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To Maan g, Ahmed and my family…!!!

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

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Stress signalling 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 signalling molecules jasmonates (JAs; Turner et al., 2002; Wasternack, 2007), ethylene (ET) and salicylic acid (SA) (Pieterse et al., 2009). 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 JAs/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 (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 signalling pathways affect each other

through extensive cross-talk occurring at different levels (Pieterse et al., 2009). Whereas SA works mainly antagonistically to JAs, ET can have either synergistic or antagonistic effects on certain subsets of genes regulated by JAs. 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 JAs and ET, whereas genes encoding proteins involved in defense against herbivorous insects, such as the acid phosphatase VSP1, are strongly induced by JAs alone and ET has a strong negative effect on the JAs 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).

Stress-induced JAs biosynthesis

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues (Turner et al., 2002; Wasternack, 2007). These signalling molecules affect a variety of plant processes including fruit ripening (Creelman and Mullet, 1997), stamen development and 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 JAs 12-oxo-phytodienoic acid (OPDA), JA, and methyl-JA (MeJA) act as active signalling molecules (Wasternack, 2007), although some of the evidence to support this notion was challenged by the discovery that the Arabidopsis *opr3* mutant used in several of the studies can synthesize JA under certain conditions (Chehab et al., 2011). A well-established bioactive JAs is (+)-7-*iso*-Jasmonoyl-L-Isoleucine (JA-Ile; Fonseca et al., 2009), which is perceived by the receptor CORONATINE INSENSITIVE1 (COI1; Fonseca et al., 2009; Katsir et al., 2008; Sheard et al., 2010).

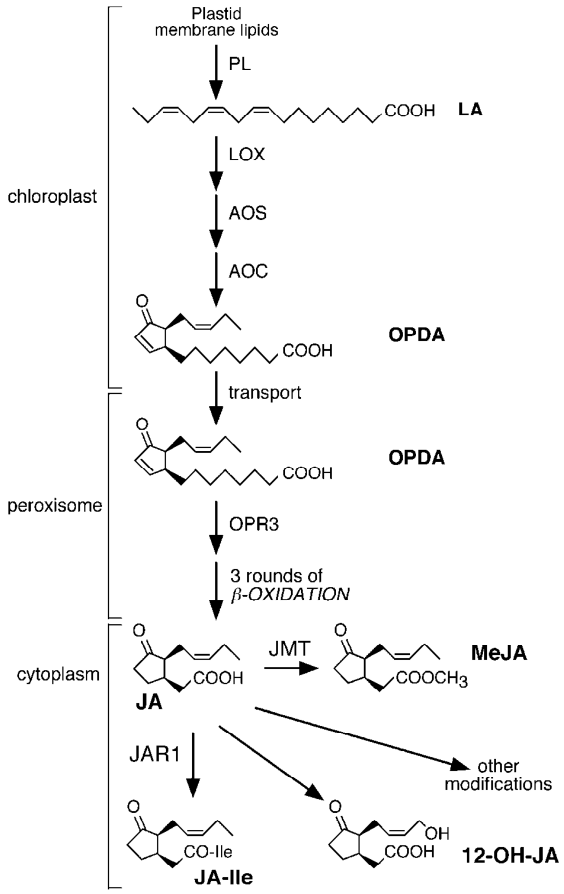

Figure 1. Schematic representation of the octadecanoid pathway leading to jasmonate biosynthesis. 12-OH-JA, 12-hydroxy-jasmonic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; JAR1, enzyme responsible for the conjugation of JA with isoleucine (JA-Ile); JMT, *S*-adenosyl-L-methionine:jasmonic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

JAs are synthesized via the octadecanoid pathway. Most of the enzymes of this pathway leading to JAs biosynthesis have now been identified by a combination of biochemical and genetic approaches (Fig. 1; Creelman and Mulpuri, 2002; Turner et al., 2002). The enzymes leading to JAs biosynthesis are

located in two different subcellular compartments (Vick and Zimmerman, 1984; Schaller, 2001; Wasternack, 2007). The octadecanoid pathway starts in the chloroplasts with phospholipase-mediated release of α-linolenic acid from membrane lipids. The fatty acid α-linolenic acid is then converted to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The second part of the pathway takes place in peroxisomes. OPDA is transported from the chloroplasts to the peroxisomes where it is reduced by OPDA reductase (OPR3) to give 3-oxo-2(2′[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), followed by three rounds of beta-oxidation involving three enzymes to yield (+)-*7-iso*-JA which equilibrates to the more stable (-)-JA. Subsequently, JA can be metabolized in the cytoplasm by at least seven different reactions (Schaller et al., 2005). Well-characterized reactions include methylation to methyl-JA (MeJA) by *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase (JMT; Seo et al., 2001), conjugation to amino acids by JA amino acid synthase (JAR1; Staswick and Tiryaki, 2004) or hydroxylation to 12-hydroxyjasmonic acid (12-OH-JA; Miersch et al., 2007). The bioactive JA-Ile is synthesized from (+)-7-*iso*-JA by JAR1-mediated conjugation to isoleucine.

How stress signals induce JAs biosynthesis is still unclear and the molecular components involved in the perception of the initial stimulus and in subsequent signal transduction resulting in JAs production are largely unknown. The control points that govern the synthesis and accumulation of JAs remain to be identified. Timing and control of JAs biosynthesis suggest several ways in which JAs signaling might be modulated during stress perception. One level of control in JAs biosynthesis and/or signaling might be the sequestration of biosynthetic enzymes and substrates inside the chloroplasts (Stenzel et al., 2003). In this way, JAs biosynthesis and signaling would only be activated by the availability of substrate upon cellular decompartmentalization during wounding or pathogen attack. However, wounding induces the expression of several JAs biosynthesis genes (Turner et al., 2002), suggesting that, at least partly, the wound-induced production of JA is a result of the increased transcription of genes encoding the JAs biosynthesis pathway enzymes and

their subsequent *de novo* protein synthesis. In addition, transcript profiling analyses showed that MeJA treatment induced the expression of several genes involved in JAs biosynthesis, such as *AOC*, *OPR1*, *OPR3*, *LOX2* and *AOS* (Sasaki et al., 2001; Pauwels et al., 2008). In addition many other reports show that JA induces transcription of the JAs biosynthesis genes *LOX2, AOS*, *OPR3*, *DAD1*, *JMT,* and *AOC* (Bell and Mullet, 1993; Laudert and Weiler, 1998; Mussig et al., 2000; Ishiguro et al., 2001; Seo et al., 2001; Stenzel et al., 2003).

Together, these results indicate the existence of a positive feedback loop for JAs biosynthesis in which JAs stimulates their own production (Fig. 3).

JAs-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 JAs responsiveness have been identified. The most common JAs-responsive promoter sequences are the GCC motif and the G-box. In addition several other JAs-responsive promoter elements have been reported.

In the promoter of the terpenoid indole alkaloid biosynthesis gene *strictosidine synthase* (*STR*) from *Catharanthus roseus* a JAs- 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*, two functionally equivalent GCC motifs (GCCGCC) are required for the JA-responsive activity of the *PDF1.2* promoter (Brown et al., 2003; Zarei et al., 2011). The GCC motif has also been shown to function autonomously as an ET-responsive element (Ohme-

Takagi and Shinshi, 1995; Fujimoto et al., 2000). The expression of the *PDF1.2* gene (Penninckx et al., 1998) and the activity of the *PDF1.2* promoter (Zarei et al., 2011) are synergistically induced by a combination of JA and ET. A tetramer of one of the GCC boxes confers JA- and ET-responsive gene expression (Zarei et al., 2011), showing that both signals converge 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 JAs 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), the Octadecanoid-derivative Responsive *Catharanthus* AP2-domain gene (*ORCA3*; Vom Endt et al., 2007) and the Jasmonate ZIM-domain 2 gene (JAZ2; Figueroa and Browse, 2011). 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). In the JAZ2 promoter the G-box is flanked at its 3′ side by 4 thymidine nucleotides which are essential for JA-responsive activity (Figueroa and Browse, 2011).

Several additional JAs-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 JAs-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 JAs-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 JAs 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 JAs-responsive elements other than the GCC motif and the G-box confer responses to bioactive JAs via COI1.

Transcription factors and JAs responses

JAZ repressors and COI1 control the activity of transcription factors

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 JAs 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 JAs-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 JAs responses (Lorenzo and Solano, 2005). COI1 is a component that is specific to the JAs pathway, whereas SGT1b and AXR1 are shared by other signalling 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 signalling pathways (Austin et al., 2002; Azevedo et al., 2002; Gray et al., 2003).

A particularly effective screen for JAs signalling 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 *MYC2* gene (Lorenzo et al., 2004), encoding a basic-Helix-Loop-Helix (bHLH) transcription factor which regulates a subset of JAs-responsive genes involved in wounding responses and resistance against insects (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007). Recombinant MYC2 binds *in vitro* to the G-box and related sequences (de Pater et al., 1997; Chini et al., 2007; Dombrecht et al., 2007; Godoy et al., 2011; Montiel et al., 2011). 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).

The gene affected in the *jai3* mutant 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. JAI3/JAZ3 and the majority of the other JAZ proteins were shown to interact *in vitro* and in yeast with MYC2 (Chini et al., 2007; Chini et al., 2009; Chung and Howe, 2009) and the releated bHLH transcription factors MYC3 and MYC4 (Fernandez-Calvo et al., 2011; Niu et al., 2011). Based on these findings it was postulated that JAZ are repressors of MYC proteins which are rapidly degraded in response to JA thereby activating MYCs (Fig. 2). Indeed JAZ1 was shown to repress the activity of MYC2 in a transient activation assay (Hou et al., 2010). JAZ can bind the general co-repressors TOPLESS (TPL) and TPL-like proteins either directly (Shyu et al., 2012) or via the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA; Pauwels et al., 2010). More recently a variety of transcription factors were shown to interact with members of the JAZ family (Pauwels and Goossens, 2011). These include the R2R3-MYB transcription factors MYB21 and MYB24 involved in stamen development and male fertility (Song et al., 2011) and the bHLH transcription factors GL3, EGL3 and TT8 involved in anthocyanin biosynthesis and trichome initiation (Qi et al., 2011).

JAZ variants lacking effective Jas domains also occur naturally in *Arabidopsis*. For JAZ10.1, two more stable variants have been described which

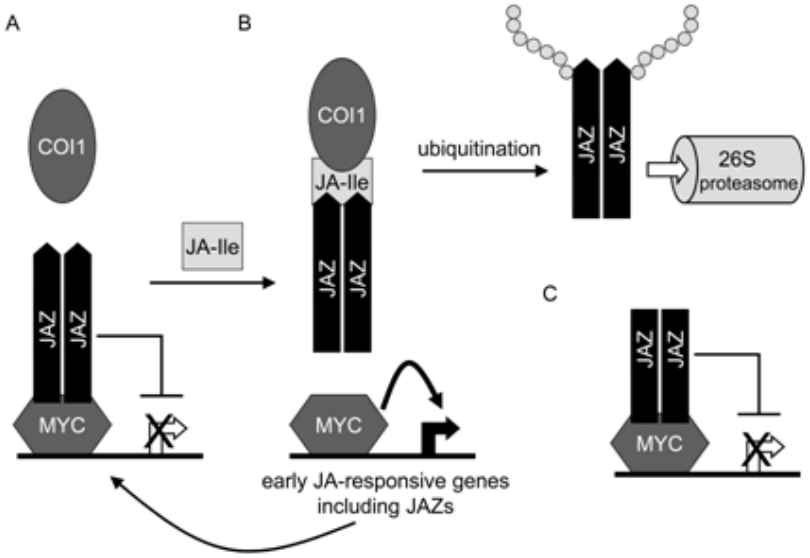

Figure 2. Model for regulation of jasmonate-responsive gene expression by MYC and JAZ proteins. Although depicted as a single protein, COI1 forms part of the putative E3 ubiquitin ligase SCF^{COI1}. (A) In the absence of JA-Ile, a (hetero) dimer of JAZ proteins interacts with MYC maintaining these transcription factors 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. MYC is liberated and activates

are translated from alternatively spliced mRNAs. JAZ10.3 misses a few amino acids at the C terminus, making it more stable in response to JAs (Chung and Howe, 2009), and therefore it has dominant-negative effects on JAs 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-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 provided evidence that COI1 is the receptor for at least certain JAs including JA-Ile, as well as for the microbial JA-Ile mimic coronatine. This notion was confirmed by binding studies and structural elucidation of recombinant COI1 co-crystallized with the Jas domain and JA-Ile (Sheard et al., 2010).

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 MYC2 (Chini et al., 2007) and MYC3 and MYC4 (Fernandez-Calvo et al., 2011; Niu et al., 2011). The model is therefore that MYC and JAZ proteins form a JAs-responsive oscillator, where JAZ proteins negatively regulate MYC activity at the protein level, JAs cause JAZ degradation and MYC activation, and MYC switches on the expression of JAZ

repressors at the gene level (Fig. 2). Homo- and heterodimerization of JAZ proteins likely play important roles in MYC 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 JAs 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 Response Factor) transcriptional activators. The F-box protein TRANSPORT INHIBITOR RESPONSE PROTEIN 1 (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 whole gene family was

screened using Northern blot expression analysis for induced expression in 2-weeks old seedlings after JA treatment (Atallah, 2005), resulting in the identification of 14 JA-responsive *AP2/ERF* genes. Transgenic Arabidopsis plants overexpressing ten of those genes in an inducible manner were screened for upregulation of a selection of JAs-responsive genes involved in a variety of JAs responses (Pré, 2006). This resulted in the identification of 4 AP2/ERF-domain proteins that affected gene expression in this screen, i.e. ORA59, ERF4, ORA33, and ORA47.

ORA59

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 (Fig. 3). 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 to non-specific expression of defense genes including *PDF1.2*, whereas ORA59 and ERF1 are *bona fide* direct regulators of *PDF1.2*.

ERF4

ERF4 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 *ERF4* 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 *ERF4* had no effect on the basal transcript level of several JA-

responsive genes in untreated plants. However, upon JA and/or ET treatment, *ERF4*-overexpressing plants showed significantly 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 *ERF4* 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 *ERF4*-overexpressing plants. This demonstrates that ERF4 plays a role in JA and ET signalling 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 *ERF4* 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 ERF4 positively regulated the expression of these genes in response to JA treatment. It is not clear how the positive effect of *ERF4* overexpression on JA signalling for this gene subset is operating at the molecular level, but assuming that ERF4 always acts as a repressor, the positive effect is hypothesized to be caused by the repression of a repressor. The ET signalling 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 MYC2 (Fig. 3; 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 MYC2 and the repressor ERF1 on JA-responsive *VSP2* expression remains to be characterized. It is possible that ERF4 antagonizes the ERF1-mediated negative effect of ET on the expression of a subset of JA-responsive genes, including *VSP* genes (Fig. 3). ERF4 and MYC2 seem to positively regulate the same subset of JA-responsive genes. However, overexpression of *MYC2* is sufficient to activate *VSP2* expression in the absence

of JAs (Lorenzo et al., 2004), which is not the case in *ERF4*-overexpressing plants (Pré, 2006).

Therefore, JA and ET synergistically induce both activators (ORA59 and ERF1) and repressors (ERF4) 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).

ORA47

The gene encoding the AP2-ERF-domain transcription factor ORA47 responds to JA treatment (Atallah, 2005; Wang et al., 2008; Pauwels et al., 2008) in a COI1-dependent manner (Atallah, 2005; Wang et al., 2008). Wang et al. (2008) reported that overexpression using the constitutive CaMV 35S promoter resulted in increased expression of *VSP2* but not of *LOX3*, and they concluded that ORA47 controls a similar gene set as MYC2. The latter conclusion was also drawn by Pauwels et al. (2008), but based on a data set contradicting the results of Wang et al. (2008). They found that both MYC2 as well as ORA47 were able to trans-activate the *LOX3* promoter in a transient assay, and they hypothesized

Conclusions

Frequently occurring JAs-responsive promoter sequences are the GCC motif, which is commonly found in promoters activated synergistically by JA and ET, and the G-box, which is commonly found in promoters activated by

JAs and repressed by ET. Important transcription factors conferring JAs-responsive gene expression in *Arabidopsis* are ORA59 (Pré et al., 2008) and MYC2 (Boter et al., 2004; Lorenzo et al., 2004) and MYC3 and MYC4 (Fernandez-Calvo et al., 2011; Niu et al., 2011), with other transcription factors acting as positive (e.g. ERF1) and negative (e.g. ERF4) modulators of the gene

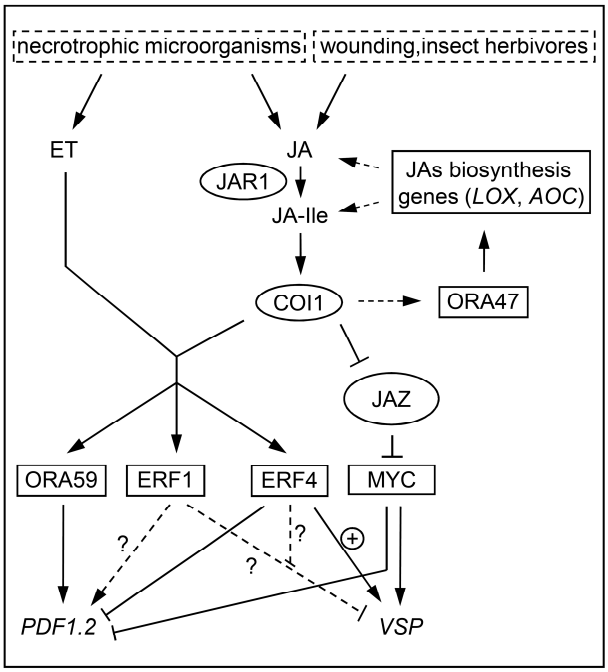

Figure 3. Model for the role of transcription factors in the stress-responsive network involving the JA and ET signaling pathways. Different types of biotic or abiotic stress, including wounding, attack by herbivorous insects and infection with necrotrophic pathogens, induce the synthesis of JA and related 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, ERF4, MYC and ORA47, 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 MYC from repression. The MYC bHLH-type transcription factors positively regulate the expression of wound-responsive genes (e.g. *VSP*) and repress other genes, including *PDF1.2*. The JAs and ET signals cooperate to induce the expression of genes encoding the AP2/ERF-domain transcription factors ORA59, ERF1 and ERF4. 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, ERF4 represses the induction of JAs/ET-responsive genes including *PDF1.2*. ERF4 also enhances the JAs-induced expression of MYC target genes including *VSP* (circled plus), possibly by repressing the negative effect of ET executed by ERF1 (dashed bar line and question mark). JAs signalling also induces the expression of the AP2/ERF-domain transcription factor ORA47, which regulates JAs biosynthesis genes including *LOX3* and *AOC2* and which is a candidate regulator of the JAs-responsive positive feedback loop for JAs biosynthesis. Figure was modified from Fig. 4 in Memelink (2009).

expression response. ORA59 interacts in vitro with the GCC box (Zarei et al., 2011) and controls the expression of genes that are synergistically induced by JAs and ET, whereas MYCs interact in vitro with the G-box and related sequences (Montiel et al., 2011; Chini et al., 2007; Dombrecht et al., 2007; Godoy et al., 2011), and control genes activated by JAs alone.

The activity of MYCs is controlled by JAZ proteins, which act as repressors (Chini et al., 2007; Hou et al., 2010). The bioactive JA-Ile (Fonseca et al., 2009) promotes the interaction between JAZ proteins and the putative ubiquitin ligase complex SCF^{COI1} (Sheard et al., 2010), presumably leading to ubiquitination of JAZ proteins and resulting in their degradation by the 26S proteasome (Chini et al., 2007; Thines et al., 2007). The question remains whether and how other JAs-responsive transcription factors such as ORA59 and ORA47 are activated by JAs 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. Alternatively and more likely, adaptor proteins may mediate the interaction between JAZ and these transcription factors.

Thesis outline

JAs are plant signaling molecules that play important roles 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. Several transcription factors have been identified that appear to be involved in JAs-responsive gene expression, including ORA59, ERF1, ORA47 and MYC2, MYC3 and MYC4. Identification of their function and their target genes, the mechanisms whereby they are activated by JAs at the protein level and of the mechanisms whereby they are regulated at the gene level is of major importance to understand how JAs act.

The studies described in this thesis were focused on the functional analysis of the JAs-responsive transcription factor ORA47 in Arabidopsis. The aim of the research was to investigate whether ORA47 regulates the positive feedback loop in JAs biosynthesis, to determine its target genes, to establish how ORA47 is regulated at the protein level, and to understand the regulation of JAs-responsive *ORA47* gene expression.

