± SE of triplicate experiments. **(c)** ZFAR1 repression is not alleviated by JA. Sixteen hrs after transformation, cell suspension protoplasts transformed with *PDF1.2*:*GUS* and overexpression vectors containing *ORA59* and *ZFAR1* ORFs were treated for 2 hours with 0.1% DMSO or 50 µM JA. Protein amounts were used to correct for differences in protein extraction efficiencies. Values represent means ± SE of triplicate experiments. Plus signs indicate DNA amounts of expression vectors used in protoplast transformations (+ = 2 µg; ++ = 6 µg).

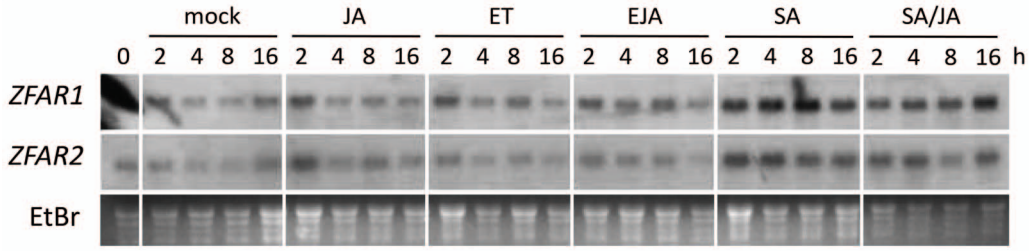

Figure 7. ZFAR1 and ZFAR2 expression is not induced by JA or ethephon but weakly by SA. Northern blot analysis of *ZFAR1* and *ZFAR2* in 14-days old *Arabidopsis* Col-0 seedlings treated with mock (C), 50 µM jasmonic acid (JA), 1 mM ethephon (E) or 1 mM salicylic acid (SA) alone or in combinations, for the number of hrs indicated. The ethidium bromide (EtBr) stained gel is shown as a control for RNA loading.

generated plants homozygous for T-DNA insertion alleles of *zfar1* and *zfar2* to circumvent possible redundant roles of these two proteins. Gene expression analysis confirmed that mutant seedlings failed to accumulate *ZFAR1* and *ZFAR2* transcripts (Figure 9a). Like the *35S*:*ZFAR1* lines, the double knockout mutant was morphologically indistinguishable from wild-type plants. Modest differences were observed in the expression of *PDF1.2, HEL*, *AN5-AT* and *ChitB* between wild-type and *zfar1zfar2* seedlings treated with 50 µM JA, 1 mM ethephon or both (Figure 9b, bands marked with asterisks). Notably, these genes were more highly induced at four hours after JA/ET combined treatment in the mutant than in the wild-type. Since the applied concentrations of inducers could rapidly and strongly induce ORA59, differences in target gene expression could have been masked by saturated levels of JA/ET-induced responses. Therefore a lower concentration of 5 µM JA was also applied in seedlings alone or in combination with 1 mM ethephon. However, both concentrations of JA induced defense gene expression to comparable levels in the seedlings resulting in the same differences between wild-type and mutant. It has been described before that SA suppressed JA-responsive expression of *PDF1.2*, and *HEL* (Glazebrook et al., 2003, Spoel et al., 2003).To address the possibility that the negative effect of SA was mediated by ZFAR proteins, *zfar1zfar2* seedlings were treated with SA alone or in combination with JA. As previously described, JA-responsive defense genes were efficiently repressed by simultaneous treatment with SA in wild-type seedlings. The cross-talk was also observed in *zfar1zfar2* mutants, indicating that ZFAR1 is not involved in SA-induced repression of defense gene expression. Intriguingly, *HEL* was weakly induced by JA and SA treatment in *zfar1zfar2* mutants, suggesting that in absence of *ZFAR1*, ORA59 was able to induce one of its target genes.

ZFAR1 negatively affects resistance against the necrotrophic fungus *Botrytis cinerea*

ORA59 was shown to be a key regulator of JA/ET-induced defense to *Botrytis* infection in

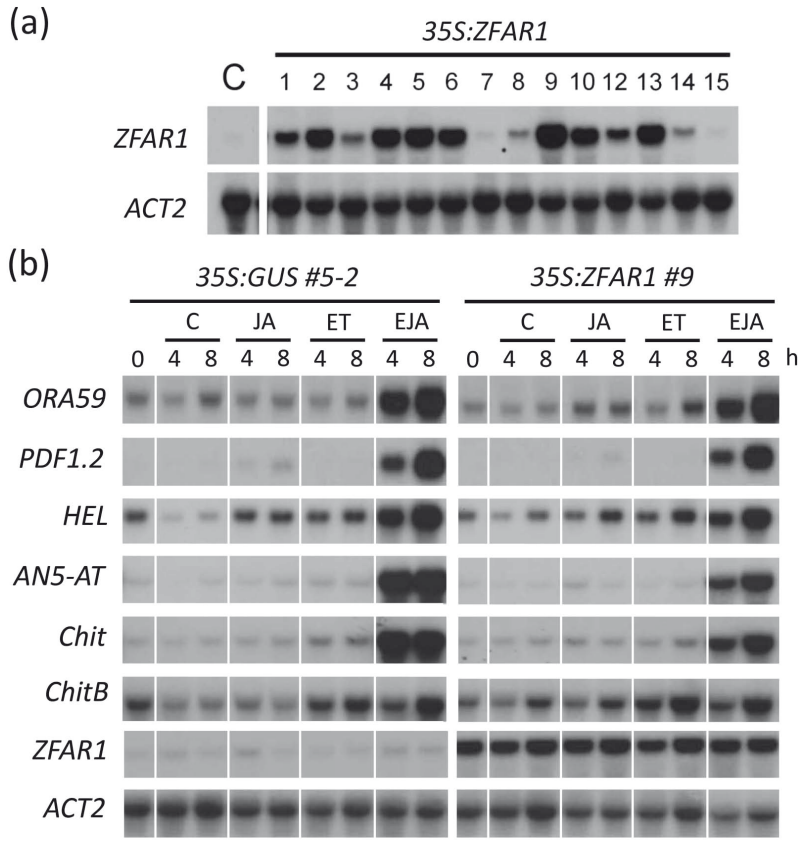

Figure 8. Overexpression of *ZFAR1* has modest effects on ORA59 target genes in stably transformed plants. **(a)** Northern blot analysis of seedlings from a control line carrying the *GUS* gene (C) or from several independent *35S*:*ZFAR1* transgenic lines (indicated by numbers). **(b)** Gene expression analysis of ORA59 target genes in the highest expressing *35S*:*ZFAR1* transgenic line #9 or the control line *35S*:*GUS* #5-2. The *ACT2* probe was used to verify RNA loading. All panels hybridized with the same probe were on the same blot and were exposed to film for the same time, therefore signal intensities can be directly compared.