Chapter 2 describes the role of ORA47 in JAs biosynthesis. Inducible overexpression of the *ORA47* gene in Arabidopsis plants resulted in induced expression of multiple JAs biosynthesis genes and in increased JAs levels. The results show that ORA47 controls JAs biosynthesis via regulation of the JAs biosynthesis genes. Probably as a result of JAs biosynthesis, several JAs-responsive defense genes are upregulated in *ORA47*-overexpressing plants. ORA47 appears to act as the regulator of the auto-stimulatory loop in JAs biosynthesis.

Chapter 3 describes the identification of candidate target genes of ORA47 using inducible ORA47 overexpression in the wildtype background and in *aos* mutant plants which are unable to synthesize JAs. Genome-wide gene expression analysis using Affymetrix ATH1 microarrays identified candidate direct target genes which were upregulated independent of JAs biosynthesis and secondary target genes which are probably expressed in response to JAs production. The putative direct target genes included the JAs biosynthesis genes identified as ORA47 targets in Chapter 2.

Chapter 4 describes the characterization of ORA47-interacting proteins identified by yeast two-hybrid screening. All 5 members of the Arabidopsis BTB-TAZ protein family except BT2 interacted with ORA47. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-activation assay, whereas JAZ1 had no effect. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of *AOC2*, indicating that BT proteins are not

the hypothetical repressors or adaptor proteins that were the target of the research described in this chapter.

Chapter 5 describes the identification of the promoter element(s) and the transcription factor(s) responsible for JAs-responsive expression of the *ORA47* gene. Based on literature data the hypothesis that *ORA47* is regulated by binding of the redundant JAs-responsive transcription factors MYC2, MYC3 and MYC4 to a G-box in the promoter was explored. The results show that the MYC proteins can bind to a single G-box in the *ORA47* promoter. Triple knockout of the *MYC* genes or overexpression of a stable JAZ1 derivative abolished JA-responsive *ORA47* expression, demonstrating the crucial role of the MYC-JAZ module in the regulation of *ORA47* expression.

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CHAPTER 2

The AP2/ERF-domain transcription factor ORA47 regulates jasmonate biosynthesis in Arabidopsis

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ABSTRACT

Jasmonates (JAs) are important signaling molecules in plant defense. The regulation of their biosynthesis is not well understood at the molecular level. The expression of genes encoding JAs biosynthetic enzymes is increased by jasmonic acid (JA), indicating that JAs biosynthesis is controlled by a positive feedback loop. Here, we report that the AP2/ERF-domain transcription factor ORA47 functions in the regulation of the JAs biosynthesis pathway. Overexpression of the *ORA47* gene conferred JAs-related phenotypes, such as inhibition of growth and anthocyanin production, and induced the expression of all biosynthesis genes of the JAs pathway tested. JAs measurements in *ORA47*-overexpressing plants showed an increase in the amounts of the JA precursor 12-oxophytodienoic acid (OPDA), JA, the bioactive form jasmonoyl isoleucine (JA-Ile) and the inactive derivative 12-hydroxy-JA. Probably as a consequence of JAs production several JAs-responsive defense genes including the gene encoding vegetative storage protein1 (VSP1) were upregulated in *ORA47*-overexpressing plants**.** Our findings indicate that ORA47 acts as the key regulator in the positive feedback loop by controlling the expression of the JAs biosynthesis genes.

INTRODUCTION

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of oxylipins that regulate plant responses to environmental and developmental cues. JAs are produced in response to mechanical and insect wounding, pathogen infection and UV irradiation (Turner et al., 2002). The biosynthetic enzymes are located in two different subcellular compartments (Fig. 1; Vick and Zimmerman, 1984; Schaller, 2001; Wasternack, 2007 Schaller and Stintzi, 2009). The first part of the pathway directs the conversion of α-linolenic acid to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The second part

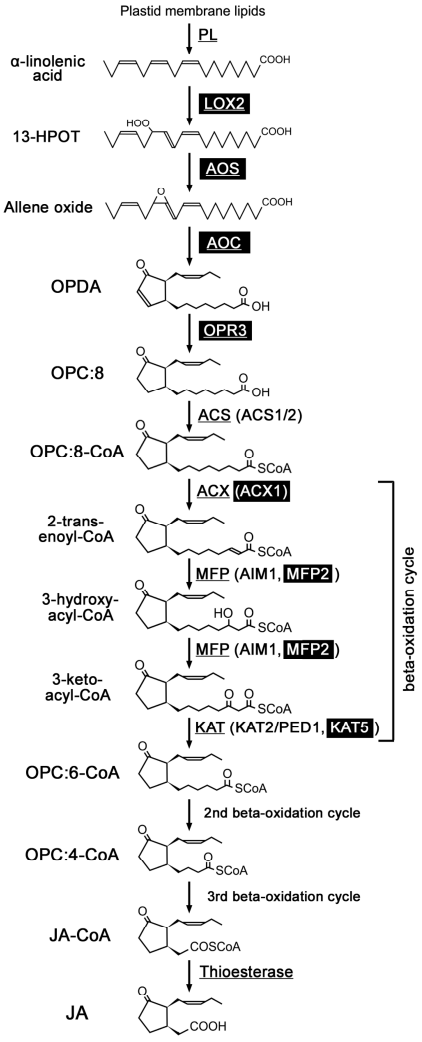

Figure 1. Octadecanoid pathway for JA biosynthesis. Abbreviations for enzyme names are underlined or in brackets. Enzymes whose corresponding genes are upregulated in *ORA47*-overexpressing plants are black-boxed. PL, phospholipase; LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, OPDA reductase; ACS, peroxisomal acyl-coenzyme A (CoA) synthetase; ACX, acyl-CoA oxidase; MFP, multifunctional protein; AIM1, abnormal inflorescence meristem 1; KAT, 3-ketoacyl-CoA thiolase; PED1, peroxisome defective 1; 13-HPOT, (9*Z*, 11*E*, 15*Z*, 13*S*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; OPDA, 12-oxo-10,15(*Z*)-octadecatrienoic acid; OPC:8, 3-oxo-2(2′(*Z*)-pentenyl)-cyclopentane-1-octanoic acid; JA, jasmonic acid

of the pathway takes place in peroxisomes, where OPDA is reduced by OPDA reductase (OPR3) to give 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), followed by three rounds of beta-oxidation involving three enzymes to yield (+)-*7-iso*-JA which equilibrates to the more stable (-)-JA (Fig. 1). Subsequently, JA can be metabolized in the cytoplasm by many different reactions (Koo and Howe, 2012), including methylation to methyl-jasmonate (MeJA) by *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase (JMT; Seo et al., 2001), conjugation to amino acids by JA amino acid synthase (JAR1; Staswick and Tiryaki, 2004) or hydroxylation to 12-hydroxyjasmonic acid (12-OH-JA; Miersch et al., 2007) (Fig. 2). The major bioactive JAs is the amino acid conjugate jasmonoyl-isoleucine (JA-Ile; Fonseca et al., 2009). Its concentrations are not only determined by JAR1-mediated synthesis but also by catabolism via an ω-oxidation pathway involving members of the CYP94 family of cytochromes P450 (Kitaoka et al., 2011; Koo et al., 2011; Koo and Howe, 2012; Heitz et al., 2012).

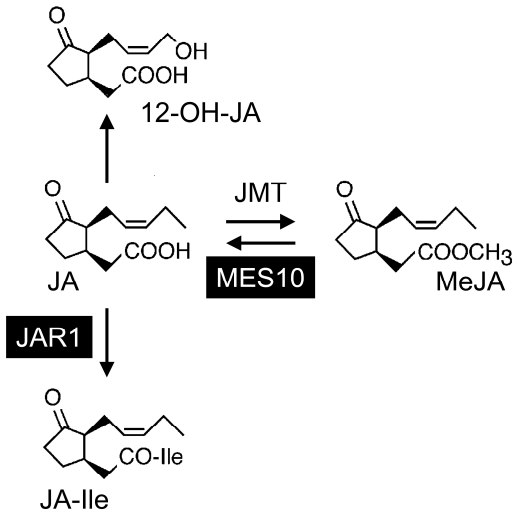

Figure 2. Metabolism of JA. Shown are several metabolic reactions relevant for this chapter. Enzymes whose corresponding genes are upregulated in *ORA47*-overexpressing plants are black-boxed. JMT, *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase; MES10, MeJA esterase 10; JAR1, JA RESISTANT 1; JA, jasmonic acid; 12-OH-JA, 12-hydroxyjasmonic acid; MeJA, methyl jasmonate; JA-Ile, jasmonoyl isoleucine.

JA-Ile is perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1) (Thines et al., 2007; Sheard et al., 2010) which is part of an SCF complex with putative E3 ubiquitin ligase activity. This leads to degradation of JAZ repressor proteins (Chini et al., 2007; Thines et al., 2007), which sets in motion defense gene expression programmes leading to the expression of a large number of defense-related proteins, including vegetative storage proteins (VSPs), the plant defensins (PDFs) or enzymes involved in the biosynthesis of protective secondary metabolites (Memelink, 2009).

The expression of JAs biosynthesis genes, including *LOX2*, *AOS*, *AOC* and *OPR3*, is induced by wounding or treatment with exogenous JA or MeJA (Turner et al., 2002; Sasaki et al., 2001; Pauwels et al., 2008). The observation that wounding induces the expression of JAs biosynthesis genes suggests that, at least partly, the wound-induced production of JAs is a result of the increased transcription of genes encoding the pathway enzymes. In addition, transcriptional activation of the JAs biosynthesis genes by (Me)JA indicates that JAs signaling is amplified by a positive feedback loop initiated by JAs (Turner et al., 2002).

The developmental regulation of JAs biosynthesis was reported to be regulated positively and negatively by different classes of TCP (TEOSINTE BRANCHED/ CYCLOIDEA/PCF) transcription factors via the regulation of *LOX* genes (Schommer et al., 2008; Danisman et al., 2012). A *LOX2* promoter with mutated TCP binding sites has a lower basal activity but still responds to MeJA treatment (Schommer et al., 2008), indicating that TCPs control JAs biosynthesis linked to development but not in response to JAs signaling. Candidate transcription factors for regulating the positive feedback loop in JAs biosynthesis were reported by Pauwels et al. (2008). In a transient assay to screen for activators the basic helix-loop-helix transcription factor MYC2 and the AP2/ERF-domain transcription factor ORA47 were able to activate the *LOX3* promoter in tobacco protoplasts. Whereas MYC2 has been extensively studied and is one of the major JAs-responsive transcription factors (Lorenzo et al., 2004, Boter et al., 2004; Dombrecht et al., 2007; Memelink, 2009; Pauwels and Goossens, 2011), little is known about the function of ORA47. The *ORA47* gene

responds to (Me)JA treatment (Wang et al., 2008; Pauwels et al., 2008) in a COI1-dependent manner (Wang et al., 2008) and to wounding (Walley et al., 2007). This has led to the hypothesis that ORA47 together with MYC2 may control the positive feedback loop in JAs biosynthesis (Pauwels et al., 2008). However, overexpression of ORA47 using the constitutive CaMV 35S promoter has been reported to result in elevated expression of the defense gene *VSP2* whereas a negative effect was found on the expression of the *LOX3* gene (Wang et al., 2008). These authors also did not report JAs-related phenotypes in the transgenic plants, such as growth inhibition or anthocyanin production.

In this study we investigated the possible function of the transcription factor ORA47 in JAs signaling. We found that plants constitutively overexpressing the *ORA47* gene showed a strong dwarf phenotype and produced anthocyanins. Inducible *ORA47* overexpression led to the activation of a large number of genes involved in JAs biosynthesis. Oxylipin measurements in plants overexpressing *ORA47* revealed increased JAs levels. In addition, many JAs-responsive defense genes, such as *VSP1*, were highly expressed in *ORA47*-overexpressing plants. Our results indicate that ORA47 is responsible for the regulation of the auto-stimulatory loop in JAs biosynthesis.

RESULTS

ORA47 overexpression causes severe dwarfism and partially phenocopies exposure to JA

To investigate the role of the transcription factor ORA47 in JA signaling, transgenic Arabidopsis plants constitutively expressing the *ORA47* gene under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter were constructed. As shown in Figure 3, three-weeks-old T1 transformants carrying a 35S:*ORA47* construct (Fig. 3A) were smaller than control plants (Fig. 3B) and accumulated anthocyanins in the shoot apex. After 5 weeks of growth, 35S:*ORA47* plants (Fig. 3C-E) were showing a severe dwarf phenotype with no stem elongation compared to control plants (Fig. 3F). Small and disorganized dark rosette leaves were initiated (Fig. 3C-E). At a later stage, two-months-old

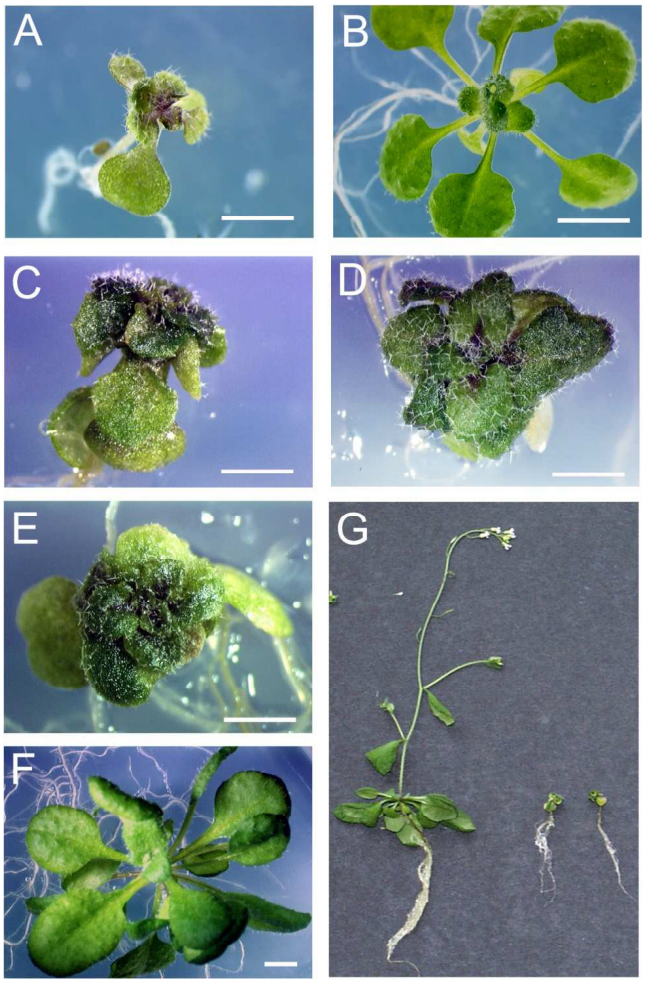

Figure 3. Arabidopsis plants constitutively overexpressing *ORA47* show dwarfism and anthocyanin production. Primary transformants carrying the 35S:*ORA47* construct (A, C, D, E, G (right)) or empty vector (B, F, G (left)) were germinated on selective medium. Bars represent 5 millimeters. (A) and (B) Three-weeks-old plants. (C-F) Five-weeks-old plants. (G) Eight-weeks-old mature control plant (left) and two independent primary transformants carrying the 35S:*ORA47* construct (right).

plants remained extremely small compared to control plants (Fig. 3G). The sterility caused by the absence of stem and flower development rendered these primary transformants difficult to use for further analyses. Therefore, the

function of ORA47 was studied using transgenic plants carrying the *ORA47* gene under the control of the estradiol-inducible XVE system (Zuo et al, 2000). T2 seeds from XVE-*ORA47* and XVE-*GUS* transformants were germinated on solid medium with or without 4 µM of the inducer estradiol. Five-days-old XVE-*ORA47* seedlings growing on non-inducing medium showed a normal phenotype similar to the XVE-*GUS* control line (Fig. 4A).

Growth of XVE-*GUS* control seedlings was similar on induction medium and normal medium, indicating that the presence of estradiol in the medium had no effect on plant growth (Fig. 4A). Estradiol-treated XVE-*ORA47* seedlings accumulated anthocyanins in the shoot apex and were smaller, with shorter and thicker roots compared to XVE-*GUS* control plants grown under the same conditions. Anthocyanin production and dwarfism were also observed when 5-days-old non-induced XVE-*ORA47* seedlings were transferred to estradiol-containing medium and grown for an additional week (Fig. 4B), indicating that these phenotypes were directly correlated to *ORA47* overexpression. These morphological traits were similar to those observed in 35S:*ORA47* seedlings constitutively overexpressing *ORA47*. In wild-type Arabidopsis seedlings, JA or methyl jasmonate (MeJA) treatment inhibits root growth (Staswick et al., 1992) and induces purple coloration in the cotyledon margins and shoot apex, due to the accumulation of anthocyanins (Fig. 4C; Feys et al., 1994). Similarly, *cev1* mutant plants having constitutively elevated levels of JA exhibit stunted growth and anthocyanin accumulation (Ellis and Turner, 2001; Ellis et al., 2002). In tobacco, exogenous JA causes a reduction in the number of flower buds (Barendse et al., 1985). Therefore, the phenotypes observed in *ORA47*-overexpressing plants resemble those of JA-treated wild-type plants, which prompted us to investigate whether ORA47 might be involved in the regulation of JAs production.

***ORA47* overexpression increases the expression of JAs biosynthesis genes**

To test whether ORA47 might transcriptionally control JAs biosynthesis, we examined the expression of the JAs biosynthesis genes in XVE-*ORA47* plants in response to estradiol treatment. RNA gel blots revealed that expression of the

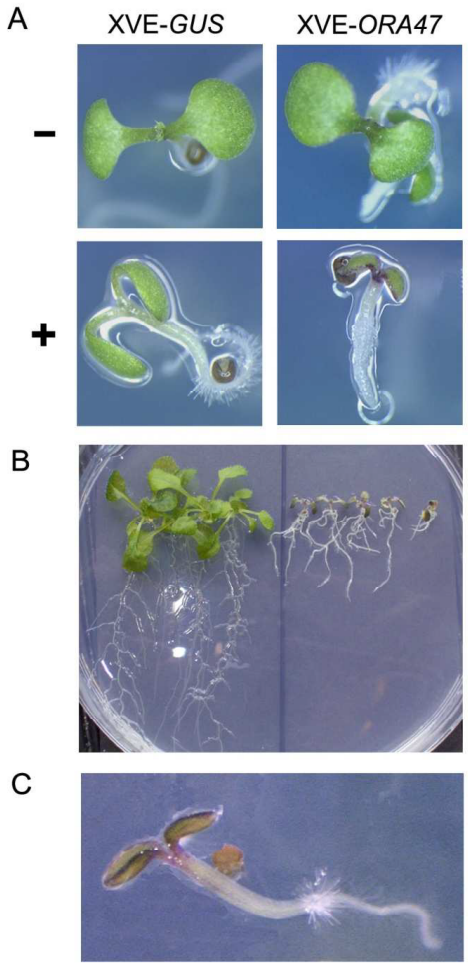

Figure 4. Growth inhibition and anthocyanin production in estradiol-induced *ORA47* overexpressing plants. (A) Transgenic seeds carrying an overexpression module with the *ORA47* or *GUS* gene under the control of an inducible promoter (XVE-*ORA47* and XVE-*GUS*, respectively) were germinated for five days on medium with 4 µM of the inducer estradiol (+) or with the solvent DMSO (-). (B) Five-days-old seedlings grown on non-inducing medium were transferred to inducing medium containing 4 µM estradiol and grown for ten additional days; left: XVE- *GUS* seedlings; right: XVE-*ORA47* seedlings. (C) Four-days-old wild-type Arabidopsis seedling grown on medium containing 50 µM jasmonic acid.

LOX2, AOS, AOC2, OPR3 and *JAR1* genes, encoding enzymes involved in the synthesis of the bioactive JA-Ile, were strongly induced in XVE-*ORA47* plants treated with estradiol (Fig. 5). Similar results were obtained in the three independent XVE-*ORA47* transgenic lines. No expression of the biosynthetic genes was observed in the XVE-*ORA47* lines without inducer, except for line 19 where *AOC2* and *OPR3* mRNAs accumulated to slightly elevated levels in the absence of estradiol. In this line, *ORA47* mRNA also showed a slightly elevated level in the non-induced condition, suggesting leaky expression of the transgene in the absence of inducer. In contrast, no *ORA47* or JAs biosynthesis gene expression was detected in the XVE-*GUS* control plants after estradiol treatment. *GUS* mRNA accumulation on the other hand was strongly induced, demonstrating the effectiveness of estradiol treatment. Hybridization with a *TUBULIN* (*TUB*) probe showed equal RNA loading. This demonstrates that the induced expression of *LOX2, AOS, AOC2*, *OPR3* and *JAR1* genes is due to the specific activation of *ORA47* transgene expression by estradiol. Whereas *LOX2*, *AOS*, *AOC2* and *OPR3* were induced by JA treatment in 10-days-old wild-type Arabidopsis seedlings (Fig. 5) consistent with other reports (Sasaki et al., 2001), *JAR1* was not upregulated at the tested time points.

Although the first enzymatic steps leading to OPC:8 synthesis are well characterized (Fig. 1), less is known about the identity and regulation of the β-oxidation genes involved in the biosynthesis of JAs. Due to their *in vitro* activity, two peroxisomal acyl-coenzyme A (CoA) synthetases, ACS1 (At4g05160) and ACS2 (At5g63380) identified within the group of 25 4-coumarate:CoA-like ligases were suggested as candidates for the coupling of a CoA group to the fatty acid chain of the OPC:8 precursor (Schneider et al., 2005). *At4g05160* gene expression was shown to be induced by MeJA treatment (Schneider et al., 2005). This prompted us to test whether the expression of these genes is controlled by ORA47. The results in Figure 5 show that, under our experimental conditions, the expression of the *At4g05160* and *At5g63380* genes did not increase in XVE-*ORA47* plants following estradiol treatment, or in wild-type plants in response to JA.

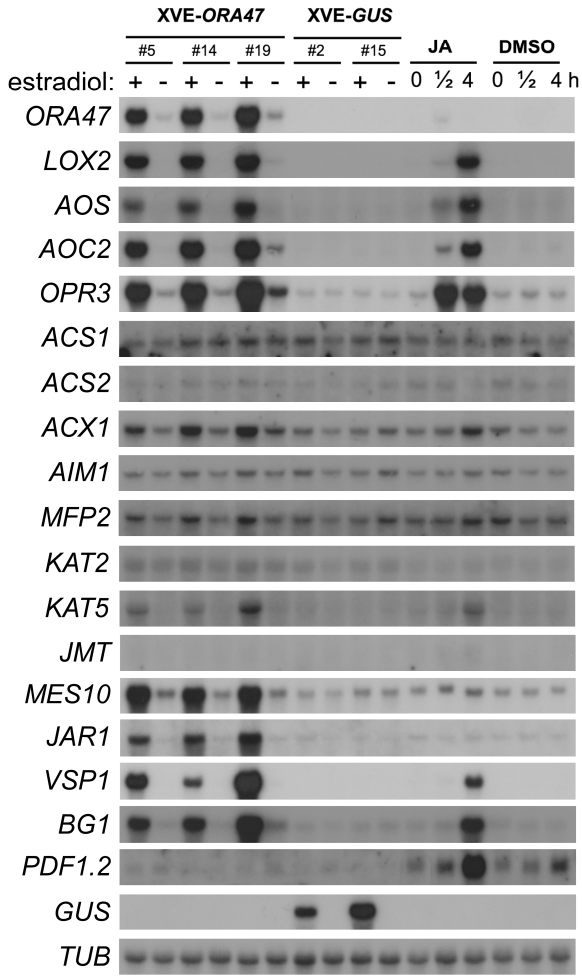

Figure 5. *ORA47* overexpression increases the expression of JAs biosynthesis genes. RNA gel blot analyses of two-weeks-old independent XVE-*ORA47* and XVE-*GUS* lines treated for 24 hours with 2 µM estradiol (+) or with the solvent DMSO (-). To study JA-responsive gene expression, two-weeks-old wild-type plants were treated with 50 µM jasmonic acid (JA) or the solvent DMSO for the number of hours (h) indicated. The *TUB* probe was used to verify RNA loading. For gene names see legends of Figs. 1 and 2. VSP1, Vegetative Storage Protein 1; BG1, Beta-Glucosidase 1; PDF1.2, Plant Defensin 1.2.

Following attachment of a CoA group, three consecutive cycles of β-oxidation are necessary to yield JA. Each round of β-oxidation requires the

concerted action of acyl-CoA oxidases (ACX), multifunctional proteins (MFP) with enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase activities, and 3-ketoacyl-CoA thiolases (KAT; Fig. 1). Wounding induces the local and systemic expression of *ACX1* and *KAT2/PED1,* whereas *ACX1* and *KAT5* transcripts accumulate in response to JA (Cruz Castillo et al., 2004). The *ped1* mutant shows a reduced JA level after wounding, indicating that KAT2/PED1 is needed for JAs biosynthesis in wounded leaves (Afitlhile et al., 2005). As shown in Figure 5, expression of the *ACX1* and *KAT5* genes, as well as the *MFP2* gene, was slightly induced in XVE-*ORA47* plants treated with estradiol as well as in JA-treated wild-type plants. In contrast, *AIM1,* encoding a multifunctional protein, and *KAT2* transcripts remained constant in all treatments.

We were unable to detect the expression of the *JMT* gene, encoding an enzyme responsible for the methylation of JA to form MeJA, suggesting that it did not respond to *ORA47* overexpression. It has been reported that JMT is expressed in mature plants but not in seedlings (Seo et al., 2001). However the *MES10* gene, encoding an enzyme that can demethylate MeJA to form JA, was strongly induced by estradiol treatment of XVE-*ORA47* plants. Similar to the *JAR1* gene the *MES10* gene was not induced by JA treatment of wildtype seedlings at the tested time points.

In addition to JAs biosynthesis genes, overexpression of the *ORA47* gene induced the expression of a large number of JAs-responsive genes including *VSP1* and *β-glucosidase1* (*BG1*) (Fig. 5). In contrast, the JAs- and ethylene-responsive defense gene *PDF1.2* was not expressed in *ORA47*-overexpressing plants. In conclusion, gene expression analysis in *ORA47*-overexpressing plants revealed that all the established JAs biosynthesis genes showed induced expression, suggesting that ORA47 overexpression might result in elevated amounts of endogenous JAs.

***ORA47* overexpression increases endogenous JAs levels**

The increase in transcript abundance of JAs biosynthesis genes in *ORA47*-overexpressing plants prompted us to determine the amounts of JA, its

precursor OPDA, the bioactive JA-Ile and the inactive 12-OH-JA in these plants. Two-weeks-old seedlings from six XVE-*ORA47* and four XVE-*GUS* lines were treated with 5 µM estradiol and tissue samples were collected after 0, 4, 8 and 24 hours. Endogenous JAs contents were determined by LC-MS analysis. Estradiol-induced expression of *ORA47* led to gradual increases over time in the target JAs levels with on average a 4-fold increase in the OPDA level, a 10-fold increase in the JA level, a 1.8-fold increase in the JA-Ile level and a 9-fold increase in the 12-OH-JA level after 24 hrs (Fig. 6A). RNA was isolated from estradiol-treated seedlings from 2 lines for each construct to monitor gene expression over time. As shown by RNA gel blot analyses, estradiol treatment effectively induced the expression of the *ORA47* and *GUS* genes in the XVE-*ORA47* and XVE-*GUS* lines, respectively, even at the shortest time point (Fig. 6B). Consistent with the results from Figure 5, the AOS, *AOC2* and *VSP1* genes were expressed exclusively in the XVE-*ORA47* lines. The JAs biosynthesis genes were switched on somewhat earlier than the defense gene *VSP1*.

12-OH-JA is inactive in defense gene induction

The defense-related genes *VSP1* and *PDF1.2* are two well-characterized markers of the JA response (Benedetti et al., 1995; Penninckx et al., 1996) and are commonly used as marker genes for the two branches of JAs-responsive signaling (Memelink, 2009). It has been reported that 12-OH-JA is inactive in inducing defense genes in potato (Miersch et al. 2008). To test the activity in Arabidopsis we compared responses to OPDA, JA and 12-OH-JA added at identical concentrations. As shown in Figure 7, OPDA and JA effectively induced the expression of *ORA47*, *VSP1* and *PDF1.2*, whereas 12-OH-JA had no effect on gene expression. Control treatments with the solvents DMSO and ethanol did not affect gene expression (data not shown).

JA activates the JAs biosynthesis genes in an *ora47* knock-down mutant and in *ORA47*-silenced plants

To further investigate the role of ORA47 in JAs signaling leading to gene expression, we analyzed the expression of the JAs biosynthesis genes in

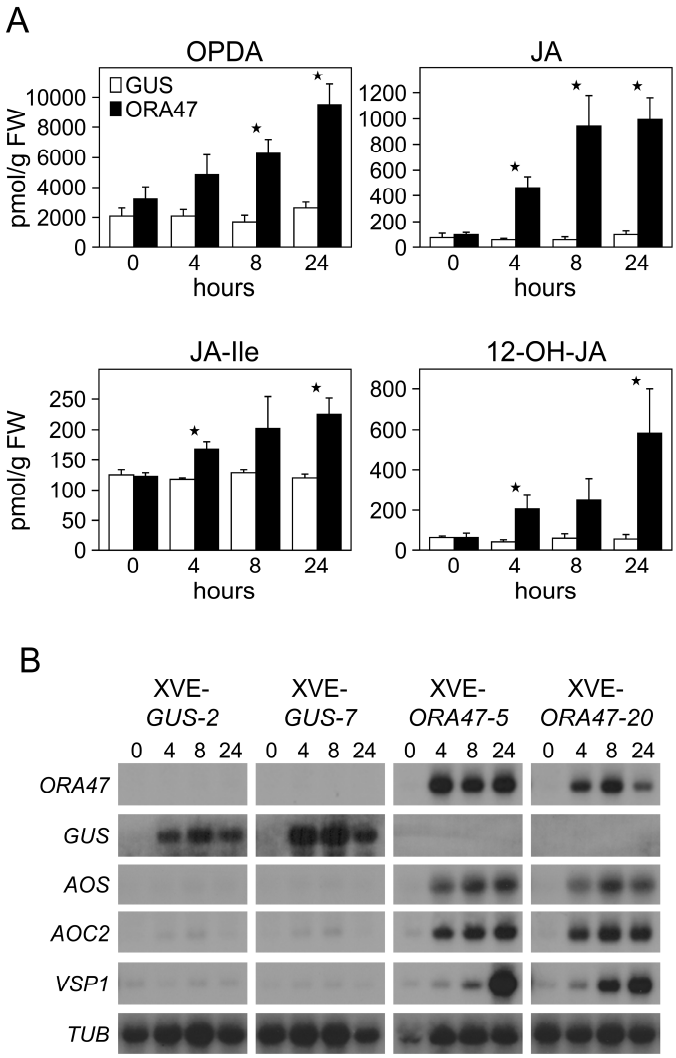

Figure 6. *ORA47* overexpression increases endogenous JAs levels. Two-weeks-old seedlings from six XVE-*ORA47* and four XVE-*GUS* lines were treated in triplicate with 5 µM estradiol and tissue samples were collected at time zero and after 4, 8 and 24 hours. (A) Levels of the indicated JAs in pmol/g fresh weight (FW). Values represent means of all lines and replicates ± SE. Significant differences between *ORA47* and *GUS* values are indicated with an asterisk (Wilcoxon test, $p < 0.05$) (B) Gene expression patterns in 2 lines for each construct. The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading. Panels with the same probe were on the same blot and exposed for the same time.

response to JA in *ora47* mutant plants. The *ora47-1* mutant line SALK_109440 contains a T-DNA inserted in the promoter region of the *ORA47* gene (155 base pairs upstream of the transcriptional start site; data not shown). As shown in Figure 8A, JA failed to induce *ORA47* gene expression in the mutant plants compared to wild-type plants. Instead, *ora47* plants exhibited a low but detectable level of *ORA47* transcript. This indicates that the promoter of the *ORA47* gene has lost the ability in the *ora47-1* mutant to respond to JA but has conserved the potential to express a basal level of the full-length *ORA47* mRNA. Expression of the JAs biosynthesis genes, such as *LOX2* and *AOC2*, was similar in the *ora47-1* mutant compared to wild-type plants in response to JA (Fig. 8A).

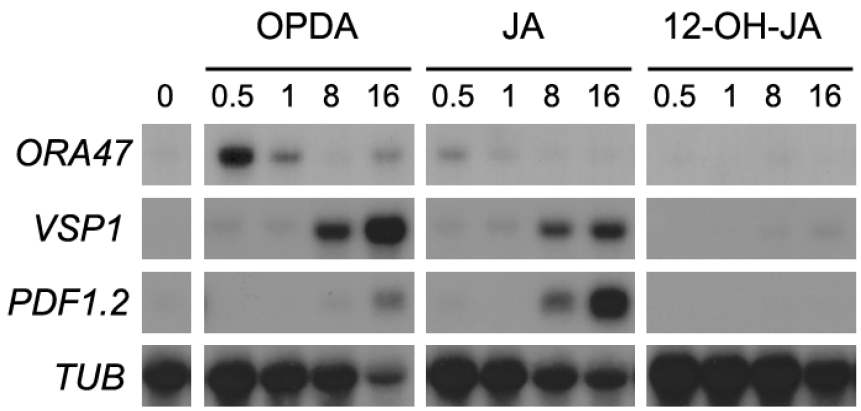

Figure 7. 12-OH-JA is inactive in defense gene induction. Two-weeks-old Arabidopsis seedlings were treated for the number of hours indicated with 50 µM OPDA, JA, or 12-OH-JA. The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading. Panels with the same probe were on the same blot and exposed for the same time

This indicates that a JA-induced increase in the *ORA47* transcript level is not required for the JA-responsive expression of the JAs biosynthesis genes *LOX2* and *AOC2*. Expression of full-length *ORA47* mRNA in the *ora47-1* mutant line suggested that this line is not a null-allele mutant line. It is likely that the functionality of the ORA47 protein is not compromised in this mutant line. Therefore, transgenic plants showing post-transcriptional silencing of the

ORA47 gene (RNAi-*ORA47*) were constructed. Expression analyses of RNAi-*ORA47* lines treated with JA showed that 27 out of 29 independent lines induced the *ORA47* gene to a similar level than in a JA-treated control line (data not shown), indicating that these transgenic lines did not effectively silence the *ORA47* gene. On the other hand, the lines RNAi-*ORA47-9* and RNAi-*ORA47-16* showed undetectable levels of *ORA47* mRNA after 15 minutes of treatment with JA (Figure 8B). Instead, hybridization with a specific probe for *ORA47* detected a prominent band corresponding to a large RNA species in the RNAi-*ORA47-9* line, independently of the treatment (Figure 8B). This RNA species is most likely the complete unspliced hairpin RNA encoded by the silencing transgene. In the RNAi-*ORA47-16* line, a smeary signal was observed with a probe specific for *ORA47.* As with the *ora47-1* mutant line, expression of the *LOX2* and *AOC2* genes in response to 8 hours treatment with JA was similar in the *ORA47*-silenced lines compared to the control line (Figure 8C).

As a third approach to silence *ORA47* expression we used the artificial microRNA (amiRNA) method (Ossowski et al., 2008; Schwab et al., 2006). The *ORA47*-specific amiRNA construct was introduced in Arabidopsis plants both under control of the constitutive CaMV 35S promoter as well as under the control of the estradiol-responsive XVE system (Zuo et al., 2000). Two-week-old seedlings of 14 independent transgenic T2 lines containing the constitutively expressed construct were treated for 15 min with 50 µM JA whereas 20 independent lines containing the inducible construct were first treated for 24 hrs with 5 µM estradiol followed by a 15 min treatment with JA. Northern blot hybridization analysis of the extracted RNAs revealed that none of the lines had an *ORA47* mRNA level that differed from wildtype seedlings or 2 XVE-GUS lines respectively (results not shown). Therefore these amiRNA lines were not further analysed.

DISCUSSION

JAs are signaling molecules that regulate certain aspects of development as well as diverse responses to stress. Little is known about the regulatory mechanisms

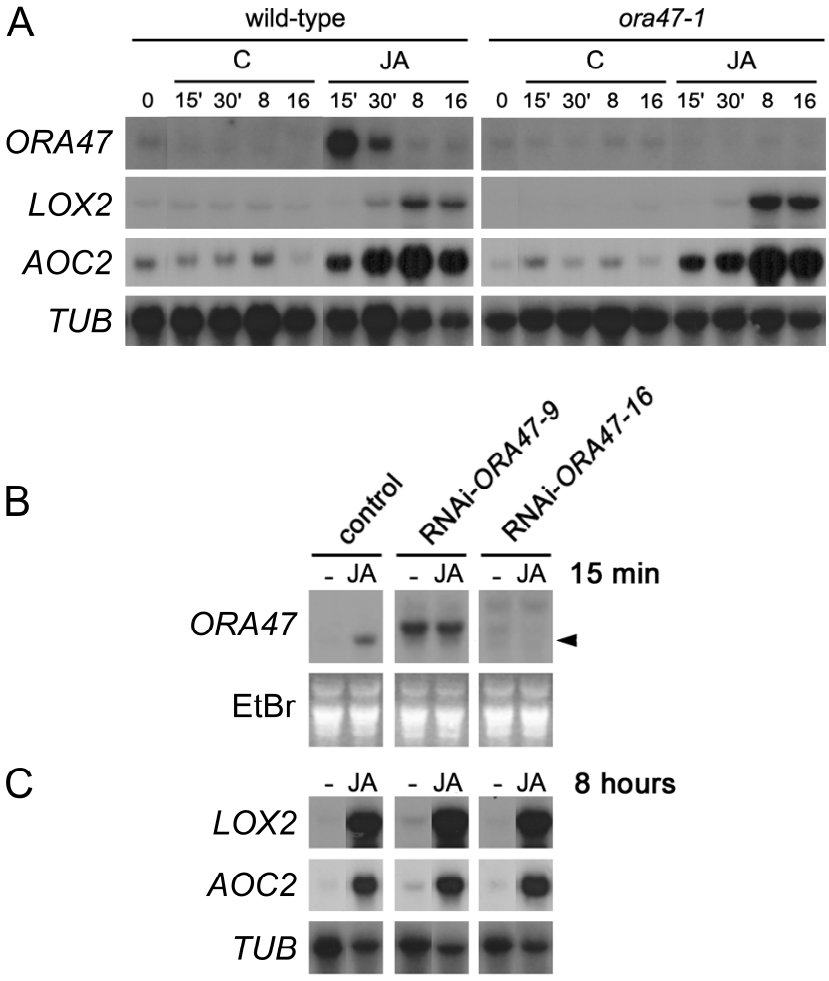

Figure 8. Expression of JAs biosynthetic genes in response to JA in the *ora47* mutant and in transgenic *ORA47*-silenced plants. (A) RNA gel blot analyses with two-weeks-old *ora47-1* mutant and wild-type seedlings treated for 15 and 30 min or for the number of hours indicated with 50 µM jasmonic acid (JA) or 0.1 % of the solvent DMSO (C). (B) and (C) RNA gel blot analyses with two-weeks-old transgenic *ORA47*-silenced lines 9 and 16 and a control line (S-GUS-6) treated for 15 minutes (B) or 8 hours (C) with 50 µM JA or 0.1 % of the solvent DMSO (-). The black arrowhead indicates the position of the *ORA47* mRNA on the RNA gel. The RNA gel blots were hybridized with the indicated probes. Equal loading was verified by ethidium bromide (EtBr) staining of the RNA gel prior to blotting (B). The *TUB* probe was used to verify RNA loading (C).

controlling JAs biosynthesis. In this report, we demonstrate that ORA47, a member of the Arabidopsis AP2/ERF-domain class of transcription factors, plays a major role in the regulation of JAs biosynthesis. We show that overexpression of the *ORA47* gene resulted in the activation of JAs biosynthesis genes, and led to elevated levels of endogenous JAs.

ORA47 positively regulates the JAs biosynthesis genes

Overexpression of *ORA47* gene in Arabidopsis activated the expression of all established genes encoding enzymes of the JAs biosynthetic pathway, such as *LOX2, AOS, AOC2*, *OPR3* and *JAR1*. Additionally, several genes encoding enzymes of the β-oxidation steps, including *ACX1, MFP2* and *KAT5,* were also expressed at moderately increased levels in *ORA47*-overexpressing plants. These findings suggest that the enzymes encoded by these genes are likely to be involved in the last steps of JA production. These genes were also induced in response to JA treatment, which is also consistent with a putative role in JA biosynthesis. In contrast, expression of two peroxisomal acyl-CoA synthetases *ACS1* and *ACS2* genes was not induced either by *ORA47* overexpression or by JA treatment. Schneider et al. (2005) reported that the A*CS1* (*At4g05160)* gene was expressed in response to MeJA treatment and that recombinant ACS1 and ACS2 were able to use the JA precursor OPC:8 as a substrate *in vitro*. These contradictory results do not allow us to clarify the role of these two enzymes in JA biosynthesis. It is possible that ACS1 and ACS2 are involved in the JA pathway with no requirement for *de novo* protein synthesis.

Our results indicate that ORA47 controls the expression of the *KAT5* gene, encoding a 3-ketoacyl-CoA thiolase, but not the homologous *KAT2* gene. Under our experimental conditions, expression of the *KAT2* (also referred to as *PED1*) gene was not induced either by *ORA47*-overexpression or by JA treatment. *KAT2* transcripts accumulate in wounded leaves and a *ped1* mutation results in lower accumulation of JA in wounded tissues (Cruz Castillo et al., 2004; Afitlhile et al., 2005), suggesting a role in wound-induced JAs production. Moreover, He et al. (2002) suggested a role for KAT2/PED1 in senescence-induced JAs synthesis. Therefore it is likely that the *KAT2/PED1* gene is

expressed in response to signals related to wounding or senescence without the requirement for ORA47, whereas JAs signaling initiating the auto-stimulatory loop would recruit ORA47 to activate the *KAT5* gene.