Arabidopsis (Pré et al., 2008). Our results from trans-activation assays and genes expression analysis of mutant plants indicated that ZFAR1 is a negative regulator of ORA59. To determine whether modulation of *ZFAR1* expression levels could result in an altered resistance phenotype against *B. cinerea*, five mature leaves of five-weeks-old WT plants, *zfar1zfar2* mutant plants and different ZFAR1 overexpression lines were inoculated with 3 µl drops of $7.5x10^5$ spores/mL suspension of *B. cinerea* and disease progression was compared between genotypes two and three days after inoculation. Leaf lesions were scored in five different classes I-V, according to disease severity, as shown in representative leaves in Figure 10a. In wild-type, approximately 65 % of infected leaves was distributed among the less severe classes I, II, and III after 3 days of

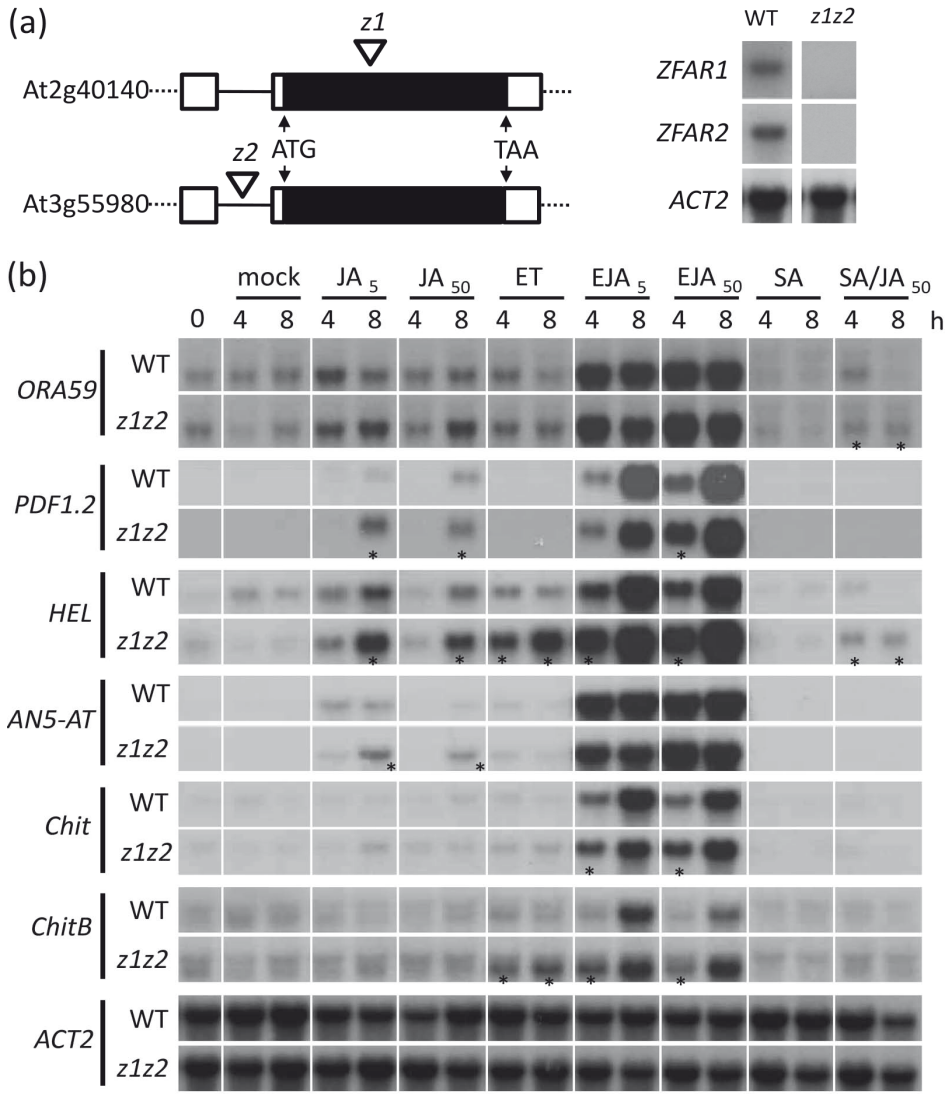

Figure 9. Double knockout mutant of *zfar1zfar2* has modest effects on ORA59 target genes in stably transformed plants. **(a)** Schematic representation of T-DNA insertions in *ZFAR1* (SALK_024800 = *z1*) and *ZFAR2* (SALK_141550 = *z2*). Triangles indicate the position of T-DNA insertions. Solid boxes and lines represent exons and introns, respectively. White boxes represent untranslated regions. Dashed lines are untranscribed regions. Northern blot analyses show that T-DNA insertions abolish *ZFAR1* and *ZFAR2* mRNA accumulation. **(b)** Gene expression analysis of ORA59 target genes in the *zfar1zfar2* T-DNA insertion mutants or wild-type Col-0. Fourteen-days old seedlings were treated with the solvents DMSO and Na-phosphate (mock), 50 µM jasmonic acid (JA), 1 mM ethephon (ET) or 1 mM salicylic acid (SA) alone or in pairwise combinations with JA, for the number of hrs indicated. The RNA gel blots were hybridized with the indicated probes. RNA loading was checked with *ACT2* probe. 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. Asterisks indicate bands discussed in the text.

infection. Interestingly, *zfar1zfar2* plants showed enhanced resistance to *Botrytis* with almost 80 % of the leaves in these classes. Three *35S*:*ZFAR1* lines showed similar percentages of lesion distribution among less severe classes as wild-type plants, but remarkable differences in the most severe class V, ranging from 18 to 23 % compared to 13 % for wild-type and 6 % for *zfar1zfar2* (Figure 10b). In addition, lesions from all different 5 classes were usually smaller on *zfar1zfar2* mutant plants than with the 2 other genotypes, indicating a delay in local disease progression (data not shown). The differences in class distribution were statistically significant and the results were consistently reproduced in two independent infection assays. Despite the clear phenotypical differences, target genes of *ORA59* involved in defense responses, including *PDF1.2*, *HEL* and *AN5-AT*, did not show significant gene expression differences with the control line in local infected leaves or systemic uninfected leaves 2 or 3 days after *Botrytis* infection. ORA59 also controls genes involved in primary metabolism leading to tryptophan biosynthesis, including *DHS1* (*3-deoxy-D-arabino-heptulosonate 7-phosphate synthase*) and *IGPS1* (*indole-3-glycerol phosphate synthase*) (Pré et al., 2008 and unpublished results), but their expression was also not changed (Figure 10c). In conclusion, modulation of *ZFAR1* expression levels result in an altered resistance phenotype against *B. cinerea*.

Discussion

Studies in plant defense responses against the necrotrophic pathogen *Botrytis* point to a complex and poorly understood, interaction between different signaling pathways. ORA59 was shown to be a key regulator of JA/ET mediated-defense responses against *Botrytis* infection in Arabidopsis (Pré et al., 2008). In addition, JA was shown to induce stabilization and nuclear localization of ORA59 (Chapter 3), indicating the existence of regulation at the protein level, probably via interaction with regulatory proteins. In this study, we identified a protein interaction between ORA59 and the CCCH zinc finger ZFAR1/AtC3H29 by yeast two-hybrid screening. Our results show that ORA59 interacts with ZFAR1 in the cytoplasm and that overexpression of ZFAR1 prevents re-localization of ORA59 upon JA stimulation. Trans-activation assays indicate that ZFAR1 acts as a repressor of ORA59. Consistent with mild gene expression differences found between wild-type and plants overexpressing or knocked-down for ZFAR1, small but significant differences in resistance against necrotrophic pathogens were observed.

Plants with disrupted *ZFAR1* and *ZFAR2* expression were found to be more resistant to the development of early disease symptoms of *Botrytis* infection whereas *ZFAR1* overexpressing lines were less resistant. However, the results contradict a previous publication where the authors concluded that a single *zfar1* mutant with exactly the same T-DNA insertion in the *ZFAR1* gene as in our double mutant was more susceptible to *Botrytis* infection (AbuQamar et al., 2006). By itself it is surprising that a phenotype was observed with the single *zfar1* mutant given the high identity of ZFAR1 and ZFAR2 and the fact that the corresponding genes have similar levels and patterns of expression. It is possible that the contradictory observations are caused