Our finding that the *LOX2* gene is upregulated in response to ORA47 overexpression fits well with the report that ORA47 can transactivate the *LOX3* promoter in tobacco protoplasts (Pauwels et al., 2008). It suggests that *LOX* genes are direct target genes of ORA47. Our finding is in disagreement with the report that the *LOX3* gene is downregulated in transgenic plants constitutively overexpressing the *ORA47* gene (Wang et al., 2008). Although we were able to obtain T1 plants after flower dip transformation, these plants were extreme dwarfs and they never flowered. Wang et al. (2008) do not mention aberrant phenotypes displayed by the transgenic plants. We hypothesize that these authors were working with plants with very low levels of ORA47 overexpression which were insufficient for induction of JAs biosynthesis genes and JAs production.

ORA47 controls JAs production

The increased expression of JAs biosynthesis genes in *ORA47*-overexpressing plants suggested that these plants might contain enhanced levels of endogenous JAs. Indeed elevated levels of OPDA, JA, JA-Ile and 12-OH-JA were found.

Several successful and unsuccessful attempts to modulate JA levels in plants have been described using transgenic approaches. Overexpression of the *AOS* gene in transgenic Arabidopsis and tobacco and of the *AOC* gene in tomato did not alter the basal level of JA, but when wounded, transgenic plants produced a higher level of JA than did wounded control plants (Laudert et al., 2000; Stenzel et al., 2003), suggesting that the production of JAs is limited by the availability of substrates (free α-linolenic acid or 13-hydroperoxyoctadecatrienoic acid), the levels of which are enhanced after wounding. In contrast, overexpression of *JMT* led to elevated levels of MeJA, while the JA content remained unchanged. Plants overexpressing *JMT* exhibited constitutive expression of JAs-responsive genes and increased resistance against *B. cinerea* (Seo et al., 2001). The *cas1* and *cet1* mutant plants contain

constitutively high levels of JA (Kubigsteltig and Weiler, 2003; Hilpert et al., 2001). These mutants exhibit a severe growth inhibition phenotype. This phenotype is likely to be due to the high JAs contents present in these mutants, as it is also observed in wild-type plants treated with exogenous JA. We speculate that the dwarf phenotype observed in plants constitutively overexpressing the *ORA47* gene is a consequence of high JAs levels and downstream gene activation. The biosynthesis of JAs is likely to induce the constitutive expression of a large number of JAs-responsive genes, thereby generating a stress condition that compromises plant development and overall fitness. Indeed, overexpression of *ORA47* induced the expression of several JAs-responsive genes, including *VSP1* and *BG1*.

ORA47 is involved in the JAs auto-stimulatory loop

Analyses of the *ora47* knock-down mutant and the *ORA47*-silenced plants showed that expression of the JAs biosynthesis genes in response to JA was not altered in these plants. The knock-down line contains a T-DNA insertion in the promoter and still expresses the *ORA47* transcript at a basal level, indicating that JAs-responsive *ORA47* gene expression is not required for activation of its target genes. The RNAi strategy yielded only two putative knock-down lines out of 29 transgenic lines. This frequency is much lower than with 3 other AP2/ERF-domain transcription factors for which we applied the RNAi strategy (Pré, 2006), including ORA59 (Pré et al., 2008). In those cases the large majority of the lines showed successful silencing. In addition lines with successful silencing always showed a prominent smear in Northern blot expression analyses using the corresponding gene as a probe. With the two putative *ORA47* knockdown lines we did not observe this smear. Therefore we hypothesize that these lines still express the *ORA47* gene at a low level sufficient for effective JA-responsive expression of its target genes. Also the amiRNA approach was not successful in generating plants with a reduced *ORA47* mRNA level. Especially in the case of the inducible amiRNA construct this was surprising, since possible lethal effects are unlikely to occur within the relatively short timeframe of induction.

Here we showed that overexpression of *ORA47*, encoding an AP2/ERF-domain transcription factor, induces the expression of all established JAs biosynthesis genes tested and results in elevated JAs levels. These results tend to place ORA47 upstream of JAs biosynthesis, in a scenario where stress signals (e.g. wounding or pathogen attack) induce *ORA47* gene expression and/or activate the ORA47 protein, leading to JAs production. However, several lines of evidence indicate that ORA47 is a downstream component in JAs signaling where ORA47 regulates the positive feedback amplification loop. First, induction of the *ORA47* gene by JA (Pauwels et al., 2008; Wang et al., 2008) requires the JA-Ile receptor COI1 (Wang et al., 2008), indicating that perception of JAs is required for *ORA47* expression in response to stress. Therefore, ORA47 is likely to act as a terminal component in JAs signal transduction, rather than as an integrator of stress signals leading to JAs production. We speculate that the function of ORA47 is to regulate the amplification loop that leads to the expression of the JAs biosynthesis genes in response to JAs. Up to now, it is still unclear how JAs production initially occurs in response to stress and what is the integrator of stress signals that leads to JAs biosynthesis.

Another hypothesis preferred by us is that an early step following perception of the stress signal involves covalent modifications of pre-existing ORA47 protein without *de novo* protein synthesis. In this context it is worth mentioning that ORA47 was detected as a phosphorylation target of MAP kinases (Popescu et al., 2009). Activation of ORA47 protein and the resulting expression of the JAs biosynthesis genes would lead to production of a small amount of JAs that would activate transcription of the *ORA47* gene and subsequent amplification of the signal by the feedback loop.

MATERIAL AND METHODS

Plant material, growth conditions and treatments

Arabidopsis thaliana ecotype Columbia (Col-0) is the genetic background for all wild-type, transgenic and *ora47-1* mutant plants. 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 grown on plates containing MA medium (Masson and Paszkowski, 1992) supplemented with 0.6% agar. Transgenic plants from T1 and T2 generations were selected on MA medium containing either 25 mg/L kanamycin for *ORA47*-silenced plants or 20 mg/L hygromycin for *ORA47*-overexpressing plants. Following stratification for 3 days at 4ºC, seeds were first germinated at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) on solid MA medium supplemented with the above mentioned appropriate antibiotics for 10 days, after which 15 to 20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml liquid MA medium without antibiotic and incubated on a shaker at 120 rpm for 4 additional days before treatment. Treatments with JA were performed by adding 50 µM (+/-)-JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.1% final concentration) to the liquid medium. As controls, seedlings were treated with 0.1% DMSO. Transgene expression in plants transformed with pER8 derivatives containing the *ORA47* or *GUS* gene was induced by adding 2-5 µM estradiol (Sigma) dissolved in DMSO (0.1% final concentration) to the liquid medium. As control, seedlings were treated with 0.1% DMSO. Alternatively, XVE-*ORA7* and XVE-*GUS* plants were germinated on solid MA medium containing 4 µM estradiol or 0.1 % DMSO as control (Figure 4A). Treatments with the different JAs were performed at a final concentration of 50 µM. The compounds JA and 12-OH-JA were dissolved in DMSO whereas OPDA was dissolved in ethanol. Plants were treated with DMSO and ethanol (0.1 % final concentration) as controls.

Binary constructs and plant transformation

The *ORA47* (*At1g74930*) open reading frame (ORF) was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-GAA GAT CTC ATA TGG TGA AGC AAG CGA TGA AG-3' and 5'-GAA GAT CTT CAA AAA TCC CAA AGA ATC AAA G-3' and, following digestion with BglII, was cloned in pIC-

20R (Marsh et al., 1984). For the construction of transgenic lines constitutively overexpressing *ORA47,* the *ORA47* insert was excised with BglII and inserted into pMOG183 (Mogen International, Leiden, The Netherlands) digested with BamHI. The pMOG183 vector is a pUC18 derivative carrying a double-enhanced Cauliflower Mosaic Virus (CaMV) 35S promoter and the *nos* terminator separated by a BamHI site. The CaMV 35S cassette containing the *ORA47* gene in sense orientation was excised with SacI/HindIII and cloned into the binary vector pCAMBIA1300 (accession number AF234296). For the construction of transgenic lines showing post-transcriptional gene silencing of the *ORA47* gene, the *ORA47* ORF was cloned into pIC-20H (Marsh *et al*., 1984) digested with BglII and into pBluescript SK+ (Stratagene, La Jolla, CA) digested with BamHI, such that the 5'-end of the *ORA47* ORF flanked the EcoRI restriction sites of the respective plasmids. The *ORA47* insert was excised from pIC-20H with EcoRI/XhoI and cloned into the pHANNIBAL vector (accession number AJ311872) to generate pHAN-ORA47as. To create an inverted repeat, the *ORA47* ORF was excised from pBluescript SK^+-ORA47 with XbaI/HindIII and cloned into pHAN-ORA47as to generate pHAN-ORA47sas. For the construction of control lines, the *GUS* ORF was excised from GusSH (Pasquali et al., 1994) with SalI/HindIII and cloned into pHANNIBAL digested with XhoI/HindIII. The pHANNIBAL expression cassettes were cloned into the binary vector pART27 (Gleave, 1992) using NotI. For the construction of plants with estradiol-responsive transgene expression, the *GUS* ORF was excised from pGUSN358→S (Clontech, Palo Alto, CA) with SalI/EcoRI and cloned into pBluescript SK+ to generate pSK-GUS. The *ORA47* ORF and the *GUS* ORF were excised from the pBluescript vector, with ApaI/SpeI and XhoI/XbaI, respectively, and cloned into the binary vector pER8 (Zuo et al, 2000) digested with ApaI/SpeI and XhoI/SpeI, respectively. Primers for artificial micro RNA (amiRNA) targeting *ORA47* mRNA were designed using the WMD (Web MicroRNA Designer) program (http://wmd.weigelworld.org). amiRNA was amplified with the 1-4 primers 5'-GAT TGA AAT TAG CAT TGC CGC GGT CTC TCT TTT GTA TTC C-3', 5'-GAC CGC GGC AAT GCT AAT TTC AAT CAA AGA GAA TCA ATG A-3', 5'-GAC CAC GGC AAT GCT TAT TTC ATT

CAC AGG TCG TGA TAT G-3', 5'-GAA TGA AAT AAG CAT TGC CGT GGT CTA CAT ATA TAT TCC T-3' and the a-b primers 5'-CTG CAA GG GAT TAA GTT GGG TAA C-3' and 5'-GCG GAT AAC AAT TTC ACA CAG GAA ACA G-3'. The amiRNA containing precursor was engineered into a 404 bp fragment containing the *MIR319a* stem-loop as described (Schwab et al., 2006; Ossowski et al., 2008). The amiRNA containing precursor cassette was cloned in pCAMBIA1300 with SphI for constitutive expression and for inducible expression the stem-loop construct was cloned in pER8 with XhoI/SpeI. The binary vector pCAMBIA1300-ORA47 was introduced into *Agrobacterium tumefaciens* strain LBA1115 (containing the Vir plasmid pSDM3010). The pART27 binary vectors were introduced into *A. tumefaciens* strain LBA4404 while pER8 binary vectors and pCAMBIA1300-amiRNA were introduced into *A. tumefaciens* strain EHA105. 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, except for pART27 transformants which were selected on 25 mg/L kanamycin.

RNA extraction and Northern blot analyses

Total RNA was isolated from frozen tissue ground in liquid nitrogen by extraction with two volumes of hot phenol buffer (1:1 mixture of phenol and 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) with the following modifications. Ten 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). Blots were prehybridized for several hours in 1 M NaCl, 10% dextran sulfate (sodium salt, Sigma), 1% SDS, and 50 µg/ml denatured salmon sperm DNA at 65°C before addition of denatured ^{32}P-labeled DNA probes. After overnight hybridization, blots were washed twice at 42°C for 30 min with 0.1 ×

SSPE (saline/sodium phosphate/EDTA) and 0.5% SDS. Finally, the blots were washed briefly with 0.1 × SSPE at room temperature. Blots were exposed on X-ray films (Fuji, Tokyo, Japan). For probe preparation, DNA fragments corresponding to the complete open reading frame of the following genes were PCR amplified using the following primer sets: 5′- CGG GAT CCG TGC GGA ACA TAG GCC ACG G- 3′ and 5′- CGG GAT CCG GAA CAC CCA TTC CGG TAA C- 3′ for *Lipoxygenase2* (*LOX2, At3g45140*); 5′- ATG GCT TCT ATT TCA ACC CC- 3′ and 5′- CTA AAA GCT AGC TTT CCT TAA CG- 3′ for *Allene oxide synthase* (*AOS* , *At5g42650*); 5′- ATG GCT CT TCA GCA GTG TC- 3′ and 5′- TTA GTT GGT ATA GTT ACT TAT AAC- 3′ for *Allene oxide cyclase2* (*AOC2, At3g25770*); 5′- ATG ACG GCG GCA CAA GGG AAC- 3′ and 5′- TCA GAG GCG GGA AGA AGG AG- 3′ for *OPDA reductase3* (*OPR3, At2g06050*); 5′- ATG GAG AAA TCC GGC TAC GG- 3′ and 5′- GCC ATG ATA CAC TCC CAT AAG- 3′ for *peroxisomal acyl-CoA synthetase1* (*ACS1, At4g05160*); 5′- CAG CCG TTT GAT TGA CCG GAG- 3′ and 5′- CGG TCG AGG CGA TTA GGT TAC- 3′ for *peroxisomal acyl-CoA synthetase2* (*ACS2, At5g63380*); 5′- AGC AAT CGA GCT CGG TTG AG- 3′ and 5′- CAG CTG CTT TGG AAC ATC CG- 3′ for *acyl-CoA oxidase1* (*ACX1, At4g16760*); 5′- TGT CAT GCC AGA GTT GCT GC- 3′ and 5′- CTT GAG AAC CCC ACT GTA TC- 3′ for *abnormal inflorescence meristem1* (*AIM1, At4g29010*); 5′- CAT CTA AGC CAG TTA AAG CTG- 3′ and 5′- GCT CCA ACA ATT CGA TCC TG- 3′ for *multifunctional protein2* (*MFP2, At3g06860*); 5′- ATG GAG AAA GCG ATC GAG AG- 3′ and 5′- TGA GAC ACC AAA GCG TTG TG- 3′ for *3-ketoacyl-CoA thiolase2* (*KAT2/PED1, At2g33150*); 5′- ATG GCT GCT TTT GGA GAT GAC- 3′ and 5′- TGC TTT AGT CTC AGG GTC CAC- 3′ for *3-ketoacyl-CoA thiolase5* (*KAT5, At5g48880*); 5′- ATG GAG GTA ATG CGA GTT CTT C- 3′ and 5′- TCA ACC GGT TCT AAC GAG CG- 3′ for *S-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase* (*JMT*, *At1g19640*); 5′-ATG TTG GAG AAG GTT GAA AC-3′ and 5′- TCA AAA CGC TGT GCT GAA G-3′ for *JAR1* (*At2g46370*); 5′-ACA CCA CAT GCA GCA ACA AC-3′ and 5′-TGA GGC TTA GTG AGC ATA GC-3′ for *MES10* (*At3g50440*); 5′- CGG GAT CCA TGA AAA TCC TCT CAC TTT- 3′ and 5′- CCC TCG AGT TAA GAA GGT ACG TAG TAG AG- 3′ for *Vegetative Storage Protein 1* (*VSP1, At5g24780*); 5′- ATG GTG

AGG TTC GAG AAG G- 3′ and 5′- CTA GAG TTC TTC CCT CAG C- 3′ for *β-Glucosidase1* (*BG1, At1g52400*); 5′- ATG GCG GCG ATA GAG GAC AG- 3′ and 5′- CTA GAC GAA GAT ACC AGA AG- 3′ for *Chlorophyllase1* (*CHL1, At5g43860*); 5′- CGG AAT TCA TGA GAG AGA TCC TTC ATA TC- 3′ and 5′- CCC TCG AGT TAA GTC TCG TAC TCC TCT TC- 3′ for *β-tubulin* (*TUB, At5g44340*); 5′-CGG GAA GGA TCG TGA TGG A-3′ and 5′-CCA ACC TTC TCG ATG GCC T-3′ for *ROC* (*At4g38740*). For *ORA47* (*At1g74930*), a specific DNA fragment that shows little homology with other *AP2/ERF* genes was PCR amplified from Arabidopsis genomic DNA using the following primer set 5′-GGG GTA CCG GAT CCT CTC CTT CTA CAT CTG CAT CTG TTG-3′ and 5′-GCT CTA GAC TCG AGT CCC AAA GAA TCA AAG ATTC-3′.

Measurements of JAs levels

For JAs measurements, six transgenic lines containing the XVE-*ORA47* expression module and four transgenic lines containing the XVE-*GUS* expression module were used. Per treatment and per line 20 two-weeks-old seedlings were incubated in 50 mL polypropylene tubes containing 10 ml MA medium. Expression of the transgene was induced by adding 5 µM estradiol dissolved in DMSO (0.05% final concentration) to the growth medium. Seedlings were collected at time zero and after 4, 8 and 24 hours, frozen and ground in liquid nitrogen. Plant material from 20 seedlings was pooled to minimize biological differences and this was done in triplicate. Quantification of OPDA, JA, JA-Ile and 12-OH-JA was done according to Balcke et al. (in preparation). Briefly, homogenized plant material (about 50 mg) was extracted with 500 µL methanol containing 0.1 ng/µL of each stable isotope-labeled internal standard ($^{2}H_{6}$-JA, $^{2}H_{2}$-(–)-JA-Ile and $^{2}H_{5}$-OPDA) using a bead mill (FastPrep24 instrument, MP Biomedicals LLC, Santa Ana, CA, USA). After centrifugation at 20,000 x g (2 min, 0°C), 450 µL of supernatant was diluted with water to 4.5 mL and subjected to solid-phase extraction (SPE) on HR-XC material (Macherey & Nagel, Düren, Germany). The fraction containing phytohormones was eluted using 900 µL acetonitrile and subjected directly to

LC-MS analysis using a 3200 Q TRAP® LC/MS/MS System hybrid QqLIT mass spectrometer (AB Sciex, Darmstadt, Germany).

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CHAPTER 3

Identification of primary target genes of ORA47

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ABSTRACT

Upon herbivore or pathogen attack plants produce the jasmonate (JAs) hormones, which set in motion defense gene expression programmes. JAs signaling also induces all known JAs biosynthesis genes in what is considered a positive feedback loop. Overexpression of the AP2/ERF-domain transcription factor ORA47 leads to elevated expression of all JAs biosynthesis genes and to elevated levels of JAs, indicating that ORA47 controls the positive feedback loop. The work described here aimed to identify direct target genes of ORA47 by comparing plants containing an inducible expression cassette under induced and non-induced conditions and quantifying the transcript abundance of 24,000 genes using the *Arabidopsis thaliana* whole genome Affymetrix gene chip (ATH1). To distinguish between direct target genes and secondary genes responding to JAs we applied the inducible ORA47 strategy in the wildtype background and in *aos* mutant plants, which are unable to produce JAs. Unexpectedly, most JAs biosynthesis genes responded to ORA47 overexpression to a much lesser degree in the mutant background. The JAs biosynthesis gene *JASMONATE RESISTANT 1* (*JAR1*) responded equally strong in the wildtype and mutant background, making it a strong candidate for an ORA47 target gene. We hypothesize that the other JAs biosynthesis genes are also direct target genes of ORA47, but that they are subject to a second layer of JAs-responsive regulation.

INTRODUCTION

Upon insect herbivory or attack by microbial pathogens plants produce jasmonate (JAs) hormones (Pieterse et al., 2009). The bioactive amino acid conjugate jasmonoyl-isoleucine (JA-Ile) is perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1) (Thines et al., 2007; Sheard et al., 2010) which is part of an SCF complex with putative E3 ubiquitin ligase activity. This leads to degradation of JAZ repressor proteins (Chini et al., 2007; Thines et al., 2007), which sets in motion defense gene expression programmes.

JAs are lipid-derived compounds that are synthesized via a series of enzymatic reactions taking place in different subcellular compartments. The first part of the pathway directs the conversion of α-linolenic acid to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The second part of the pathway takes place in peroxisomes, where OPDA is reduced by OPDA reductase (OPR3) followed by three rounds of beta-oxidation involving three enzymes to yield jasmonic acid (JA). Subsequently, JA can be further metabolized in the cytoplasm. Methylation yields the volatile methyl-jasmonate (MeJA). Conjugation to the amino acid isoleucine by JA amino acid synthase (JAR1; Staswick and Tiryaki, 2004) yields the bioactive form JA-Ile (Fonseca et al., 2009).

In Arabidopsis genes involved in defense against wounding and insect herbivory are controlled by the JAs-responsive basic Helix-Loop-Helix transcription factor MYC2 (Lorenzo et al., 2004) and the related proteins MYC3 and MYC4 (Fernandez-Calvo et al., 2011; Niu et al., 2011). JAZ can bind to MYC (Chini et al., 2007; Chini et al., 2009; Fernandez-Calvo et al., 2011). JAZ1 was shown to repress the activity of MYC2 (Hou et al., 2010) and JAZ can bind to the corepressors TOPLESS (TPL) and TPL-related proteins either directly (Shyu et al., 2012) or via the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA; Pauwels et al., 2010). More recently a variety of transcription factors were reported to interact with members of the JAZ family (Pauwels and Goossens, 2011).

JAs signaling also induces all known JAs biosynthesis genes including *LOX2*, *AOS*, *AOC2* and *OPR3* (Sasaki et al., 2001; Pauwels et al., 2008) in what is considered a positive feedback loop. Overexpression of the AP2/ERF-domain transcription factor ORA47 induces the expression of all JAs biosynthesis genes, leads to elevated levels of JA and JA-Ile and causes severe growth inhibition (Chapter 2). ORA47 can activate the *LOX3* promoter in a transient assay in tobacco protoplasts (Pauwels et al., 2008), indicating that *LOX3* is a direct target gene of ORA47.

The aim of the work described here was to identify direct target genes of ORA47 by comparing plants containing an inducible expression cassette under induced and non-induced conditions and quantifying the transcript abundance of 24,000 genes using the *Arabidopsis thaliana* whole genome Affymetrix gene chip (ATH1). Our working hypothesis was that two types of genes would be switched on by ORA47 overexpression, the direct target genes and genes that respond to JAs produced in response to ORA47 overexpression. To distinguish between these two types we applied the inducible ORA47 strategy in the wildtype background and in *aos* mutant plants, which are unable to produce JAs. Unexpectedly, most JAs biosynthesis genes responded to ORA47 overexpression to a much lesser degree in the mutant background. The *JASMONATE RESISTANT 1* (*JAR1*) gene encoding the JA-Ile conjugating enzyme was a JAs biosynthesis gene that responded equally strong in the wildtype and mutant background, making it a strong candidate for an ORA47 target gene. We hypothesize that the other JAs biosynthesis genes are also direct target genes of ORA47, but that they are subject to a second layer of JAs-responsive regulation.

RESULTS

Validation of transgenic lines containing the inducible XVE-ORA47 cassette

AOS is a single-copy gene encoding a cytochrome P450 enzyme essential for the biosynthesis of JAs. An *aos* T-DNA knockout mutant does not produce any JAs (Park et al., 2002). To be able to distinguish between primary target genes of ORA47 and secondary JAs-responsive genes, transgenic plants containing the estradiol-responsive XVE-ORA47 cassette were crossed with the *aos* mutant.

As a validation of the hypothesis that expression of the XVE-ORA47 cassette in the *aos* mutant background would not lead to JAs biosynthesis we observed the phenotypes of seedlings after estradiol treatment. As shown in Figure 1, germination of wildtype seedlings containing the XVE-ORA47 cassette on medium containing estradiol was severely inhibited. Mutant *aos* seedlings containing the cassette developed normally on estradiol-containing medium.

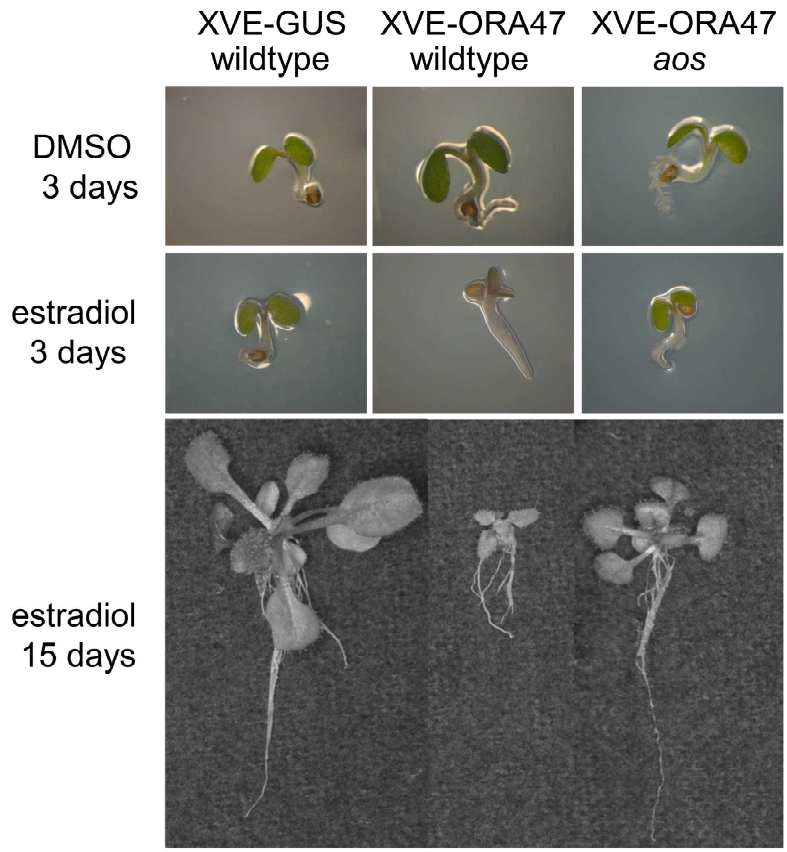

Figure 1. The *aos* mutation counteracts growth inhibition caused by ORA47 overexpression. Seedlings with the genotypes indicated at the top were germinated for 3 days on medium supplemented with 0.1% of the solvent DMSO or 5 µM estradiol. Seedlings germinated on DMSO-containing medium were then transferred to estradiol-containing medium and grown for another 15 days. Experiments were carried out with 2 XVE-GUS lines, 3 XVE-ORA47 lines in the wild-type background and 3 XVE-ORA47 lines in the *aos* mutant background. Representative pictures are shown (WT-GUS line 2.1; WT-ORA47 line 16C; *aos*-ORA47 line 16C2).

When seedlings germinated on control medium were transferred 3 days after germination to estradiol-containing medium, wildtype seedlings containing the XVE-ORA47 cassette showed severe growth retardation compared to control seedlings. Mutant *aos* seedlings containing the XVE-ORA47 cassette grew largely the same as control seedlings. However in general they were slightly

smaller, indicating that the dwarf phenotype of wildtype seedlings overexpressing ORA47 is largely but not entirely due to JAs production and that overexpression of ORA47 has additional minor growth-retarding effects.

To further validate the lines for use in genome-wide expression analysis, the functionality of the inducible cassette and the expression of selected JAs biosynthesis and JAs-responsive genes in response to JA and estradiol treatments were tested in the wildtype and *aos* mutant backgrounds (Fig. 2). Wildtype and *aos* seedlings that did not contain the XVE-ORA47 cassette showed the same responses to JA treatment and did not respond to estradiol treatment. The *aos* mutant seedlings still expressed an *aos* transcript in a JA-responsive manner. The amount of transcript was lower and its size was smaller than the wildtype transcript. Wildtype seedlings containing the XVE-ORA47 cassette showed leaky expression of the estradiol-inducible cassette. This resulted in elevated expression of several JA-responsive genes after treatment with the solvent DMSO. Treatment with estradiol resulted in elevated expression of all selected genes to levels that were similar to those after JA treatment. The *aos* seedlings expressed the *ORA47* gene at a high level after estradiol treatment, indicating that the XVE-ORA47 cassette was functional. However all selected genes except *AOC2* were not induced to levels detectable in this analysis. *AOC2* was induced by estradiol treatment but at a much lower level than in wildtype seedlings containing the XVE-ORA47 cassette. All genes responded to JA at a similar level as in non-transgenic *aos* seedlings.

Based on the observations that the XVE-ORA47 cassette was responsive to estradiol and that the *AOC2* gene was upregulated, we concluded that our *aos* lines were suitable for micro-array analysis.

Genome-wide analysis of gene expression in response to ORA47 overexpression

The micro-array analysis was designed such that three independent wildtype lines and three independent *aos* lines containing the XVE-ORA47 construct were treated with the solvent DMSO or with 5 µM estradiol (Fig. 3). ATH1 micro-arrays were used to evaluate the effect of estradiol treatment and hence ORA47

overexpression on gene expression. In the wildtype background 367 genes were upregulated and 27 genes were downregulated. In the *aos* mutant background 65 genes were upregulated and 4 genes were downregulated (Fig. 6; Table 1). When the genes were grouped according to gene ontology (GO) annotation, it appeared that in the wildtype the largest groups of genes with annotated functions were involved in metabolic processes and in responses to stimuli (Fig. 4). Zooming in on the metabolic processes group, it turned out that relatively

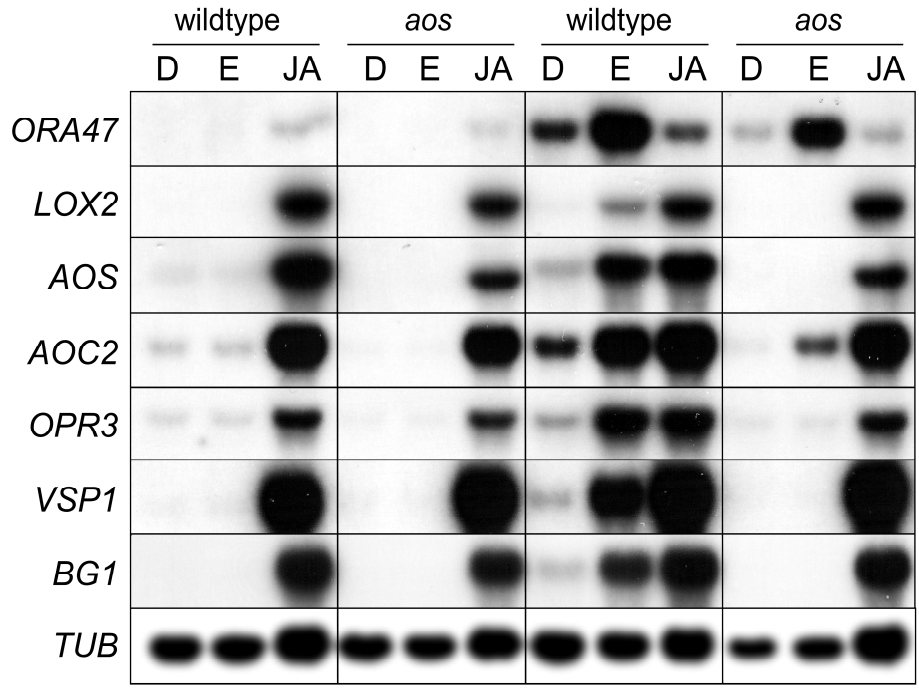

Figure 2. Expression of selected JAs biosynthesis and JAs-responsive genes in response to ORA47 overexpression in the wildtype and *aos* mutant background. Two-week-old wildtype or *aos* seedlings that did or did not contain the estradiol-responsive XVE-ORA47 cassette were treated for 8 hrs with 0.1 % of the solvent DMSO (D), 50 µM JA or 5 µM estradiol (E). The RNA gel blots were hybridized with the indicated probes. The *Tubulin* (*TUB*) probe was used to verify RNA loading. All panels for each probe were on the same blot and exposed to film for the same time allowing direct comparison of expression levels.

large parts were involved in amino acid, phenylpropanoid and oxylipin metabolism. Zooming in on the response to stimulus group, oxylipin metabolism surfaced again as an important process. In addition the *JAZ* family formed an important group and a large group of genes was annotated as involved in defense including wounding responses.

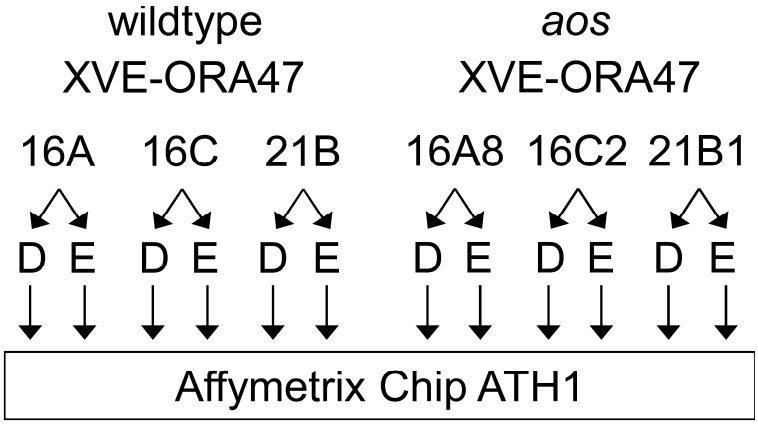

Figure 3. Micro-array design. The *aos* mutant was crossed with two T3 offspring from XVE-ORA47 line 16 and with T3 offspring from line 21. Homozygous *aos* mutants containing the XVE-ORA47 cassette were selected. Two-week-old wildtype or *aos* seedlings containing the estradiol-responsive XVE-ORA47 cassette were treated for 8 hrs with 0.1 % of the solvent DMSO (D) or 5 µM estradiol (E) and RNA was isolated.

The genes induced in the *aos* background had a variety of functional annotations including several stress-responsive and defense genes. Two groups of genes involved in a common process emerged, one group involved in JAs biosynthesis and one group involved in biosynthesis of the defense metabolites glucosinolates. The JAs biosynthesis group included *LOX2*, *LOX3*, *AOC1*, *AOC2*, *OPR3* and *JAR1*. It also included *MES10*, encoding MeJA esterase converting MeJA to JA. The glucosinolate group included genes encoding the transcription factor MYB29, regulating aliphatic glucosinolate biosynthesis (Hirai et al., 2007), the transcription factor MYB34, regulating aromatic glucosinolate biosynthesis (Celenza et al., 2005), the enzyme flavin-

Table 1. List of genes differentially expressed in the *aos* background. Fold change in response to estradiol treatment is indicated in a linear scale.

AGI code	Gene symbol	Description	Fold
At1g74930	ORA47	ERF/AP2 transcription factor	17,4
At1g17420	LOX3	Lipoxygenase	15,2
At2g03020, At4g16540	-	Heat shock protein	13,0
At3g25760, At3g25770	AOC1, AOC2	Allene oxide cyclase	7,8
At4g22620	-	SAUR-like auxin-responsive protein	7,7
At5g47330	-	alpha/beta-Hydrolases	6,4
At2g33380	RD20	Putative calcium-binding EF-hand protein	5,5
At2g46370	JAR1	Jasmonate-amido synthetase	5,5
At3g45140	LOX2	Lipoxygenase	4,9
At2g34930	-	Disease resistance family protein	4,8
At2g42540	COR15A	Cold-regulated protein cor15a precursor	4,6
At1g32860	-	Glycosyl hydrolase	4,4
At5g54170	-	Polyketide cyclase/dehydrase	4,4
At4g30560	ATCNGC9	Cyclic nucleotide gated channel protein	4,3
At5g55180	-	O-Glycosyl hydrolase	3,9
At4g17350	-	Unknown protein	3,8
At4g36900	RAP2.10	ERF/AP2 transcription factor	3,8
At4g27570	-	UDP rhamnose--anthocyanidin-3-glucoside rhamnosyltransferase	3,7
At1g20070	-	Unknown protein	3,6
At5g09530	-	Periaxin	3,5
At5g50720	HVA22E	HVA22 homolog	3,5
At3g05640	-	Protein phosphatase 2C	3,4
At1g01470	LEA14	Late-embryogenesis abundant protein	3,3
At3g13310	-	Chaperone DnaJ-domain protein	3,3
At3g04010	-	O-Glycosyl hydrolases	3,1
At2g06050	OPR3	12-oxophytodienoate	3,1
At2g31940	-	Unknown protein	3,0

At4g24960	HVA22D	ABA- and stress-inducible protein	3,0
At5g07690	ATMYB29	Myb-like transcription factor	2,9
At5g44210	ERF9	ERF/AP2 transcription factor family	2,9
At2g15830	-	Unknown protein	2,8
At3g50440	MES10	Methyl jasmonate esterase	2,7
At5g19875	-	Unknown protein	2,7
At1g75040	PR5	Thaumatin-like protein	2,7
At5g47240	atnudt8	Nudix hydrolase homolog 8	2,6
At2g44130, At2g44140	-	Galactose oxidase	2,6
At5g09520	-	Hydroxyproline-rich glycoprotein	2,6
At1g43910	-	P-loop containing nucleoside triphosphate hydrolase	2,5
At2g36830	GAMMA-TIP	Tonoplast intrinsic protein	2,4
At4g38080	-	Hydroxyproline-rich glycoprotein	2,4
At5g22500	FAR1	Alcohol-forming fatty acyl-CoA reductase	2,3
At2g22080	-	Unknown protein	2,3
At4g38810	-	EF-Hand containing protein	2,3
At1g52400	BGLU18	Glycosyl hydrolase	2,3
At5g60890	MYB34	Myb-like transcription factor	2,3
At2g29450	ATGSTU5	TAU glutathione S-transferase	2,3
At3g22540	-	Unknown protein	2,3
At1g33790	-	Jacalin lectin protein	2,3
At4g15210	BAM5	Beta-amylase	2,3
At3g17130	-	Plant invertase/pectin methylesterase inhibitor protein	2,2
At5g64700	-	Nodulin MtN21	2,2
At3g21420	-	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	2,2
At2g22330	CYP79B3	Cytochrome P450	2,1
At1g21550	-	Calcium-binding EF-hand family protein	2,1
At1g65860	FMO GS-OX1	Flavin-monooxygenase	2,1
At2g32240	-	Unknown protein	2,1
At5g65300	-	Unknown protein	2,1
At3g47380	-	Plant invertase/pectin methylesterase inhibitor protein	2,1

At5g44530	-	Subtilase	2,0
At5g59740	-	UDP-N-acetylglucosamine (UAA) transporter	2,0
At3g02230	RGP1	UDP-arabinose mutase	2,0
At2g28570	-	Unknown protein	2,0
At5g24420	-	6-phosphogluconolactonase 5	2,0
At4g15290	ATCSLB05	Cellulose synthase like protein	-2,1
At5g46900, At5g46890	-	Bifunctional inhibitor/LTP/seed storage 2S albumin	-2,3
At4g19030	NLM1	Aquaporin	-2,6

monooxygenase glucosinolate S-oxygenase 1 (FMO-GSOX1) involved in aliphatic glucosinolate biosynthesis, and the cytochrome P450 enzyme CYP79B3 involved in aromatic glucosinolate biosynthesis (Sonderby et al., 2010). In addition a gene encoding myrosinase-binding protein involved in the formation of toxic glucosinolate breakdown products was induced (Table 1).

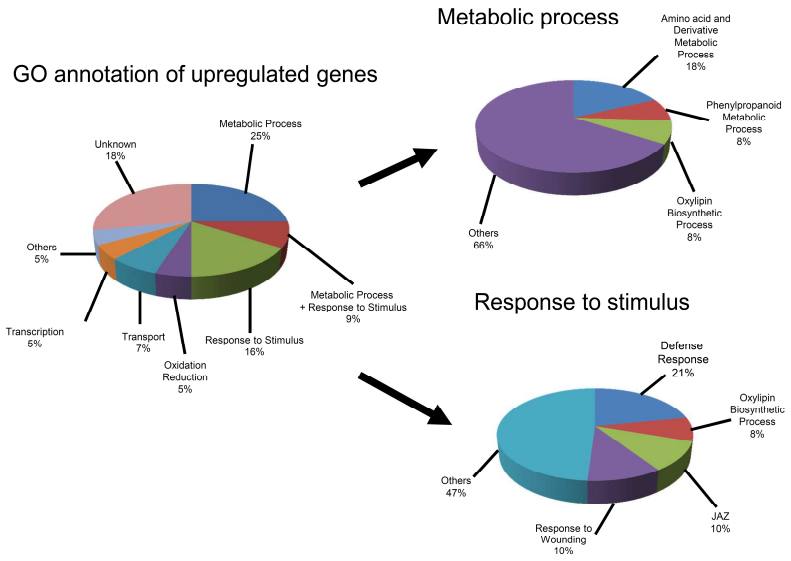

Figure 4. GO annotation of genes induced by ORA47 overexpression in the wildtype background.