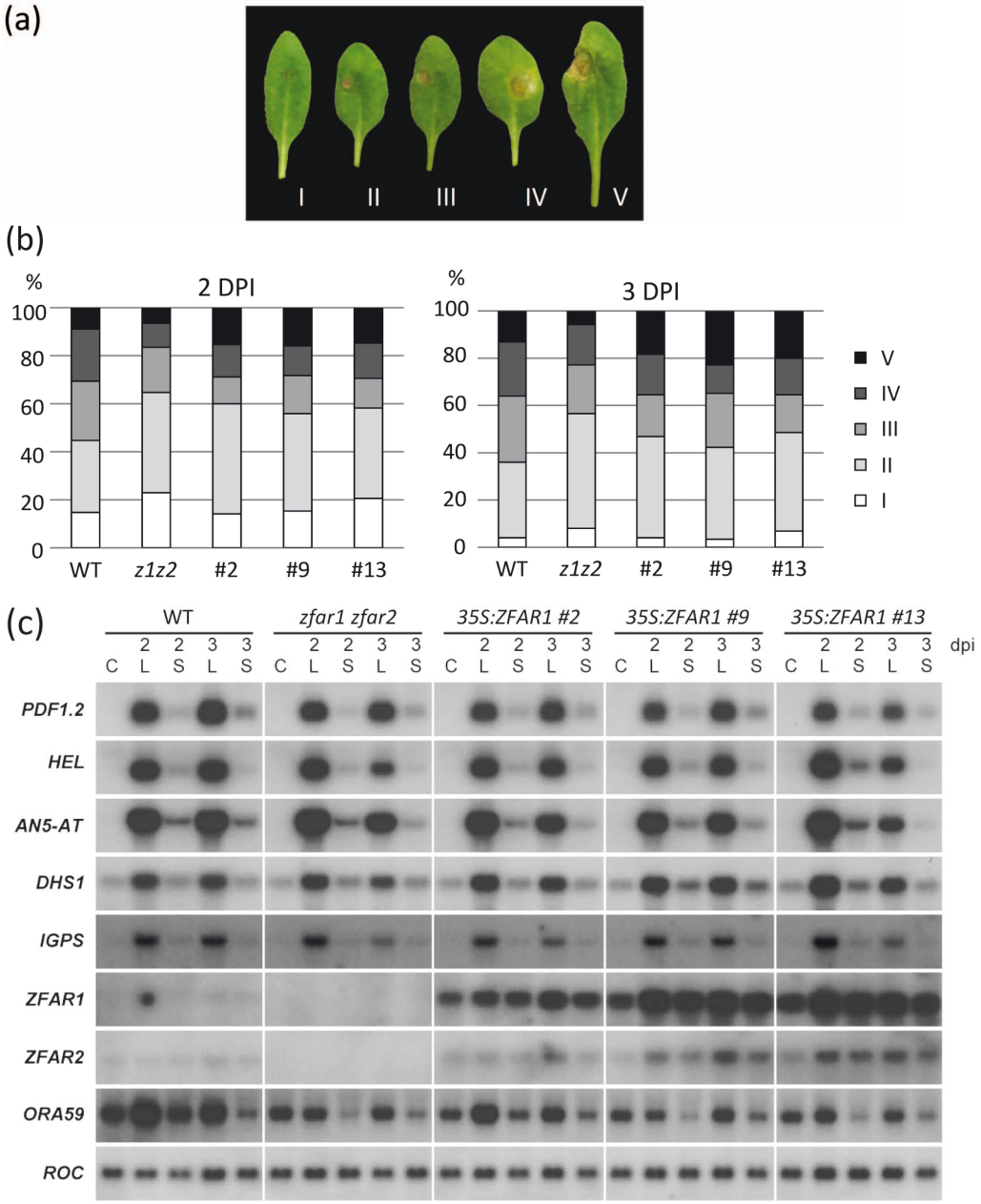

Figure 10. Modulation of *ZFAR1* expression levels affects resistance against the necrotrophic fungus *Botrytis cinerea*. Disease severity was scored in *Arabidopsis* wild-type plants, *zfar1zfar2* mutants and transgenic plants overexpressing the *ZFAR1* gene. Disease ratings were performed 2 and 3 days after inoculation. **(a)** Representative disease symptoms at 4 days after inoculation. Class I, no visible disease symptoms; II, non-spreading lesion; III, spreading lesion; IV, spreading lesion surrounded by a chlorotic halo; and V, spreading lesion with extensive tissue maceration. **(b)** Distribution of disease severity classes. Disease severity is expressed as the percentage of leaves falling in disease

by differences in damage assessment and by the infection method. We evaluated resistance on mature leaves spotted with *Botrytis* at 2 and 3 days after infection by classification of damage progression in all inoculated leaves. AbuQamar et al. sprayed whole plants with fungal spores and conclusions were drawn after an extended incubation of 12 dpi. The authors also reported that disease symptoms were developing after 3 days, but with no clear differences observed. Their conclusions were not based on quantitative measurements of damage and no statistical analysis was performed.

We demonstrate here that the ZFAR1 region containing the two zinc finger repeats was responsible for transcriptional activation in yeast and for interaction with the C-terminal part of ORA59. Similar binding domain analysis in yeast with the cotton CCCH-ZF GhZFP1 demonstrated that the two zinc finger motifs and the 40 amino acids N-terminal region are essential for mediating interactions with the defense-related proteins GZIRD21A (GhZFP1 interacting and responsive to dehydration protein 21A) and GZIPR5 (GhZFP1 interacting and pathogenesis-related protein 5) (Guo et al., 2009). Recently Addepalli and Hunt (2008) suggested that RNA nuclease activity may be a common characteristic of Arabidopsis CCCH-containing proteins after confirming this activity in five CCCH proteins (not including ZFAR1 and 2) randomly selected from Arabidopsis. Although a role as ribonucleases cannot be excluded since we did not test ribonuclease activity, we present evidence for a completely different function of ZFAR1 and ZFAR2 as repressors of the transcription factor ORA59.

In localization studies of ZFAR1 and ZFAR2 fused to GFP we found that both proteins have a cytosolic distribution in Arabidopsis protoplasts. Previously ZFAR2 (atSZF1) was reported to be nuclear in onion cells (Sun et al., 2007), despite the absence of a predicted nuclear localization signal (NLS). However more recently all five members of the CCCH ankyrin-containing group, including ZFAR1 and ZFAR2, were demonstrated to accumulate in cytoplasmic foci in maize protoplasts (Pomeranz et al., 2010b). These authors also showed that the Arabidopsis AtTZF1/AtC3H23 protein trafficks between nucleus and cytoplasmic foci, and they proposed that CCCH proteins play roles in signal transduction events as nucleocytoplasmic shuttle proteins (Pomeranz et al., 2010a), in analogy to the mammalian CCCH proteins ZFP36L1 and ZFP36L2 from the tristetrapolin (TTP) protein family (Phillips et al., 2002; Frederick et al., 2008). Since we observed ZFAR1 and ZFAR2 also in the nucleus, it is possible that the nucleocytoplasmic shuttling of ORA59 is affected by interaction with ZFAR proteins. Since ZFAR1 interacts with the C-terminal region of ORA59 containing the NES and NLS, interaction with ZFAR1 could mask these signals and thereby affect cellular localization.

severity classes. Data represent 170 leaves of 35 plants per genotype. Disease resistance tests were performed at the same time for all genotypes and were independently performed twice with the same results. The differences between genotypes were analyzed by Pearson Chi-square test. **(c)** Non-infected control plants (C), infected local (L) and non-infected systemic (S) leaves from several inoculated plants of each genotype were collected at day 0, day 2 and day 3 after inoculation (dpi) with *B. cinerea* and RNA was extracted. The RNA gel blot was hybridized with the indicated probes. The *ROC* probe was used to verify RNA loading.