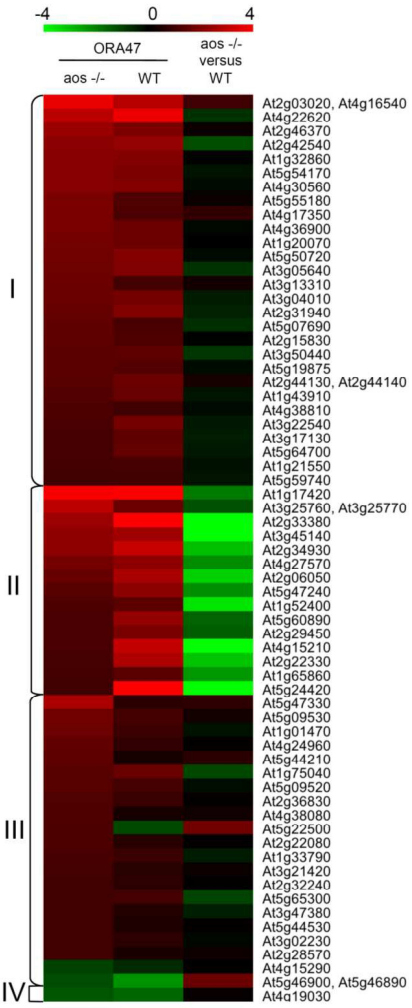

Figure 5. Heat maps of genes expressed differentially in the *aos* mutant with and without ORA47 overexpression. Relative expression of these genes in the wildtype background with and without ORA47 induction and the relative expression in the *aos* mutant versus the wildtype background both overexpressing ORA47 are also shown. Each individual square in the heat map is the average of the three lines analyzed for each condition. Color coding is shown in a log2 scale ranging from -4 to +4. Genes are grouped according to expression pattern in *aos* versus wildtype induced condition. Group I are genes that were similarly induced in both backgrounds. Group II were induced in both groups but to a lesser extent in the *aos* mutant. Group III are genes that were up- or down-regulated in the *aos* background but did not respond to ORA47 overexpression in the wildtype background. Group IV consists of a single gene that was downregulated in both backgrounds.

A more detailed look at the genes differentially expressed in the *aos* background showed that around 70% are also differentially expressed in the wildtype background (Fig. 6). Four patterns of gene expression could be distinguished (Fig. 5). Group I containing 30 genes was upregulated to a similar level in the wildtype and *aos* background by estradiol treatment. Within this group are the JA-Ile biosynthesis genes *JAR1* and *MES10*. Group II consisting of 16 genes was also upregulated in both backgrounds but at a lower level in the *aos* mutants. It contains the JAs biosynthesis genes *LOX2*, *LOX3*, *AOC1*, *AOC2* and *OPR3*. Group III contains 22 genes showing altered expression in the *aos* background but not in the wildtype with 19 upregulated and 3 downregulated genes. Finally, 1 gene (At4g19030), encoding an aquaporin, was downregulated by ORA47 overexpression in both backgrounds (group IV).

Validation of the ATH1 micro-array data

The expression levels determined from ATH1 arrays were confirmed by Northern blot hybridization expression analysis. Figure 7 shows the expression of 2 genes selected from group I (genes equally upregulated in both backgrounds), 3 group II genes (upregulated in both backgrounds but stronger in the wildtype) and 2 genes from the group that were only upregulated in the wildtype background. The expression of *MES10* and *JAR1* was induced very similarly by estradiol in both backgrounds confirming the microarray result. The expression of the JAs biosynthesis genes *LOX2*, *AOC2* and *OPR3* responded to estradiol treatment in the *aos* mutant, but to a much lower level than in the wildtype. The expression of the *MYC2* gene and of its likely target gene *VSP1* responded strongly in the wildtype but not in the mutant background. It can be concluded that the Northern blot results confirmed the micro-array data.

DISCUSSION

The goal of the studies described here was to identify candidate direct target genes of ORA47. To this end we used an inducible ORA47 overexpression strategy. Since ORA47 overexpression leads to JAs biosynthesis (Chapter 2), we

reasoned that upregulated genes in a wildtype background would probably consist of direct ORA47 target genes and secondary JAs-responsive genes. Therefore we applied the inducible strategy using the *aos* mutant which is unable to make any JAs (Park et al., 2002).

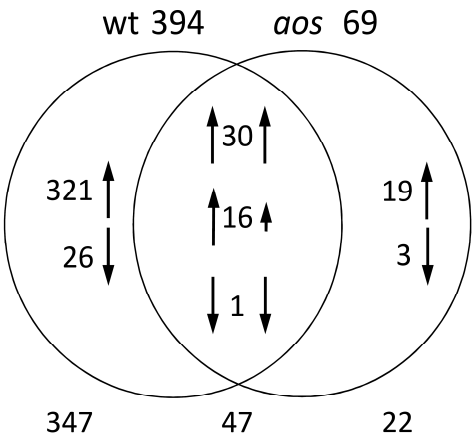

Figure 6. Venn diagram of numbers of genes differentially expressed upon ORA47 induction in wildtype and *aos* backgrounds. Different arrow sizes indicate a response differing in amplitude between the two genotypes. Arrows pointing up and down indicate genes up and down regulated by ORA47 induction, respectively.

With 69 genes differentially expressed in the *aos* mutant background we found a relatively small number of candidate direct target genes. For example in a comparison of wildtype plants versus plants constitutively overexpressing the AP2/ERF-domain transcription factor ERF1 (Lorenzo et al., 2003), 164 genes were found to be differentially expressed using a gene chip that contained 3-fold less gene probes than the ATH1 chip used in our study. Using a gene chip that contained 4-fold less gene probes than the ATH1 micro-array, 268 genes were found to be differentially expressed in a comparison of wildtype plants and plants constitutively overexpressing the transcription factor AtMYB102 (de Vos et al., 2006). The fact that we found 10-20-fold less differentially expressed genes is likely due to two reasons. We eliminated a large number of secondary genes by using a JAs-negative mutant. Transcription factors studied by others may have a similar but unknown effect on the generation of signaling

molecules causing secondary effects. The second reason is that we used an inducible system to overexpress the transcription factor for a relatively short time instead of constitutively overexpressing it. In a study using a similar inducible approach with 4 different AP2/ERF-domain transcription factors that all caused strong upregulation of the *PDF1.2* gene when constitutively overexpressed, it was observed that using inducible overexpression only two transcription factors caused increased *PDF1.2* expression (Pré et al., 2008). Those same two transcription factors were able to trans-activate the *PDF1.2* promoter in a transient assay in protoplasts, whereas the two others were not. This indicates that the inducible approach preferentially increases the expression of direct target genes. Consistent with this notion only 86 differentially expressed genes were found using plants inducibly overexpressing the AP2/ERF-domain transcription factor ORA59 using the Agilent Arabidopsis 3 Oligo array carrying 26,000 gene probes (Pré et al., 2008). This indicates that less than 100 direct target genes for a single transcription factor is a more realistic estimate than the around 1000 genes suggested by other studies. The insight that the use of a constitutive overexpression strategy results in a group of upregulated genes of which 90% are secondary response genes may also explain the lack of correlation between upregulation by transcription factor overexpression and the presence of consensus binding sites for that transcription factor in the promoters reported by some studies (Chakravarthy et al., 2003).

As expected we found the JAs biosynthesis genes among the genes differentially expressed in the *aos* background, suggesting that they are direct target genes. However whereas *JAR1* and *MES10* were upregulated to a similar level in the wildtype and *aos* background, most JAs biosynthesis genes were upregulated much less in the *aos* mutant. We hypothesize that genes equally responding to ORA47 overexpression in both backgrounds are bona fide candidates for being direct target genes of ORA47. We think that genes that respond in both backgrounds but at a lower level in the *aos* background are also serious candidates for being ORA47 target genes. For example *AOC2* and *LOX3* are among the Group II genes. The *LOX3* promoter was found to be activated

by ORA47 in a transient assay in tobacco protoplasts (Pauwels et al., 2008), strongly suggesting that it is a direct target gene. The *AOC2* gene is induced by JA in a cycloheximide-independent manner (Zarei, 2007), and a GCC-box like sequence from the *AOC2* promoter is bound in vitro by recombinant ORA47 protein (Zarei et al., 2011), suggesting that *AOC2* is also a direct target gene.

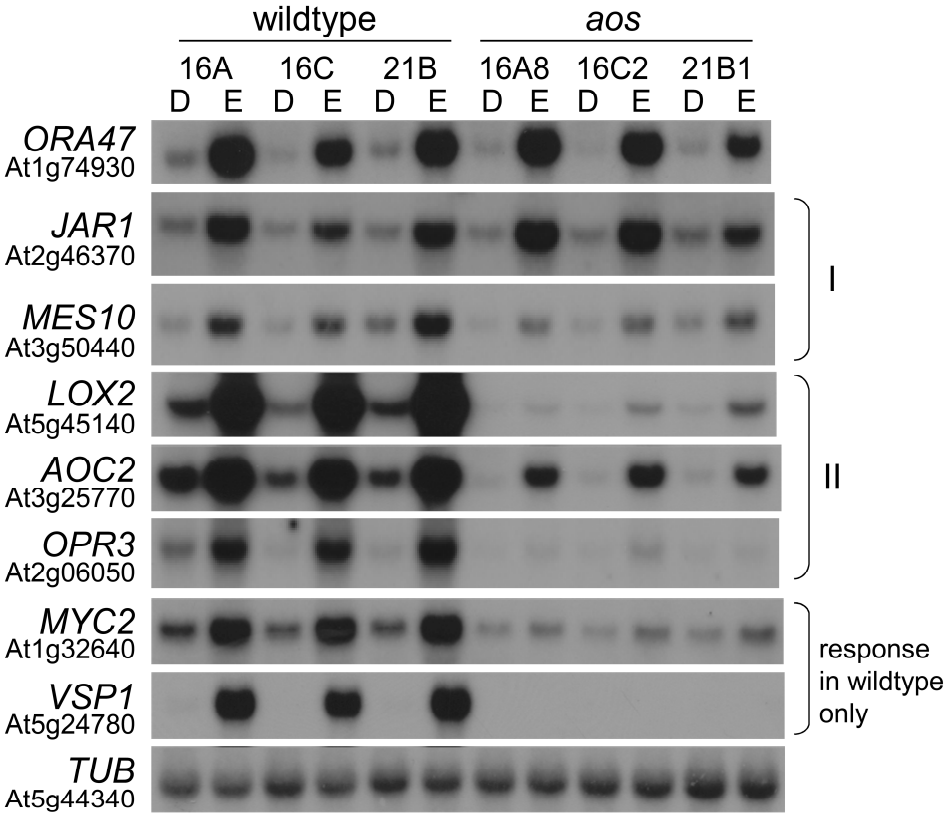

Figure 7. Expression of selected JAs biosynthesis and JAs-responsive genes in response to ORA47 overexpression in the wildtype and *aos* mutant background. Two-week-old wildtype or *aos* seedlings that did or did not contain the estradiol-responsive XVE-ORA47 cassette were treated for 8 hrs with 0.1 % of the solvent DMSO (D) or 5 µM estradiol (E). The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

We consider genes that respond to ORA47 overexpression in the wildtype background but not in the *aos* background as bona fide secondary response genes which are expressed as a result of JAs production. An unexpected category of genes were those that were only upregulated in the *aos* background. We hypothesize that these genes are direct target genes of ORA47. A reason why these genes are not upregulated in the wildtype background might be that they are repressed because of secondary effects due to JAs production.

An interesting group of genes with common annotations upregulated in the *aos* background are related to glucosinolate biosynthesis. The glucosinolate-myrosinase system is a major defense mechanism in Arabidopsis (Burow et al., 2010; Müller et al., 2010). Genes involved in regulation and biosynthesis of both aliphatic and indole glucosinolates were upregulated in the *aos* background. In the wildtype background an additional 6 genes involved both in aliphatic and aromatic glucosinolate biosynthesis were upregulated bringing the total number of upregulated genes involved in glucosinolate biosynthesis or its regulation up to 10. Therefore it seems likely that ORA47 overexpressing wildtype plants have increased levels of both aliphatic and indole glucosinolates and it would be interesting to confirm this experimentally and to test the effect of ORA47 overexpression on insect feeding behavior.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

Arabidopsis thaliana wild-type plants, the *aos* mutant, and all transgenic plants are in the genetic background of ecotype Col-0. *aos* seeds were obtained from the Nottingham Arabidopsis Stock Center. Pollen from XVE-ORA47 transgenic plant (Chapter 2) flowers was used to pollinate flowers from male-sterile *aos* mutant plants (Park et al., 2002). F1 plants were selected on solid MA medium (Masson and Paszkowski, 1992) containing 25 µg/ml hygromycin, were selfed, and hygromycin-resistant F2 plants were screened by PCR to select for homozygous *aos* mutants. To obtain large amounts of F3 seeds flowers of F2

plants were treated with MeJA to restore fertility. Surface-sterilized seeds were grown for 10 days at 21°C in a growth chamber (16 h light/8 h dark photoperiod at 200 µE m^{-2} s^{-1} at 70% relative humidity) on solid MA medium containing 25 µg/ml hygromycin. Fifteen to 20 seedlings per sample were transferred to 50 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 mL MA medium and incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated with 5 µM estradiol (Sigma-Aldrich, St. Louis, MO) or 50 µM JA (Sigma). As control, seedlings were treated with the solvent DMSO (0.1%).

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. DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5′-GAA GAT CTC AAT GGA AGA AGA ATC GGG TTT AGT A-3′ and 5′-GAA GAT CTC ATC AAA AAT CCC AAA GAA TCA-3′ for *ORA47* (*At1g74930*), 5′- ATG GCT TCT ATT TCA ACC CC- 3′ and 5′- CTA AAA GCT AGC TTT CCT TAA CG- 3′ for *AOS* (*At5g42650*), 5′-ATG TTG GAG AAG GTT GAA AC-3′ and 5′- TCA AAA CGC TGT GCT GAA G-3′ for *JAR1* (*At2g46370*), 5′-ACA CCA CAT GCA GCA ACA AC-3′ and 5′-TGA GGC TTA GTG AGC ATA GC-3′ for *MES10* (*At3g50440*), 5′-GTC GAC TTC ATG AAA TTA AAA TGT TTC TC-3′ and 5′-GTC GAC CCA AAA GAT TAC AAA GAC TTT TC-3′ for *AOC2* (*At3g25770*), 5′-CGG GAT CCG TGC GGA ACA TAG GCC ACG G-3′ and 5′-CGG GAT CCG GAA CAC CCA TTC CGG TAA C-3′ for *LOX2* (*At3g45140*), 5′-ATG ACG GCG GCA CAA GGG AAC-3′ and 5′-TCA GAG

GCG GGA AGA AGG AG-3′ for *OPR3* (*At2g06050*), 5′-ATG ACT GAT TAC CGG CTA CAA-3′ and 5′-CCG ATT TTT GAA ATC AAA CTT G-3′ for *MYC2* (*At1g32640*), 5′-CGG GAT CCA TGA AAA TCC TCT CAC TTT-3′ and 5′-CCC TCG AGT TAA GAA GGT ACG TAG TAG G-3′ for *VSP1* (*At5g24780*), 5′-ATG GTG AGG TTC GAG AAG-3′ and 5′-CTA GAG TTC TTC CCT CAG-3′ for *BG1* (*At1g52400*) and 5′-CGG AAT TCA TGA GAG AGA TCC TTC ATA TC-3′ and 5′-CCC TCG AGT TAA GTC TCG TAC TCC TCT TC-3′ for *TUB* (*At5g44340*).

Micro-array hybridization and data analysis

For micro-array analysis, total RNA was extracted using the RNeasy plant mini kit (Qiagen) according to manufacturer's protocol. The Affymetrix micro-arrays (*Arabidopsis* ATH1 genome array) contain probe sets representing around 24,000 genes on a single array. RNA labeling and hybridization on the ATH1 micro-arrays (one sample per chip) were performed according to the manufacturer's instructions (www.affymetrix.com/support/technical/manual/expression_manual.affx) by ServiceXS (www.servicexs.com). The probe arrays were scanned and data analysis was performed using matlab software (v.7.6). The probe level intensities from the CEL files were normalized using the Robust Multichip Average algorithm. A test statistic was computed for each gene to evaluate the evidence for differential expression. P-values were generated by the permuted two-tailed t-test using the "mattest" function of matlab. The "mavolcanoplot" function was used to identify genes with expression changes. A cutoff value of 2-fold and a p-value < 0.05, which are commonly used for micro-array analysis, were adopted to discriminate genes that were differentially expressed. Annotation of the genes represented on the micro-array was obtained from The Arabidopsis Information Resource (TAIR). The heat map was built using the MultiExperiment Viewer software (v4.8). To test the hybridization quality, "Arabidopsis control genes" coding for GAPDH, actin, tubulin, ubiquitin, and several ribosomal RNAs (25S, 5S), spotted by the manufacturer, were verified. The expression ratios [DMSO/estradiol] of the control genes were consistently in the range of 0.81–1.29.

ACKNOWLEDGEMENTS

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CHAPTER 4

Identification and analysis of ORA47-interacting proteins

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ABSTRACT

Upon herbivore or pathogen attack plants produce the jasmonate (JAs) hormones, which are perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1). This leads to degradation of JAZ repressor proteins, which sets in motion defense gene expression programmes. JAs signaling also induces all known JAs biosynthesis genes including *ALLENE OXIDE CYCLASE 2* (*AOC2*) in what is considered a positive feedback loop. The JAs-responsive AP2/ERF-domain transcription factor ORA47 controls all JAs biosynthesis genes. *AOC2* is a primary JAs-responsive gene, indicating that the activity of ORA47 is regulated by a repressor protein that is degraded in a JAs-responsive and COI1-dependent manner. The work described here aimed to identify proteins interacting with ORA47 and to determine their effect on ORA47 activity. Via yeast two-hybrid screening and pull-down assays we identified all 5 members of the Arabidopsis BTB-TAZ protein family except BT2 as ORA47 interactors. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-activation assay, whereas JAZ1 had no effect. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of *AOC2*. We conclude that BT proteins are not the hypothetical repressors or adaptor proteins that we were looking for. We hypothesize that ORA47 is regulated via interaction of its C-terminal domain with an adaptor protein that recruits certain members of the JAZ family.

INTRODUCTION

Upon insect herbivory or attack by microbial pathogens plants produce jasmonate (JAs) hormones (Pieterse et al., 2009). The bioactive amino acid conjugate jasmonoyl-isoleucine (JA-Ile) is perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1) (Thines et al., 2007; Sheard et al., 2010) which is part of an SCF complex with putative E3 ubiquitin ligase activity. This

leads to degradation of JAZ repressor proteins (Chini et al., 2007; Thines et al., 2007), which sets in motion defense gene expression programmes.

Genes involved in defense against wounding and insect herbivory are controlled by the JAs-responsive basic Helix-Loop-Helix transcription factor MYC2 (Lorenzo et al., 2004) and the related proteins MYC3 and MYC4 (Fernandez-Calvo et al., 2011; Niu et al., 2011). JAZ were shown to bind to MYC (Chini et al, 2009; Fernandez-Calvo et al., 2011). JAZ1 can repress the activity of MYC2 (Hou et al., 2010) and JAZ can bind to the corepressors TOPLESS (TPL) and TPL-related proteins either directly (Shyu et al., 2012) or via the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). More recently a variety of transcription factors were shown to interact with members of the JAZ family (Pauwels and Goossens, 2011).

JAs signaling also induces all known JAs biosynthesis genes including *ALLENE OXIDE CYCLASE 2* (*AOC2*) (Sasaki et al., 2001; Wasternack, 2007) in what is considered a positive feedback loop. The JAs-responsive AP2/ERF-domain transcription factor ORA47 controls all JAs biosynthesis genes (Chapter 2). *AOC2* is a COI1-dependent cycloheximide-independent primary JAs-responsive gene (Chapter 3; Zarei, 2006; Wang et al., 2008), indicating that the activity of ORA47 is regulated by a repressor protein that is degraded upon JAs signaling and recognition by the SCF^{COI1} complex.

The aim of the work described in this chapter was to identify proteins interacting with ORA47 and to determine their effect on ORA47 activity. Via yeast two-hybrid screening and pull-down assays we identified all 5 members of the Arabidopsis BTB-TAZ (Bric-à-brac, Tramtrack, Broad - Transcriptional Adaptor Zinc finger) protein family except BT2 as ORA47 interactors. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-activation assay, whereas JAZ1 had no effect. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of *AOC2*.

RESULTS

Determination of activation domains in ORA47 in yeast and Arabidopsis cells

For yeast two-hybrid screening it is important that the bait protein does not contain an activation domain. To determine whether ORA47 contains activation domains active in yeast, it was fused to the GAL4 DNA-binding domain (BD) and the activation of the GAL4-dependent *His3* selection gene was monitored. Expression of full-length ORA47 fused to GAL4BD in the vector pAS2.1 auto-activated the expression of the *His3* selection gene in yeast strain PJ69-4A (Fig. 1). Auto-activation was not repressed by addition of 5-50 mM 3-aminotriazole (3-AT), a competitive inhibitor of the HIS3 enzyme. Auto-activation is a frequently occurring problem with transcription factor baits and can be circumvented by removal of the activation domain. Testing a series of C-terminal deletion derivatives for auto-activation showed that derivatives extending until aa position 140 caused no auto-activation. Longer derivatives caused varying degrees of growth inhibition as well as auto-activation. An internal deletion derivative lacking aa 140-180 gave rise to normal growth and caused auto-activation with up to 50 mM 3-AT in the medium (Fig.1; ORA47Δ8). It can be concluded that the 140-160 region causes growth inhibition by deletion derivatives but not by the full-length protein or derivative ORA47Δ8. An activation domain is located in the 160-180 region which does not completely alleviate growth inhibition. Another activation domain is located in the C-terminal 15 aa (Fig. 1; ORA47Δ5).