We hypothesize that ZFAR1 could be part of a mainly cytosolic complex that represses ORA59 via cytoplasmic retention. Tight regulation of key transcription factors involved in defense responses at the protein level is a common strategy in plants. The JA-responsive transcription factor AtMYC2 interacts with GATA-type ZF proteins called JAZ (Chini et al., 2007). The JA-Ile-responsive SCF^{COI1}-mediated degradation of JAZs is thought to activate AtMYC2 (Chini et al., 2007). A clear difference with ORA59/ZFAR1 is that the interaction between AtMYC2 and JAZ occurs in the nucleus. Several examples of regulation of transcription factor activity via a nucleocytoplasmic shuttling mechanism exist in plants. NPR1 (Nonexpressor of Pathogenesis-Related gene 1) is retained in the cytoplasm of healthy plants as protein oligomers but in response to SA NPR1 monomers are formed, which re-localize to the nucleus where they act as co-activators of TGA transcription factors (Kinkema et al., 2000; Mou et al., 2003; Pieterse and van Loon, 2004). Perhaps the nucleocytoplasmic shuttling of the activator of hypersensitive cell death bZIP10 offers the best similarity to ORA59 regulation. Interaction with the zinc finger protein LSD1 (Lesions Simulating Disease1) negatively regulates bZIP10 activity by retaining it partially in the cytoplasm and thereby modulates basal defense responses against biotrophic pathogens (Kaminaka et al, 2006). Similar mechanisms of transcription factor regulation have been studied extensively and in great detail in mammals. The paradigm for regulation of transcription factor activity by nucleocytoplasmic shuttling is NF-kB, a major regulator of I inflammatory responses (Hayden and Ghosh, 2008). The regulation is brought about by cytoplasmic retention of NF-kB by interaction with IkB and degradation of IkB in response to inflammatory signals results in movement of NF-kB to the nucleus. The gene expression response is shut down by nucleocytoplasmic shuttling of IkB and re-localization of the NF-kB-IkB complex in the cytoplasm. In this model, degradation of IkB, NF-kB, protein-protein interactions and protein-DNA interactions are regulated by extensive protein modifications (including phosphorylation, ubiquitination, acetylation) (Chen and Greene, 2004; Hayden and Ghosh, 2008), illustrating the possible complexity of such regulatory mechanisms.

The research described in this chapter provides novel insight in the regulation of the key transcription factor ORA59 in JA/ET-mediated responses. This also reports a novel function of CCCH-type ZF proteins, i.e. interaction with a transcription factor. Our data can be combined into an attractive, but certainly oversimplified, model. In the absence of colonization by necrotrophic pathogens, ZFAR1 interacts with and partially retains ORA59 in the cytoplasm. After perception of JA and ET, ORA59 is activated, presumably due to its dissociation from ZFAR1 and NLS-mediated transport into the nucleus. Inside the nucleus, ORA59 induces the expression of defense-related target genes. Because ORA59 shuttles between the nucleus and the cytoplasm, the quantitative output in target gene expression depends on the relative intracellular amounts of ZFAR1 and ORA59, the retention activity of ZFAR1 and the ORA59 export rate. This mechanism enables fine-tuning of ORA59-related target gene expression and, therefore, exact adjustment of the JA-mediated responses. Since protein modifications and degradation could also play an important role in fine-tuning of ORA59 activity, it is of interest to identify other ORA59 and ZFAR1 interacting proteins to understand the biochemical mechanism by which ORA59 is regulated and

to determine whether this interaction represents a cross node between different types of stress signaling pathways.

Material and Methods

Yeast two-hybrid assays

Full-length *ORA59* and *ORA59 deletion* derivative 81-244 cloned in pAS2.1 (acc. No. U30497) were co-transformed with empty pACT2 (acc. No. U29899) to yeast strain PJ64-4A (James et al., 1996). For auto-activation assays, transformants were plated on minimal synthetic defined (SD)-glucose medium supplemented with Met/Ura/His and lacking Leu and Trp (-LT). Ability to activate transcription in yeast was evaluated by monitoring growth after 7 days on selective SD medium lacking Leu, Trp and His (-LTH) supplemented with increasing 3-AT concentrations ranging from 0 to 50 mM. *ORA59* deletion derivative *81-244* cloned in pAS2.1 was used as bait for the screening. Using the Stratagene cDNA synthesis kit amplified cDNA libraries representing $2x10^6$ primary transformants were prepared from an equal mixture of RNAs from 10 days old ecotype Col-0 seedlings treated with 50 µM JA and 1 mM ethephon for 30 min and 4 hrs in the lambda vector HybriZAP-2.1 (Stratagene) and from an equal mixture of RNAs from stems, leaves and flowers of mature ecotype *Landsberg erecta* plants in the vector λACTII. The HybriZAP library was converted to a pAD-GAL4-2.1 plasmid library according to the HybriZAP manual. The λACTII library was converted in a pACT2 (Clontech) plasmid library via Cre-lox excision in *E. coli* strain BNN132. Co-transformation of bait and cDNA library at a ratio of 1:1 was performed into yeast strain PJ64-4A according to a yeast transformation protocol modified from Gietz et al. (1992). Transformed cells were plated on SD medium containing 20 mM 3-AT and lacking Trp, Leu and His. *ZFAR1* (At2g40140) was digested from pACT2 with SmaI and *Xho*I, and cloned in pAS2.1 digested with SmaI and *Sal*I. *ZFAR1 deletion* derivatives were PCR amplified with the following primer sets: 5'-CAG TGG CCA TGG AGG CCA TGT GCG GTG CAA AGA GCA AC-3' and 5'-GTC AGG ATC CTG CAT TCT CAC CAG GAT GAA C-3' for *ZF1-Δ1*; 5'-CAG TGG CCA TGG AGG CCG ATT CTC GGT TTG TTC CTA AC-3' and 5'-GTC AGG ATC CTT ATG CCA CAA TCT GCT GCT CAT GG-3' for *ZF1-Δ2*; and 5'-CAG TGG CCA TGG AGG CCC GGG ATG AGT TAA GAC CGG TT-3' and 5'-GTC AGG ATC CTT ATG CCA CAA TCT GCT GCT CAT GG-3' for *ZF1-Δ3* and cloned in pGEM-T Easy vector (Promega). Fragments were digested with *Sfi*I and *Bam*HI and cloned in pAS2.1 or pACT2 digested with *Sfi*I and *Bam*HI. *ZFAR2* (At3g55980) was amplified by PCR on a At Col0 cDNA template with 5'-GAG CTC GGA TCC AAA TGT GCA GTG GAC CAA AGA G-3' and 5'-CTG CAG CTC GAG AGA TCT TTA CAC CAC AGT CTG CTC CTT C-3' and cloned in pGEM-T Easy. For pAS2.1 cloning, *ZFAR2* was PCR amplified with primers 5'-GAT CCA TAT GTG CAG TGG ACC AAA GAG CAA TC-3' and 5'-GTC AGG ATC CTT ACA CCA CAG TCT GCT CCT TCT C 3', digested with *Nde*I/ *Bam*HI and cloned in pAS2.1 digested with *Nde*I/ *Bam*HI. For pACT2 cloning, *ZFAR2* was PCR amplified with primers 5'-CGG GAT CCC GAT GTG CAG TGG ACC AAA GAG C-3' and 5'-GGA TCC CTC GAG CAC CAC AGT CTG CTC CTT C-3', digested with *Bam*HI/*Xho*I and cloned in pAS2.1 digested with *Bam*HI/*Xho*I. Interaction

assays were performed by co-transformation of bait and prey plasmids into yeast strain PJ64-4A and plated on SD-LT medium. As control, empty pAS2.1 and pACT2 were used. Transformants were allowed to grow for 4-5 days. Subsequently, cells were incubated for 16 hours in liquid SD-LT and 5 µl of 100-fold dilutions were spotted on solid SD-LTH supplemented with increasing 3-AT concentrations. Yeast cells were allowed to grow for 7 days at 30°C.

Phylogenetic tree

Protein sequences were aligned by clustalW and tree was constructed with PHYLIP 3.60 package programs. A distance matrix was made with Protdist (Jones-Taylor-Thornton), followed by Neighbor (Neighbor-joining). The tree was drawn with Drawtree and adjusted with Retree.

Plant materials, growth conditions and chemical treatments

Arabidopsis thaliana wild-type plants, *zfar1zfar2*, and *ZFAR1*-overexpressing plants are in the genetic background of ecotype Col-0. T-DNA knockout lines *zfar1* (SALK_024800) and *zfar2* (SALK_141550) were obtained from NASC. Pollen from homozygous *zfar2* plants were used to pollinate emasculated homozygous *zfar1* flowers. F1 seedlings were grown without selection and genotyped with LBb1for 5'-GCG TGG ACC GCT TGC TGC AAC T-3' and SALK-zfar1R 5'-GAC GGA TAG TGG TTC ATC TGA G-3' or SALK-zfar2R 5'-CTT CCT TTT GCC TTG ATT CG-3' to identify double homozygous individuals.