To study whether the activation domains found in yeast also function in plant cells, the potential of deletion derivatives to activate the 600 bp *AOC2* promoter was studied in transfected Arabidopsis protoplasts. As shown in Figure 2, full-length ORA47 activated the promoter almost 6-fold. Derivative ORA47Δ3 lacking the C-terminal 55 aa was completely inactive. Addition of the 140-160 and 160-180 regions increased the activation potential in a stepwise manner to about 3-fold. Contrary to the yeast experiment, addition of the 180-195 region had no effect, indicating that although it acted as a potent activation

domain in yeast, it had no activity in Arabidopsis protoplasts. It can be concluded that the whole 140-195 C-terminal domain is necessary for the full activation potential of ORA47 in Arabidopsis protoplasts.

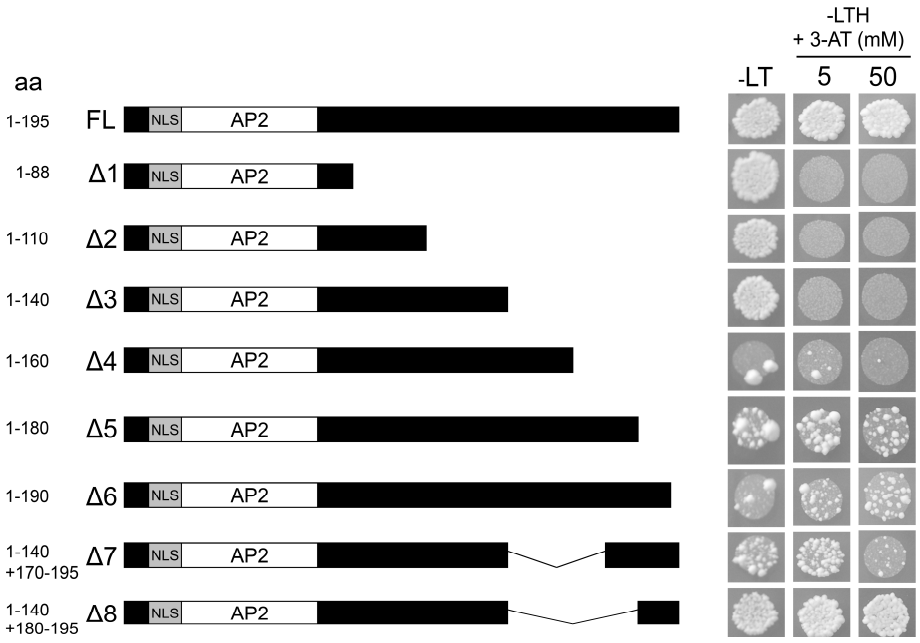

Figure 1. The C-terminal part of ORA47 contains regions that confer transcriptional activation and/or toxicity in yeast. ORA47 derivatives were fused to the GAL4 DNA-binding domain in the plasmid pAS2.1 and introduced in yeast strain pJ69-4A together with the empty vector pACT2. Cultures were spotted on minimal SD medium without Leucine and Tryptophan (-LT) to select for the plasmids and on medium additionally lacking Histidine complemented with 5 mM 3-aminotriazole (-LTH + 3-AT) to select for transcriptional activation of the *His3* gene. Growth was recorded after 7 days.

Identification of proteins that interact with ORA47

To identify proteins that interact with ORA47, yeast two-hybrid screenings were performed using derivative ORA47Δ2 extending from aa 1-110 as bait. Screening of 1.8 x 10^6 yeast transformants obtained with an Arabidopsis cDNA library generated from untreated above-ground parts of mature ecotype *Landsberg erecta* plants in the vector pACT2 resulted in 85 colonies that were able to grow on medium lacking histidine of which 74 were also able to grow

on medium lacking adenine. From 41 colonies prey plasmids were recovered. Only 14 plasmids conferred growth on selective medium after re-transformation. Eleven of these plasmids contained a cDNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase, 1 cDNA encoded the transcriptional repressor RGA1 (AT2G01570) but was not in frame with the GAL4AD, 1 cDNA encoded a protein with unknown function with AGI code AT5G50410, and 1 cDNA encoded the protein BT5 (AT4G37610). RbcS was not further analyzed because RbcS is a chloroplast protein and ORA47 is a nuclear protein, and RGA1 was discarded because it was not in frame. The analysis concentrated on AT5G50410 and BT5.

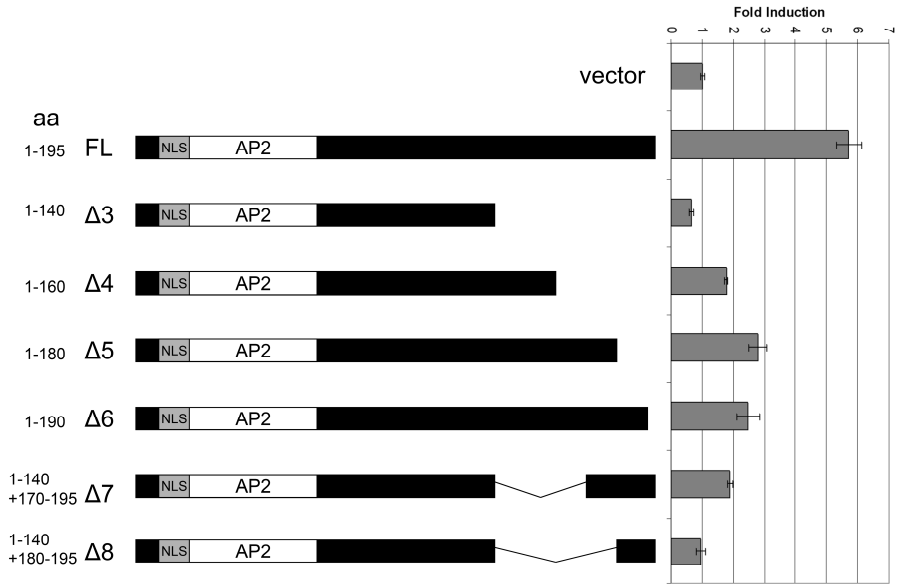

Figure 2. The C-terminal part of ORA47 contains an activation domain active in Arabidopsis protoplasts. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying a 0.6 kb derivative of the *AOC2* promoter fused to GUS and effector plasmids without or with *ORA47* derivatives driven by the CaMV 35S promoter. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the vector control.

ORA47 activity is not affected by AT5G50410

As a first step, interaction between ORA47 and AT5G50410 was tested using the in vitro pull-down technique. Radiolabelled AT5G50410 protein translated in

vitro was mixed with resin-bound recombinant Strep/His-tagged ORA47 or MYC2 proteins produced in *E. coli*. As shown in Figure 3, AT5G50410 was pulled down by ORA47-containing resin but not by empty resin. AT5G50410 also was pulled down by MYC2-coated beads, but much less efficiently.

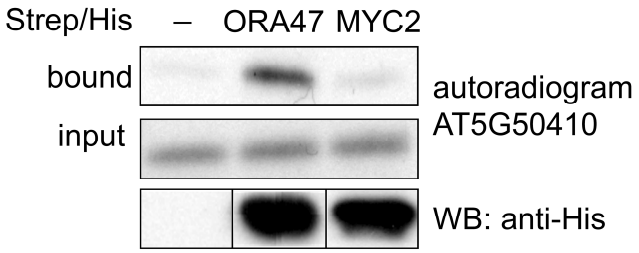

Figure 3. ORA47 interacts with AT5G50410 protein in vitro. Pull-down experiments were carried out with in vitro synthesized radiolabeled AT5G50410 protein and resin-bound recombinant Strep/His-tagged ORA47 or MYC2. Shown are autoradiograms of the input and pull-down reactions and western blots of the pull-down reactions with anti-His antibodies. The samples were analyzed on the same western blot allowing direct comparison of signals.

Next we tested whether AT5G50410 had an effect on the activation activity of ORA47. As a first step we determined the dose-response relationship for trans-activation of gene expression via the 600 bp *AOC2* promoter by ORA47. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying the *AOC2* promoter fused to GUS and varying amounts of effector vectors without or with *ORA47* driven by the CaMV 35S promoter. As shown in Figure 4, 100 ng of ORA47 effector plasmid activated the *AOC2* promoter 4-fold. Fold activation increased with an increasing amount of effector plasmid to 7-fold with 3 µg plasmid. Based on the titration series we tested ORA47 activity with an equal amount of AT5G50410 effector plasmid and with a 5-fold mass excess. Figure 5 shows that AT5G50410 did not significantly affect ORA47 activity.

ORA47 interacts with BT proteins

Next we investigated the BT5 interactor. *BT5* belongs to a small family of 5 *BT* genes in Arabidopsis. The encoded proteins are characterized by an N-terminal

BTB (Bric-à-brac, Tramtrack, Broad) or POZ (POx virus Zinc finger) domain, a TAZ (Transcriptional Adaptor Zinc finger) domain and a C-terminal CaMBD (CalModulin Binding) Domain. The BTB/POZ and TAZ domains mediate protein-protein interactions, and the CaMBD mediates calmodulin binding in a Ca^{2+}-dependent manner. The interaction of all 5 BT proteins with ORA47 was tested in yeast. As shown in Figure 6 ORA47 interacted with BT1, BT3, BT4 and BT5. The interaction was weak since yeast growth was inhibited by addition of 5 mM 3-AT in the medium. Only BT5 showed stronger interaction allowing growth, consistent with its recovery in the screening procedure.

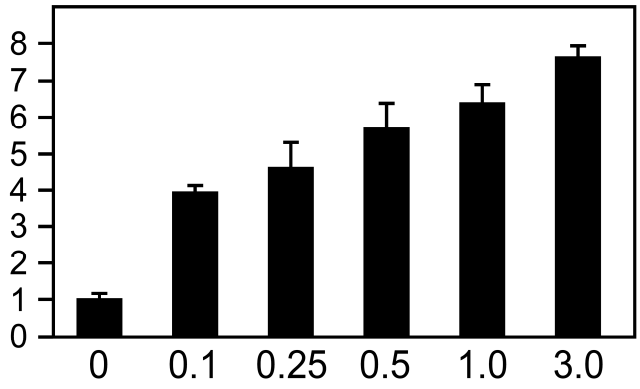

Figure 4. Dose-response relationship for trans-activation of gene expression via the *AOC2* promoter by ORA47. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying a 0.6 kb derivative of the *AOC2* promoter fused to GUS and varying microgram amounts as indicated of overexpression vectors without or with *ORA47* driven by the CaMV 35S promoter. Total effector plasmid amounts were equalized in all reactions by addition of empty overexpression vector. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the zero ORA47 effector control set at 1.

To confirm the interaction we used the in vitro pull-down technique. Radiolabelled BT4 and BT5 proteins translated in vitro were mixed with resin-bound recombinant Strep/His-tagged ORA47, MYC2 or ERF4 proteins produced in *E. coli*. As shown in Figure 7, BT4 and BT5 were pulled down by ORA47-containing resin but not by empty resin or by MYC2- or ERF4-coated resin.

ORA47 does not interact with JAZ proteins

The observation that *AOC2* is a COI1-dependent, cycloheximide-independent primary JA-responsive gene (Chapter 3; Wang et al., 2008; Zarei, 2006) indicates that pre-existing ORA47 is activated via COI1-mediated protein degradation. The most straightforward model is that a protein repressing ORA47 activity is degraded analogous to the MYC2-JAZ model. Besides interacting with MYC2, JAZ also interact with MYC3 and MYC4 and several other transcription factors belonging to different classes (Pauwels and Goossens, 2011). JAZ were not isolated in the two-hybrid screen with ORA47. However a deletion derivative was used for screening leaving open the possibility that JAZ interact with full-length ORA47. We tested this option in a yeast two-hybrid assay using MYC2 as a positive control. As shown in Figure 8 MYC2 interacted with all JAZ proteins except JAZ4 and JAZ7 consistent with previous reports (Chini et al., 2009; Fernandez-Calvo et al., 2011). On the other hand, none of the JAZ proteins interacted with ORA47.

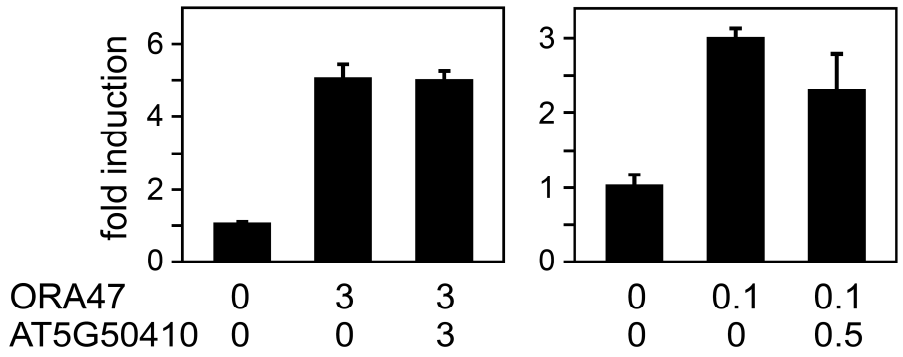

Figure 5. ORA47 activity is not affected by AT5G50410. Arabidopsis cell suspension protoplasts were transiently co-transformed with a *GUS* reporter gene driven by a 0.6 kb derivative of the *AOC2* promoter, and combinations of effector plasmids carrying *ORA47* or AT5G50410 in microgram amounts as indicated. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the zero ORA47/AT5G50410 effector control set at 1.

BT proteins interact with JAZ in yeast

Another option that would still implicate JAZ proteins in regulation of ORA47 activity would be indirect binding via an adaptor protein. BT proteins are ideal candidates for such an adaptor role due to the presence of two protein-protein interaction domains. Therefore we tested interaction between the BT proteins and the JAZ family in a two-hybrid assay in yeast. As shown in Figure 9B, except for BT2, all BT proteins interacted with 2 or more JAZ proteins.

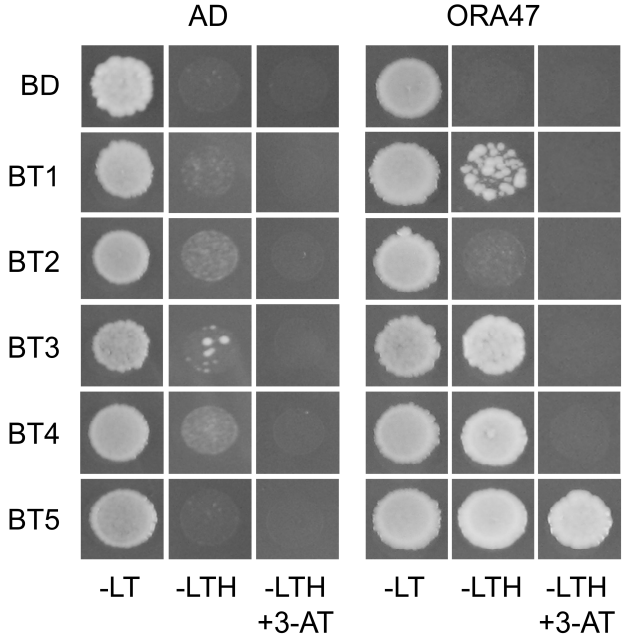

Figure 6. ORA47 interacts with BT proteins in yeast. Yeast cells expressing deletion derivative ORA47Δ2 fused to GAL4BD and BT fused to GAL4AD were spotted on minimal SD medium without Leucine and Tryptophan (-LT) to select for the plasmids and on medium additionally lacking Histidine without (-LTH) or with 5 mM 3-aminotriazole (-LTH + 3-AT) to select for transcriptional activation of the *His3* gene. Growth was recorded after 5 days. Yeast cells transformed with the empty plasmids pAS2.1 and pACT2, expressing the binding domain (BD) and activation domain (AD) of GAL4, respectively, were used as controls.

Interaction in most cases was weak since yeast growth was inhibited by addition of 5 mM 3-AT in the medium (Fig. 9C). Only yeast cells expressing BT4

and JAZ1 or JAZ8 were able to grow under these conditions. Interestingly, these same JAZ proteins were the only ones showing interaction with BT5 on selection medium without 3-AT (Fig. 9B). In summary, the in vitro pull-down and yeast interaction results are compatible with a scenario where BT4 and BT5 serve as adaptors to connect JAZ1 or JAZ8 to ORA47.

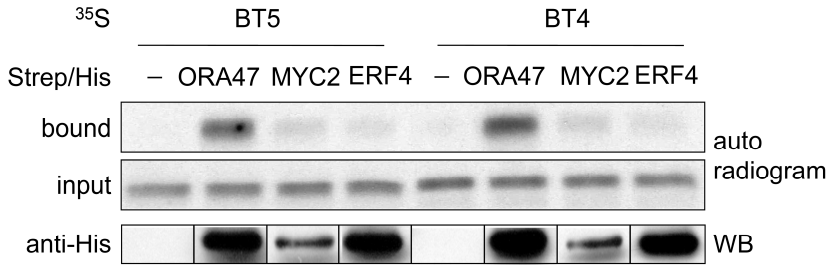

Figure 7. ORA47 interacts with BT4 and BT5 in vitro. Pull-down experiments were carried out with in vitro synthesized radiolabeled BT4 and BT5 proteins and resin-bound recombinant Strep/His-tagged ORA47, MYC2 or ERF4. Shown are autoradiograms of the input and pull-down reactions and western blots of the pull-down reactions with anti-His antibodies. The samples were analyzed on the same western blot allowing direct comparison of signals.

BT4 and BT5 proteins repress ORA47 activity

We tested whether BT4, BT5, JAZ1 or combinations thereof had an effect on the activation activity of ORA47. Arabidopsis cell suspension protoplasts were transiently co-transformed with a *GUS* reporter gene driven by a tetramer of the GCC box from the *AOC2* promoter, and combinations of effector plasmids carrying *ORA47*, BT4, BT5 and *JAZ1*. As shown in Figure 10, JAZ1 had no effect on the activity of ORA47. On the other hand, BT4, BT5 or both combined reduced ORA47 activity about 2-fold. Co-expression of JAZ1 with the BT proteins did not have an additional negative effect on ORA47 activity.