Seeds were surface-sterilized by incubation for 1 min in 70 % ethanol, 15 min 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 grown on plates containing MA (Masson and Paszkowski, 1992) medium supplemented with 0.6% agar. 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 at 12 h light/12 h dark light regime for pathogen assays.

For treatments, seedlings were first grown on solid MA medium for 10 days, supplemented with 20 mg/L hygromycin for overexpressing lines. Twenty to 25 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml MA medium and incubated on a shaker for 4 additional days before treatment. Seedlings were treated for different time periods with 50 µM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.05% final concentration), 1 mM of the ET-releasing compound ethephon (Sigma) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration), 1 mM SA dissolved in water (pH 6.2), or a combination of JA and ethephon or JA and SA. As controls, seedlings were treated with 0.05% DMSO and 0.5 mM sodium phosphate pH 7.

Plasmid construction and protoplast assays

The *ORA59* (At1g06160) open reading frame (ORF) was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-CGG GAT CCA TAT GGA ATA TCA AAC TAA CTT C-3' and 5'-CGG GAT CCT CAA GAA CAT GAT CTC ATA AG-3', digested with *Bam*HI and cloned into pRT101 (Töpfer

et al., 1987). The *ORA47* (At1g74930) ORF was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-GAA GAT CTC ATA TGG TGA AGC AAG CGA TGA AG-3' and 5'-GAA GAT CTT CAA AAA TCC CAA AGA ATC AAA G-3' and following digestion with *Bgl*II cloned in pIC-20R (Marsh et al., 1984). The *ORA47* insert was excised with *Bgl*II and inserted into pMOG183 (Mogen International, Leiden, The Netherlands) digested with *Bam*HI. *ZFAR1* was digested from pACT2 with SmaI and *Spe*I or with *Bam*HI and *Spe*I and cloned in pRT101 digested with SmaI and *Xba*I and in pTH2^{BN} (a derivative of pTH2) with *Bgl*II and *Spe*I, respectively. For the construction of GFP-ORA59, the *ORA59* open reading frame (ORF) was excised from pBluescript SK+-ORA59 with *Eco*RI/*Spe*I respectively and cloned into pTH2^{BN} digested with *Eco*RI/*Spe*I respectively. For C-terminal GFP fusionsof full-length *ZFAR1* and deletion derivatives DNA fragments were PCR amplified with the primer sets 5'-ATC ATG TGC GGT GCA AAG AGC AAC C-3' and 5'-CAG TGG ATC CTG CCA CAA TCT GCT GCT CAT GGT C-3' for *ZFAR1*; 5'- ATC ATG TGC GGT GCA AAG AGC AAC C -3' and 5'-CAG TGG ATC CTG CAT TCT CAC CAG GAT GAA C-3' for *ZF1-Δ1*; 5'-CAG TGG CCA TTA TGG CCG ATT CTC GGT TTG TTC CTA AC-3' and 5'-CAG TGG ATC CTG CCA CAA TCT GCT GCT CAT GGT C-3' for *ZF1-Δ2*; 5'-GTC AAG ATC TAT GCG GGA TGA GTT AAG ACC GGT T-3' and 5'-CAG TGG ATC CTG CCA CAA TCT GCT GCT CAT GGT C-3'for *ZF1-Δ3*, digested at one end with *Bam*HI and cloned in pRT101 digested with *Eco*RI (filled in with T4 DNA polymerase) and *Bam*HI. Inserts were excised with *Sph*I and *Xba*I and cloned in pTH2Δ*Eco*RI (another derivative of pTH2) digested with *Sph*I and *Xba*I. *ZFAR2* was PCR amplified with primers 5'-TCA ATG TGC AGT GGA CCA AAG AGC-3' and 5'-CAG TGG TAC CCT CGA GTA CCA CAG TCT GCT CCT TCT C-3', digested at one end with *Kpn*I and cloned in pRT101 digested with *Xho*I (filled in with T4 DNA polymerase) and *Kpn*I. insert was excised with *Sph*I and *Xho*I and cloned in pTH2 (Niwa et al., 1999; Chiu et al., 1996) digested with *Sph*I and *Sal*I. Primer sets used for BiFC cloning were: 5'-GTC AAC TAG TAT GTG CGG TGC AAA GAG CAA CC-3' and 5'-CAG TGG ATC CTT ATG CCA CAA TCT GCT GCT CAT GG-3' for *ZFAR1* cloning with *Spe*I and *Bam*HI in pRTL2-YNEE and -YCHA; 5'-GTA CGC GGC CGC TTA TGT GCG GTG CAA AGA GCA ACC-3' and 5'-GCA AGC GGC CGC GTT GCC ACA ATC TGC TGC TCA TGG TC-3' for *ZFAR1* cloning with *Not*I in pRTL2-EEYN and -HAYC; 5'-GAT CGT CGA CAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CAG TAG ATC TTC AAG AAC ATG ATC TCA TAA GC-3 for *ORA59* cloning with *Sal*I and *Bgl*II in pRTL2-YNEE and -YCHA; 5'-GAT CGT CGA CAA TGG AAT ATC AAA CTA ACT TC-3' and 5'-CGA AGC GGC CGC GTA GAA CAT GAT CTC ATA AGC TC-3' for *ORA59* cloning with *Sal*I and *Bgl*II in pRTL2-EEYN and -HAYC; 5'-GTC ACA TAT GAG ATG ACT GAT TAC CGG CTA CAA CC-3' and 5'-CAG TAG ATC TTT AAC CGA TTT TTG AAA TCA AAC TTG C-3' for *AtMYC2* cloning with *Nde*I and *Bgl*II in pRTL2-YNEE and -YCHA; 5'-GTA CGC GGC CGC TTA TGA CTG ATT ACC GGC TAC AAC C-3' and 5'-GCA AGC GGC CGC GTA CCG ATT TTT GAA ATC AAA CTT GC-3' for *AtMYC2* cloning with *Not*I in pRTL2-EEYN and -HAYC; 5'-GAT CGT CGA CAA TGT CGA GTT CTA TGG AAT GTT C-3' and 5'-GAC TCA TAT GTT CAT ATT TCA GCT GCT AAA CCG AGC-3' for *JAZ1* cloning with *Sal*I and *Nde*I in pRTL2-YNEE and -YCHA; 5'-GAT CGT CGA CAA TGT CGA GTT CTA TGG AAT GTT C-3' and 5'-GCA AGC GGC CGC GTT ATT TCA GCT GCT AAA CCG AGC-3' for *JAZ1* cloning with *Sal*I and *Not*I in pRTL2-EEYN and –HAYC. PCR-amplified inserts were digested with the restriction enzymes mentioned above and cloned in the mentioned pRTL2 derivatives (Bracha-Drori et al.,

2004) digested with the corresponding enzymes.

Protoplasts were isolated from Arabidopsis cell suspension ecotype Col-0 and plasmid DNA was introduced by polyethylene glycol (PEG)-mediated transfection as previously described (Schirawski et al., 2000). Co-transformation with plasmids carrying *PDF1.2*-promoter-*GUS* or *AOC2*-promoter-*GUS* and effector plasmids carrying *ZFAR1*, *ORA59*, *ERF1* or *ORA47* fused to the *CaMV 35S* promoter were carried out. To study a possible effect of ZFAR1 interaction with the transcription factors, a ratio of 2:2:2 or 2:6:2 (µg GUS:ZFAR1:effector plasmid) was chosen. As controls, co-transformations of *PDF1.2*-promoter-*GUS* with the empty pRT101 expression vector were used. Protoplasts were incubated at 25°C for at least 16 hrs prior to harvesting by centrifugation and immediately frozen in liquid nitrogen. GUS activity assays were performed as described (van der Fits and Memelink, 1997). GUS activities from triplicate transformations were normalized against total protein content to correct for differences in protein extraction efficiencies. For nuclear localization studies, protoplasts expressing GFP-ORA59 were treated with 0.1% DMSO or 50 µM JA for 2 hours. For each data point at least 150 GFP-expressing protoplasts were analyzed by confocal microscopy and cytosolic and nuclear localization were scored as a percentage of the total number of GFP-expressing cells analysed. Images of transfected protoplasts were acquired with a Leica DM IRBE confocal laser scanning microscope equipped with an Argon laser line of 488 nm (excitation) and a band pass emission filter of 500-550 nm.

Plant vectors and transformation

For the construction of transgenic lines constitutively overexpressing *ZFAR1*, the Cauliflower Mosaic Virus (*CaMV*) *35S* cassette containing the *ZFAR1* ORF in sense orientation was digested from pRT101 with *Sph*I and cloned in pCAMBIA1300 (acc No. AF234296) digested with *Sph*I. The binary vector pCAMBIA1300-*ZFAR1* was introduced into *Agrobacterium tumefaciens* strain EHA105 (containing the Vir plasmid pSDM3010) (Hood et al., 1993). Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin. Transgenic plants from T2 generations were selected on MA medium containing only 20 mg/L hygromycin.

***Botrytis cinerea* pathogen assay**

B. cinerea was grown on potato dextrose agar plates for 2 weeks at 22°C. Spores were harvested as described by Broekaert et al. (1990). Plant seedlings germinated on plates were transferred to individual pots containing sterile soil and randomly distributed in trays. Seedlings were cultivated for another 3 weeks in a growth chamber with an 8 h day (1400 lux at 24°C) and 16 h night (20°C) cycle at 65% relative humidity. For inoculation with fungal pathogens, 3 µL droplets of spore suspension were deposited on 5 mature leaves of each plant. Inocula consisted of 7.5×10^5/mL *B. cinerea* spores incubated in half-strength potato dextrose broth for 2 hours prior to inoculation. After inoculation, plants were maintained under high relative humidity with the same temperature and photoperiod conditions. In each experiment, 45 plants per

genotype were inoculated. Control plants were not inoculated but kept under the same growing conditions.

Disease ratings were assessed at day 2 and day 3 after inoculation with *B. cinerea*. Disease ratings were assigned to the inoculated leaves of each plant, as described by Ton et al. (2002) with minor modifications. Briefly, intensity of disease symptoms and lesion size were classified: I, no visible disease symptoms; II, non-spreading lesion; III, spreading lesion; IV, spreading lesion surrounded by a chlorotic halo; and V, spreading lesion with extensive tissue maceration. For gene expression analysis, 5 infected and several non-infected leaves from 5 inoculated plants of each genotype were collected at day 2 and day 3 after *B. cinerea* infection. Leaf tissues were pooled and frozen in liquid nitrogen and after stored at –80°C.

RNA extraction and Northern blot analyses

Total RNA was extracted from pulverized frozen tissue by phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride, washed with 70 % ethanol, and resuspended in water. For RNA-blot analysis, 10 µg RNA samples were subjected to electrophoresis on 1.5% agarose/1% formaldehyde gels, and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). All probes were ^{32}P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed on X-ray films (Fuji, Tokyo, Japan).

DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5'-AAT GAG CTC TCA TGG CTA AGT TTG CTT CC-3' and 5'-AAT CCA TGG AAT ACA CAC GAT TTA GCA CC-3' for *PDF1.2* (At5g44420); 5'-ATG GCT CTC ACA AAA ATC TTC-3' and 5'-TTA GCA AGT TAT GTT GGC GC-3' for *Chit* (At2g43580); 5'-GCT TCA GAC TAC TGT GAA CC-3' and 5'-TCC ACC GTT AAT GAT GTT CG-3' for; 5'-CGG GAT CCA TAT GAA GAT CAG ACT TAG CAT AAC-3' and 5'-CGG GAT CCT CAA ACG CGA TCA ATG GCC GAA AC-3' for *HEL* (At3g04720); 5'-TGT CCC ACT CTC GTT CTT TG-3' and 5'-TCA AGT CCG GCT GGA ACA TTG-3' for *AN5-AT* (At5g61160); 5'-GCA ATT CTC GAT CCG AGC TC-3' and 5'-CTC TAC TTG GAG AAG CCT TC-3' for *IGPS* (At2g04400); 5'-TCC ACC AGA TCT ATC TAC GG- 3' and 5'- GCA GCG TAA CCT CCA GTG GC- 3' for *DHS1* (At4g39980); 5'-CTG TGC CAA TCT ACG AGG GTT-3' and 5'-GGA AAC CTC AAA GAC CAG CTC-3' for ACT2 (At3g18780); 5'-CGG GAA GGA TCG TGA TGG A-3' and 5'-CCA ACC TTC TCG ATG GCC T-3' for *ROC* (At4g38740). *ORA59* (At1g06160) open reading frame was digested with *Eco*RI and *Sal*I from pGEM-T Easy. *ZFAR1* (A2g40140) and *ZFAR2* (At3g55980) were digested with *Sac*I and *Xho*I from pGEM-T Easy. *ChitB* (At3g12500) was digested with *Eco*RI from pGEM-T Easy.

Protein extraction and Immunoblot analysis

Protoplasts were ground in 50 µl of CCLR protein extraction buffer (25 mM Na-phosphate buffer pH 7.5, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1% triton X-100, 10% glycerol). 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.

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) in TBST with 5% non-fat milk. After 1 hr incubation at room temperature the blots were washed 4x with TBST. After incubation with anti-GFP antibodies, blots were incubated for 1 hr with anti-rabbit IgG antibodies linked to peroxidase (1:10000; Sigma) 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% H_2O_2) 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).

Acknowledgements

We are grateful to Antony Champion for the construction of the JA-treated library and cloning of ORA59 in pAS2.1 and to Solange Villette for pGEM T-Easy clonings of ZFAR1 deletion derivatives. A.P.K. was partially supported by a van der Leeuw grant from the Netherlands Organization for Scientific Research (NWO) awarded to J.M.

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SUMMARY

Summary

Fitness and survival of plants depend on efficient mechanisms to cope with adverse conditions present in natural environments. The initiation of defense responses against attacking organisms depends on the action of several endogenously produced phytohormones, including jasmonic acid (JA) and related jasmonates (JAs) and ethylene (ET). JAs play a major role in defense against wounding, insects and necrotrophic pathogens. The current knowledge of the octadecanoid pathway for biosynthesis of JAs and of the different components of the JA signaling pathway are reviewed in **Chapter 1.**

In defense against necrotrophic pathogens, the JA and ET signaling pathways synergize to activate a specific set of defense genes, including the *PDF1.*2 gene encoding the small antimicrobial protein PLANT DEFENSIN 1.2. The APETALA2 (AP2)-domain transcription factor OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2-domain protein 59 (*ORA59)* acts as the integrator of the JA and ET signaling pathways in *Arabidopsis thaliana* and is the key regulator of JA- and ET-responsive *PDF1.2* expression. The mechanisms by which ORA59 activates its target genes were not previously investigated. The studies described in this thesis focused on the functional analysis of the JA/ET-responsive transcription factor ORA59 in Arabidopsis.

Studies described in **Chapter 2** aimed at dissecting the interaction of ORA59 and the related transcription factor ETHYLENE-RESPONSIVE FACTOR 1 (ERF1) with the *PDF1.2* promoter. ERF1 had been previously suggested by others to also integrate JA and ET signaling pathways, but it plays a minor role if any since plants which lack *ORA59* expression do not express JA- and ET-responsive genes. We showed that 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 of these proteins. We did not observe a synergistic effect between ORA59 and ERF1 in trans-activating the *PDF1.2* promoter, indicating that each transcription factor acts independently on the *PDF1.2* promoter. Using the chromatin immunoprecipitation technique we were able to show that ORA59 binds to the *PDF1.2* promoter *in vivo*. In stably transformed plants single mutation of either GCC box completely abolished the expression of the *PDF1.2* promoter in response to JA alone or to the combination of JA with the ET-releasing agent ethephon. A tetramer of a single GCC box conferred JA/ethephon-responsive gene expression, demonstrating that the JA and ET signaling pathways converge to a single GCC box. Therefore ORA59 and two functionally equivalent GCC box binding sites form the module that enables the *PDF1.2* gene to respond synergistically to simultaneous activation of the JA and ET signaling pathways.

Defense responses need to be suppressed under normal growth conditions but when required should be quickly activated, a process which involves tight regulation of the activity of key transcription factors. Studies on the effects of JA on the activity of ORA59 protein are described in **Chapter 3.** The results show that JA caused stabilization as well as nuclear localization of ORA59. The re-localization of ORA59 depended on nuclear localization (NLS) and export (NES) signals in the protein. Besides the NLS and NES at least two other protein domains also affected ORA59 localization as well as stabilization. Interestingly, JA-responsive nuclear localization of ORA59 did not require the JAs receptor COI1. Based on the results in **Chapter 3** we postulate that Arabidopsis cells have a JAs receptor distinct from COI1, an F-box protein that targets ORA59 for degradation, and a repressor protein that sequesters ORA59 in the cytoplasm.

Therefore we set out to identify and functionally characterize ORA59-interacting proteins.

Chapter 4 describes the characterization of the ORA59-interacting CCCH zinc finger protein ZFAR1 identified as an interacting protein in yeast two-hybrid screening. A closely related protein called ZFAR2 also interacted with ORA59 in yeast. Bimolecular Fluorescent Complementation (BiFC) assays showed that ORA59 and ZFAR1 interacted in the cytoplasm of Arabidopsis cell suspension protoplasts. Re-localization studies of ORA59 showed that ZFAR1 interfered with JA-induced nuclear localization of ORA59. Moreover, ZFAR1 repressed ORA59 activity in trans-activation assays. Plant infection assays with the necrotrophic fungus *Botrytis cinerea* showed that transgenic plants overexpressing ZFAR1 showed accelerated disease progression, while a *zfar1zfar2* double knockout mutant was less severely affected than wild-type plants. The significant differences in resistance levels were not associated with major changes in the expression levels of JA/ET-dependent defense marker genes such as *PDF1.2*. In conclusion, our results indicate that ZFAR1 acts as a repressor protein that sequesters ORA59 in the cytoplasm to fine-tune basal resistance against pathogens.

The identification of the mechanisms whereby the transcription factor ORA59 is activated by JA at the protein level, the interaction of ORA59 with other transcription factors, and identification of the binding sites in the promoters of ORA59 target genes is of major importance to understand how JAs mediate defense responses. A model summarizing the main results presented in this thesis is depicted in Figure 1. ORA59 is present at very low levels in unelicited cells due to degradation via the 26S proteasome. Further control of the background expression level of target genes is achieved by retention of ORA59 in the cytoplasm via interaction with ZFAR1, which masks the NLS in ORA59 (Figure 1a). Upon infection with a necrotrophic fungus, JAs and ET are produced, which initiate signaling pathways leading to the release of ORA59 from the repressor ZFAR1 and causing ORA59 stabilization. ORA59 then moves to the nucleus, where it activates defense genes including *PDF1.2* leading to resistance (Figure 1b).

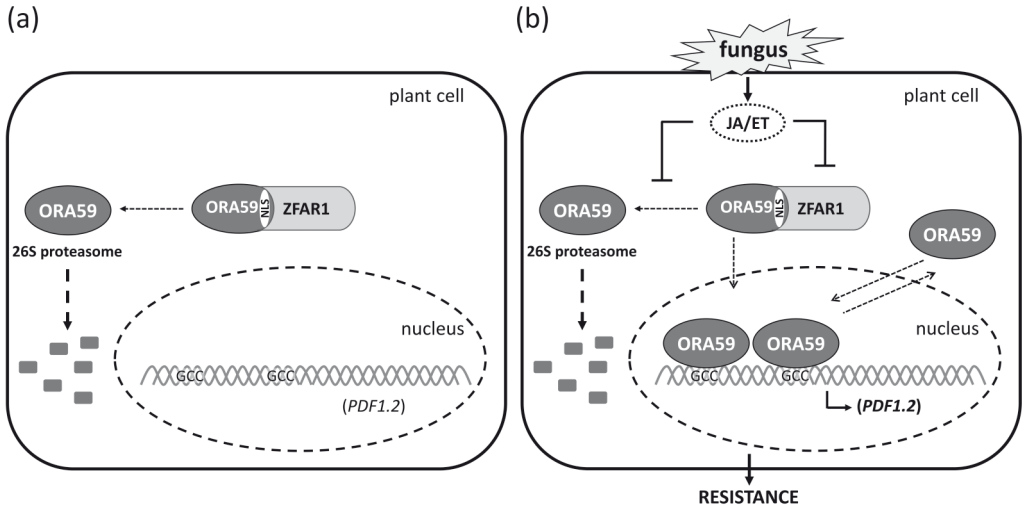


Figure 1. Model of ORA59 regulation. See text for details.

Samenvatting

Samenvatting

De fitness en overleving van planten hangt af van efficiënte mechanismen om te reageren op bedreigende omstandigheden aanwezig in de natuurlijke leefomgeving. Bij het aanschakelen van de verdediging tegen aanvallers spelen endogeen geproduceerde plantenhormonen, zoals jasmonzuur (JA) en gerelateerde jasmonaten (JAs) en ethyleen (ET), een sleutelrol. JAs spelen een hoofdrol in de verdediging tegen verwonding, insecten en necrotrofe micro-organismen. De huidige inzichten in de biosynthese van JAs via de octadecanoïd route en in de verschillende componenten van de JA signaaltransductieroute worden beschreven in **Hoofdstuk 1**.

In de verdediging tegen necrotrofe pathogenen werken de JA en ET signaaltransductieroutes op synergistische wijze samen teneinde een specifieke set van genen aan te schakelen, zoals het *PDF1.2* gen dat codeert voor het kleine antimicrobiële eiwit PLANT DEFENSIN 1.2. De APETALA2 (AP2)-domein transcriptiefactor OCTADECANOÏD-RESPONSIEF ARABIDOPSIS AP2-domein eiwit 59 (*ORA59)* integreert de JA en ET signaaltransductieroutes in *Arabidopsis thaliana* (zandraket) en is de hoofdregulator van de expressie van het *PDF1.2* gen in respons op JA en ET. De wijze waarop ORA59 zijn doelwitgenen aanschakelt werd nog niet eerder bestudeerd. Het onderzoek beschreven in dit proefschrift was er op gericht om de werking van de JA/ET-responsieve transcriptiefactor ORA59 in Arabidopsis op te helderen.

Het onderzoek beschreven in **Hoofdstuk 2** had als doel om de interactie van ORA59 en de verwante transcriptiefactor ETHYLEEN-RESPONSIEVE FACTOR 1 (ERF1) met de *PDF1.2* promoter uiteen te rafelen. Andere onderzoekers hebben eerder gesuggereerd dat ERF1 ook als integrator van de JA en ET signaaltransductieroutes optreedt, maar als het al een rol speelt is deze miniem, aangezien planten die geen expressie van ORA59 vertonen niet in staat zijn om JA- en ET-responsieve genen aan te schakelen.

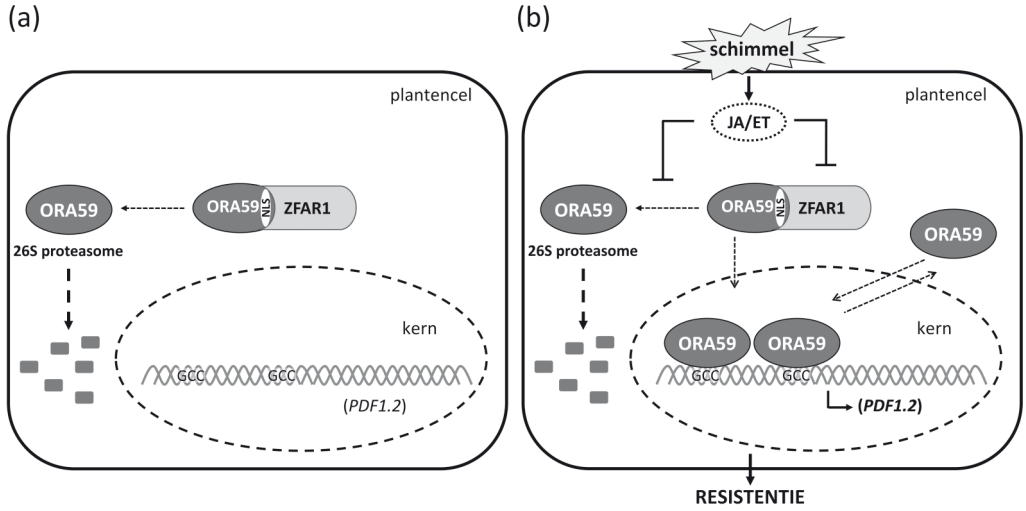
Zoals beschreven in **Hoofdstuk 2** hebben wij aangetoond dat twee GCC motieven in de *PDF1.2* promoter belangrijk zijn voor *trans*-activering door ORA59 en ERF1 in transiënte testen in protoplasten en voor binding van deze eiwitten *in vitro*. We hebben geen synergistische samenwerking tussen ORA59 en ERF1 kunnen constateren, wat aangeeft dat deze transcriptiefactoren onafhankelijk van elkaar de *PDF1.2* promoter aanschakelen. Met behulp van de chromatine immunoprecipitatie techniek hebben we kunnen aantonen dat ORA59 *in vivo* bind aan de *PDF1.2* promoter. In stabiel getransformeerde planten doet mutatie van één van beide GCC motieven de activiteit van de PDF1.2 promoter in respons op toediening van JA alleen of samen met de ET-genererende verbinding ethephon volledig teniet. Een tetrameer van één enkel GCC motief bracht JA/ethephon-responsieve genexpressie tot stand, wat aantoont dat de JA en ET signaaltransductieroutes convergeren naar één enkel GCC motief. Uit deze resultaten kan geconcludeerd worden dat ORA59 en twee functioneel equivalente GCC motieven de module vormen die de synergistische respons van het *PDF1.2* gen op gelijktijdige activering van de JA en ET signaaltransductieroutes bewerkstelligt.

Verdediging moet onder normale groeiomstandigheden uit staan, maar moet als het nodig is snel aangezet kunnen worden. Dit vereist een strikte regulatie van de activiteit van de

sleuteltranscriptiefactoren. Onderzoek naar de effecten van JA op de activiteit van ORA59 eiwit zijn beschreven in **Hoofdstuk 3**. De resultaten laten zien dat JA zowel stabilisatie als nucleaire lokalisatie van ORA59 veroorzaakt. De herverdeling van ORA59 was afhankelijk van nucleaire lokalisatie (NLS) en nucleaire export (NES) signalen in het eiwit. Naast de NLS en NES waren nog minimaal twee andere eiwitdomeinen betrokken bij herverdeling en stabilisatie van ORA59. Een intrigerende ontdekking was dat de herverdeling van ORA59 na toediening van JA ook plaats vond in cellen die de JAs receptor COI1 missen. Gebaseerd op de resultaten in **Hoofdstuk 3** postuleren we dat Arabidopsis cellen in het bezit zijn van een JAs receptor die verschilt van COI1, van een F-box eiwit dat ORA59 markeert voor afbraak, en van een repressoreiwit dat de lokalisatie van ORA59 beperkt tot het cytoplasma.

Dit bracht ons er toe om op zoek te gaan naar eiwitten die interactie vertonen met ORA59. **Hoofdstuk 4** beschrijft de karakterisering van het CCCH zinkvingereiwit ZFAR1, dat interactie vertoonde met ORA59 in tweehybride screening in gist. Een zeer verwant eiwit dat ZFAR2 is genoemd bond ook aan ORA59 in gist. Bimoleculaire Fluorescentie Complementatie (BiFC) testen toonden aan dat de binding tussen ORA59 en ZFAR1 plaats vond in het cytoplasma van Arabidopsis celsuspensieprotoplasten. Co-expressie van ZFAR1 verhinderde de nucleaire lokalisatie van ORA59 in Arabidopsis protoplasten na toediening van JA. Het remde ook de activiteit van ORA59 op de *PDF1.2* promoter in *trans*-activeringstesten. Infectietesten met de necrotrofe schimmel *Botrytis cinerea* (grauwe schimmel) lieten zien dat transgene planten met een verhoogde expressie van ZFAR1 meer ziekteverschijnselen vertoonden, terwijl een *zfar1zfar2* dubbele knock-out mutant minder ziekteverschijnselen vertoonde dan wildtype planten. De significante verschillen in de ontwikkeling van ziekteverschijnselen ging niet gepaard met grote veranderingen in de expressie van JA/ET-afhankelijke merkergenen voor verdediging zoals *PDF1.2*. Samen geven de resultaten aan dat ZFAR1 een repressor is dat die lokalisatie van ORA59 beperkt tot het cytoplasma om zo de basale resistentie tegen pathogenen nauwkeurig te regelen.

De identificatie van mechanismen waardoor de transcriptiefactor ORA59 op eiwitniveau geactiveerd wordt door JA, de interactie van ORA59 met andere transcriptiefactoren, en de identificatie van bindingsplaatsen in de promoters van doelwitgenen van ORA59 zijn van groot belang om te kunnen begrijpen hoe JAs de verdediging van planten reguleren. Figuur 1 toont een model dat de belangrijkste resultaten van het onderzoek beschreven in dit proefschrift samenvat. ORA59 is in zeer lage hoeveelheden aanwezig in gezonde cellen doordat het afgebroken wordt door het 26S proteasoom. De achtergrondexpressie van de doelwitgenen wordt ook nog eens verlaagd doordat ORA59 vastgehouden wordt in het cytoplasma door ZFAR1, dat de NLS in ORA59 maskeert (Fig. 1a). Na infectie met een necrotrofe schimmel produceren de plantencellen JAs en ET, die signaaltransductieroutes op gang brengen die leiden tot het loslaten van ORA59 van de repressor ZFAR1 en tot stabilisatie van ORA59. ORA59 gaat dan naar de kern, waar het genen betrokken bij verdediging zoals *PDF1.2* aan schakelt, wat vervolgens leidt tot resistentie (Fig. 1b).



Figuur 1. Model voor regulatie van ORA59. Zie tekst voor details.

Curriculum vitae

Ana Paula Körbes was born on August 11, 1979 in Porto Alegre, State of Rio Grande do Sul (RS), Brazil. She attended primary and high school at Colégio São José in São Leopoldo. In 1998 she started the study of Biological Sciences at Universidade do Vale do Rio dos Sinos (UNISINOS) in São Leopoldo. From August 2000 until November 2002 she did a research internship in tissue culture techniques and soybean transformation under the supervision of Dr. Annette Droste at Laboratório de Cultura de Tecidos Vegetais at UNISINOS. In 2002 she obtained her BSc in Biology with honorable mention. She entered the Master program of Genetics and Molecular Biology at Universidade Federal do Rio Grande do Sul (UFRGS) in Porto Alegre in April 2004. Her master project focused on the characterization of eucalyptus genes conferring drought tolerance and was carried out under the supervision of Dr. Giancarlo Pasquali at Centro de Biotecnologia of UFRGS. She obtained her MSc diploma in March 2006. From April 2006 until April 2010 she worked as a PhD student under the supervision of Prof. Dr. Johan Memelink in the Institute of Biology Leiden (IBL) at Leiden University in the Netherlands. Since May 2010 she is working as a post-doc with Dr. Annelies Schulte at ExPlant Technologies in Leiden.