Quadruple *bt* mutants have wildtype levels of JA-responsive *AOC2* expression

To test whether BT proteins affect the activity of ORA47 and hence the expression of its target genes in plants, quadruple *bt* knockout plants were

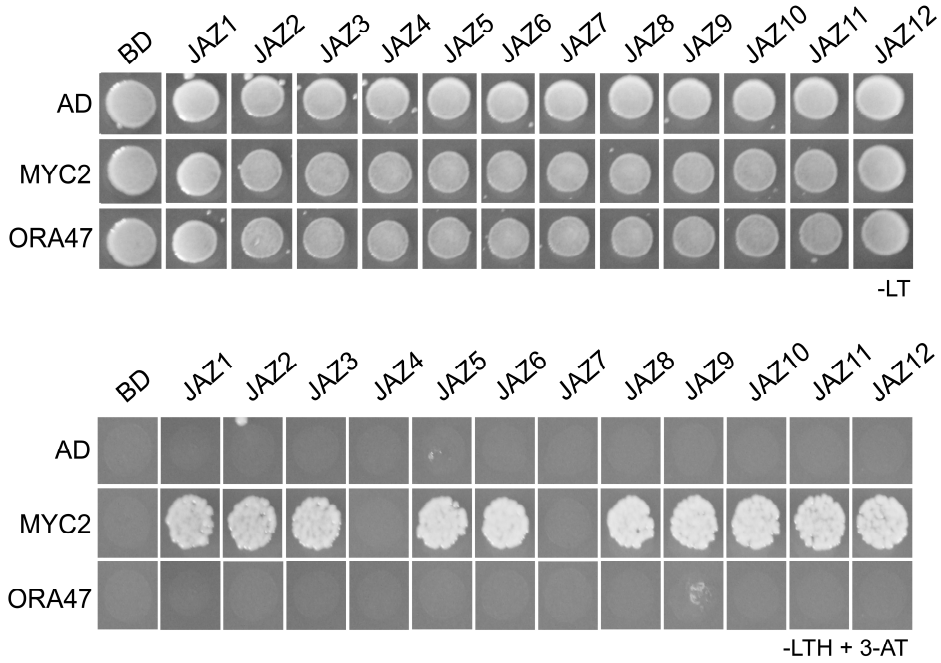

Figure 8. ORA47 does not interact with JAZ repressors in yeast. Yeast cells expressing ORA47 or MYC2 fused to GAL4AD and JAZ fused to GAL4BD were spotted on minimal SD medium without Leucine and Tryptophan (-LT) to select for the plasmids and on medium additionally lacking Histidine complemented with 5 mM 3-aminotriazole (-LTH + 3-AT) to select for transcriptional activation of the *His3* gene. Growth was recorded after 5 days. Yeast cells transformed with the empty plasmids pAS2.1 and pACT2, expressing the binding domain (BD) and activation domain (AD) of GAL4, respectively, were used as controls.

analyzed for JA-responsive *AOC2* expression. The mutant *bt* genes contain T-DNA or transposon insertions and do not give rise to the normal full-length transcripts (Robert et al., 2009). Since a quintuple *bt* mutant is not viable (Robert et al., 2009), we analyzed quadruple mutants which either have a wildtype *BT2* or a wildtype *BT3* gene. BT2 showed no interaction with ORA47 or JAZ proteins in yeast two-hybrid assays (Figs. 6 and 9B). Wildtype and mutant seedlings were treated for various times with 5 or 50 µM JA or the solvent DMSO and the expression of the *AOC2* gene was analyzed. From Figure 11

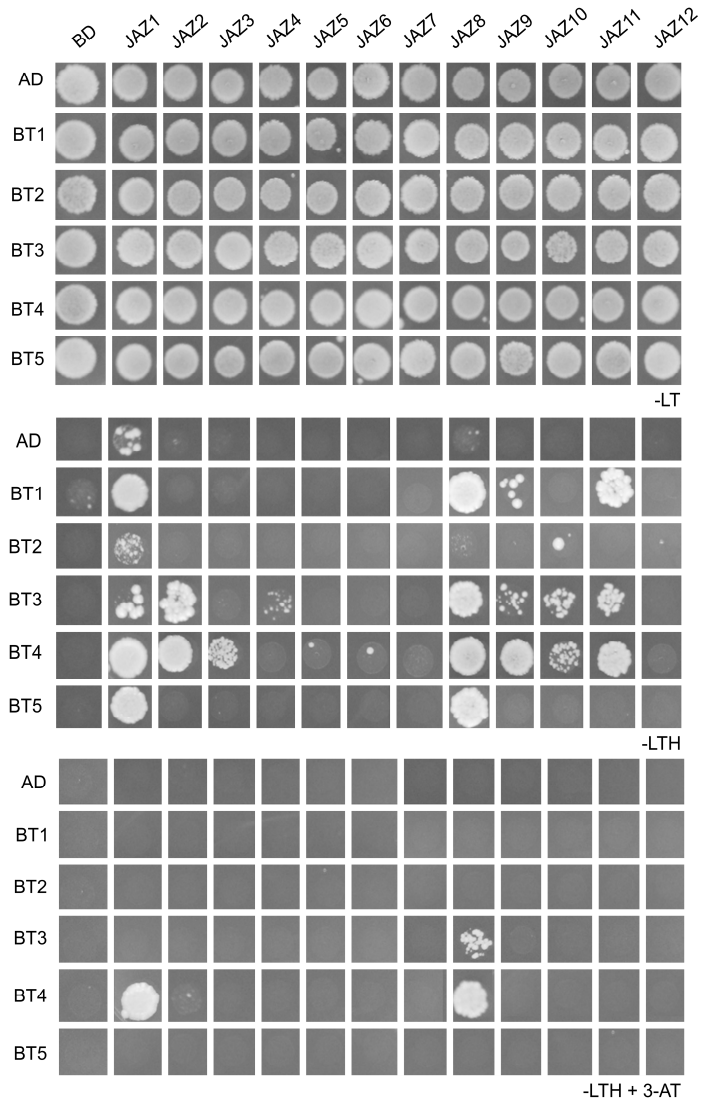

Figure 9. BT proteins interact with JAZ repressors in yeast. Yeast cells expressing BT proteins fused to GAL4AD and JAZ fused to GAL4BD were spotted on minimal SD medium without Leucine and Tryptophan (-LT) to select for the plasmids and on medium additionally lacking Histidine without (-LTH) or with 5 mM 3-aminotriazole (-LTH + 3-AT) to select for transcriptional activation of the His gene. Growth was recorded after 5 days. Yeast cells transformed with the empty plasmids pAS2.1 and pACT2, expressing the binding domain (BD) and activation domain (AD) of GAL4, respectively, were used as controls.

showing the result of a northern blot hybridization experiment it can be concluded that quadruple *bt* knockout did not change the basal expression level of the *AOC2* gene. Although a slight variation was observed between plant lines after JA treatment, there was also not a consistent effect of quadruple *bt* knockout on JA-responsive *AOC2* expression.

DISCUSSION

Based on the hypothesis that ORA47 activity is regulated by a repressor protein that is degraded upon JA signaling via recognition by the SCF^{COI1} complex, the aim of the work described in this chapter was to identify proteins interacting with ORA47 by yeast two-hybrid screening and to determine their effect on ORA47 activity.

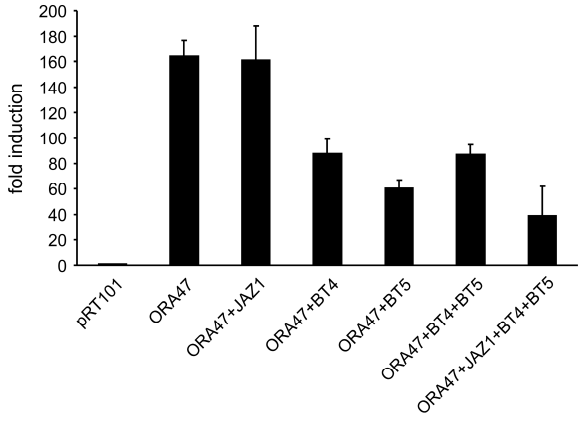

Figure 10. BT4 and BT5 proteins repress ORA47 activity. Arabidopsis cell suspension protoplasts were transiently co-transformed with a *GUS* reporter gene driven by a tetramer of the GCC box from the *AOC2* promoter, and combinations of effector plasmids carrying *ORA47*, BT4, BT5 and *JAZ1* as indicated. Total effector plasmid amounts were equalized in all reactions by addition of empty overexpression vector. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the vector control.

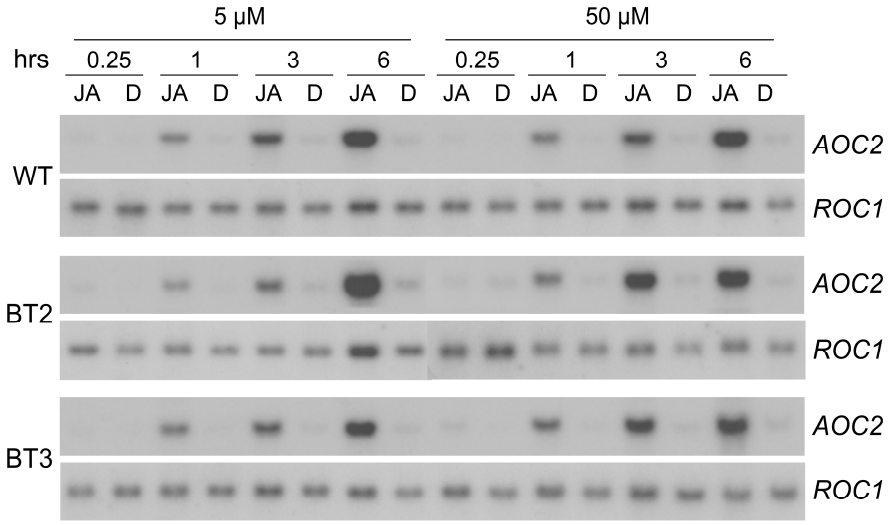

Figure 11. Quadruple *bt* mutants have wildtype levels of JA-responsive *AOC2* expression. RNA was extracted from 14-days-old wild-type or mutant Arabidopsis seedlings treated with 5 or 50 µM jasmonic acid (JA), or with the solvent DMSO (D), for the number of hours indicated. The RNA gel blot was hybridized with an *AOC2* probe. The *ROC1* probe was used to verify RNA loading. All panels for each probe were on the same blot and exposed to film for the same time allowing direct comparison of expression levels.

Screening was performed with an ORA47 derivative lacking the C-terminal half due to the fact that this region contained several domains that activated transcription in yeast. Also in plant cells the C-terminal region was required for the activity of ORA47. Screening resulted only in 2 clones that were considered worthwhile to pursue. One encoded the protein AT5G50410 with unknown function and the other encoded the BTB-TAZ protein BT5. Interaction was confirmed by in vitro pull-down experiments. AT5G50410 did not affect the activity of ORA47 in a transient trans-activation assay in Arabidopsis protoplasts. All 5 members of the BT protein family except BT2 interacted with ORA47 in yeast. BT5 showed the strongest interaction. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-

activation assay, whereas JAZ1 had no effect. The combination of BT4, BT5 and JAZ1 had the same partially repressing effect as the BT4 and BT5 combination. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of the ORA47 target gene *AOC2*.

We conclude that although BT proteins interact specifically with ORA47 and have a negative effect on its activity, they are not the hypothetical repressor or adaptor proteins that we were looking for. It is possible that the hypothetical repressor or adaptor interacts with the C-terminal domain that is missing in the ORA47 derivative used as a bait for screening. In MYC proteins for example, the relative orientation of the DNA-binding domain and the activation domain is reversed, and JAZ were shown to bind to an N-terminal region close to the putative activation domain (Fernandez-Calvo et al., 2011). Although experimental evidence is missing, the current model is that the MYC-JAZ complex while bound to its target promoters recruits the co-repressor TPL and TPL-like proteins either directly (Shyu et al., 2012) or via the adaptor protein NINJA (Pauwels et al., 2010). Therefore, it seems worthwhile to perform the search for ORA47 interactors with another method allowing the use of full-length ORA47. One such method is the TAP-tag method which allows the determination of the composition of native complexes formed in planta (Pauwels et al., 2010; Fernandez-Calvo et al., 2011).

Until now, the only proteins known to be degraded in a COI1-dependent manner upon JA signaling are the JAZ proteins (Chini et al., 2007; Thines et al., 2007). However, COI1 has been reported to interact with histone deacetylase 6 (HDA6) in yeast and in planta (Devoto et al., 2002), although JA-responsive HDA6 degradation was not established. In auxin signaling, which has many similarities to JA signaling, the transcriptional repressors Aux/IAA are the only known targets for SCF^{TIR1}-mediated degradation (Chapman and Estelle, 2009). Assuming that COI1 control of ORA47 activity proceeds via JAZ degradation, the most plausible model is that the C-terminal part of ORA47 interacts with certain members of the JAZ family via an adaptor protein.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

Arabidopsis thaliana wild-type plants and the mutants *bt1BT2bt3bt4bt5* and *bt1bt2BT3bt4bt5* are in the genetic background of ecotype Col-0. Surface-sterilized seeds were grown for 10 days at 21°C in a growth chamber (16 h light/8 h dark photoperiod at 200 µE m^{-2} s^{-1} at 70% relative humidity) on solid MA medium (Masson and Paszkowski, 1992). Fifteen to 20 seedlings per sample were transferred to 50 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 mL MA medium and incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated with 5 or 50 µM JA (Sigma-Aldrich, St. Louis, MO). As control, seedlings were treated with the solvent DMSO (0.1%).

Yeast two-hybrid screening

Full-length *ORA47* and deletion derivatives 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 Ade/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. *ORA47* deletion derivative 1-110 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 stems, leaves and flowers of mature ecotype *Landsberg erecta* plants in the vector λACTII. 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 5 mM 3-AT and

lacking Trp, Leu and His. BTs were amplified with the primers 5′-GGA TCC GTA TGG CTA TAA CCG CCA CTC A-3′ and 5′- GGA TCC CTA TAT AAT TCG ACC GAC CA-3′ for *BT1* (*At5g63160*), 5′- GGA TCC GTA TGG AAG CTG TTC TTG TCG C-3′ and 5′- GGA TCC TTA AAC CCC TTG TGC TTG TT-3′ for *BT2* (*At3g48360*), 5′- GGA TCC GTA TGT CTA GTA GTA CCA AGA AC-3′ and 5′- GGA TCC CTA TAT CAA ACC AGA AGA AC-3′ for *BT3* (*At1g05690*), 5′-GAG GAT CCC AAT GCA GGG AAG AGA AGA TAA GC-3′ and 5′-GAG GAT CCC ATT AAC AGT TTG TCA CCG GTA-3′ for *BT4* (*At5g67480*) and 5′-GAG GAT CCC AAT GGA GAA CAT GGA CGA TTT CT-3′ and 5′-GAG GAT CCC ATC ATA AAG TAA CAT CAA TTG CT-3′ for *BT5* (*At4g37610*). JAZs were amplified with the primers 5′-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC-3′ and 5′-CGG GAT CCC GTC GAC TCA TAT TTC AGC TGC TAA ACC G-3′ for *JAZ1* (*At1g19180*), 5′-CGG GAT CCG TCG ACG AAT GTC GAG TTT TTC TGC CGA GT-3′ and 5′-CGG GAT CCC GTC GAC TTA CCG TGA ACT GAG CCA AGC T-3′ for *JAZ2* (*At1g74950*), 5′-CGC GTC GAC GTA TGG AGA GAG ATT TTC TCG G-3′ and 5′-CGG TCG ACG TTT TAG GTT GCA GAG CTG AGA G-3′ for *JAZ3* (*At3g17860*), 5′-CGC GTC GAC GTA TGG ATT GGT CAT TCT CAA G-3′ and 5′-CGG TCG ACG TTT TAG TGC AGA TGA TGA GCT G-3′ for *JAZ4* (*At1g48500*), 5′-CGG GAT CCG TCG ACG AAT GTC GTC GAG CAA TGA AAA TGC and 5′-CGG GAT CCC GTC GAC CTA TAG CCT TAG ATC GAG ATC T-3′ for *JAZ5* (*At1g17380*), 5′-CGG GAT CCG TCG ACG AAT GTC AAC GGG ACA AGC G-3′ and 5′-CGG GAT CCC GTC GAC CTA AAG CTT GAG TTC AAG GTT-3′ for *JAZ6* (*At1g72480*), 5′-CGC GTC GAC GTA TGA TCA TCA TCA TCA AAA ACT G-3′ and 5′-CGG TCG ACG TTC TAT TCG GTA ACG GTG GTA A-3′ for *JAZ7* (*At2g34600*), 5′-GAA GAT CTC TCG AGG AAT GAA GCT ACA GCA AAA TTG TG-3′ and 5′-GAA GAT CTC TCG AGC ATT ATC GTC GTG AAT GGT ACG-3′ for *JAZ8* (*At1g30135*), 5′-CGG GAT CCG TCG ACG AAT GGA AAG AGA TTT TCT GGG T-3′ and 5′-CGG GAT CCC GTC GAC TCA TAA GCC TCT CTT TGC G-3′ for *JAZ9* (*At1g70700*), 5′-CGG GAT CCG TCG ACG AAT GTC GAA AGC TAC CAT AGA ACT-3′ and 5′-CGG GAT CCC GTC GAC TTA GGC CGA TGT CGG ATA GT-3′ for *JAZ10* (*At5g1322*), CGC GTC GAC GTA TGG CTG AGG

TAA ACG GAG A-3′ and 5′-CGG TCG ACG TTT CAT GTC ACA ATG GGG CTG G-3′ for *JAZ11* (*At3g43440*) and 5′-CGG GAT CCG TCG ACG AAT GAC TAA GGT GAA AGA TGA GC-3′ and 5′-CGG GAT CCC GTC GAC CTA AGC AGT TGG AAA TTC CTC-3′ for *JAZ12* (*At5g20900*). Interaction assays were performed by co-transformation of bait and prey plasmids into yeast strain PJ64-4A and plating 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 5 mM 3-AT. Yeast cells were allowed to grow for 7 days at 30°C.

In vitro pull-down assays

ERF4 (*At3g15210*) was amplified with the primers 5′-CGG AAT TCA ATG GCC AAG ATG GGC TTG AAA CCC-3′ and 5′-CGG TCG ACC CTT GGG CCT GTT CCG ATG GAG-3′ and cloned in EcoRI/SalI digested pASK-IBA45 (IBA Biotagnology, Göttingen, Germany). MYC2 in pASK-IBA45 (Montiel et al., 2011) and ORA47 in pASK-IBA45plus (Zarei et al., 2011) were described previously. *BT4* (*At5g67480*) was amplified with the primers 5′-GAG GAT CCC AAT GCA GGG AAG AGA AGA TAA GC-3′ and 5′-GAG GAT CCC ATT AAC AGT TTG TCA CCG GTA-3′ and *BT5* (*At4g37610*) was amplified with the primers 5′-GAG GAT CCC AAT GGA GAA CAT GGA CGA TTT CT-3′ and 5′-GAG GAT CCC ATC ATA AAG TAA CAT CAA TTG CT-3′. PCR fragments were digested with BamHI and cloned in pBluescript II SK+ plasmid. In vitro synthesis of full-length BT4 and BT5 proteins was achieved using a pBluescript II SK+ plasmid template with the TNT Coupled Wheat Germ Extract System (Promega) in the presence of [^{35}S]methionine. Recombinant Strep/His-tagged proteins were expressed in *Escherichia coli* BL21 (DE3) pLysS cells. Crude protein extracts containing about 5 µg of Strep/His-tagged protein were mixed with 100 µL of 50% Strep-Tactin resin slurry, incubated for 1 hr at 4 ^{o}C with mixing and washed twice. For each pull-down experiment 10-15 µl of in vitro translated protein reaction in 500 µl of incubation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.2% Nonidet P40, 1 mM

phenylmethylsulphonyl fluoride, protease inhibitor cocktail (Roche)) was added. After 60 min rotation at room temperature, the samples were washed 6 times. The washed resin was resuspended in 30 µl of SDS-PAGE loading buffer. Following boiling for 5 min, 20 µl of the samples were subjected to 10% SDS-PAGE. The gel was incubated in Amplify reagent (Amersham), dried and exposed to X-ray film (Fuji, Tokyo, Japan). For western blot analysis 5 µl of the samples were subjected to 10% SDS-PAGE and transferred to Protran nitrocellulose (Schleicher&Schuell) by semidry blotting. Detection was done by incubation with Penta-His HRP antibody conjugate (Qiagen 1:20000), following blocking with Penta-His HRP blocking agent, washing and incubation 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.

Plasmid construction and protoplast assays

A 600 bp *AOC2* promoter fragment was amplified on Arabidopsis genomic DNA with the primer set 5'-GCT CTA GAA TAA AAA TCA GTG TTC TAT CC-3' and 5'-TGG TCG ACT GAT AAA AAT AAA ATA AAA AG-3', digested with XbaI and SalI and cloned in plasmid GusSH (Pasquali et al., 1994). The *ORA47* (At1g74930) open reading frame (ORF) was PCR-amplified 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', digested with BglII and cloned into BamHI digested pRT101 (Töpfer et al., 1987). The *BT4* (*At5g67480*) ORF was PCR-amplified using the primer set 5'-GAG GAT CCC AAT GCA GGG AAG AGA AGA TAA GC-3' and 5'-GAG GAT CCC ATT AAC AGT TTG TCA CCG GTA-3', digested with BamHI and cloned in pRT101. The *BT5* (*At4g37610*) ORF was PCR-amplified using the primer set 5'-GAG GAT CCC AAT GGA GAA CAT GGA CGA TTT CT-3' and 5'-GAG GAT CCC ATC ATA AAG TAA CAT CAA TTG CT-3', digested with BamHI and cloned in pRT101. The *JAZ1* (*At1g19180*) ORF was PCR-amplified using the primer set 5'-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC-3' and 5'-

CGG GAT CCC GTC GAC TCA TAT TTC AGC TGC TAA ACC G-3', digested with SalI and cloned in pRT101. 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 *AOC2*-promoter-*GUS* and effector plasmids carrying *ORA47* fused to the CaMV 35S promoter were carried out. To study a possible effect of BT interaction with the transcription factors, a ratio of 2:2:2 or 2:6:2 (µg GUS::effector plasmid) was chosen. As controls, co-transformations of *AOC2*-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.

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. DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5'-GTC GAC TTC ATG AAA TTA AAA TGT TTC TC-3' and 5'-GTC GAC CCA AAA GAT TAC AAA GAC TTT TC-3' for *AOC2 (At3g25770)* and 5,-CGG GAA GGA TCG TGA TGG A-3' and 5'- CCA ACC TTC TCG ATG GCC T-3' for *ROC1 (At4g38740).*

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CHAPTER 5

MYC2, MYC3 and MYC4 control the jasmonate-responsive expression of the *ORA47* gene

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ABSTRACT

Upon herbivore or pathogen attack plants produce the jasmonate (JAs) hormones. This leads to degradation of JAZ repressor proteins thereby activating transcription factors including MYC2, MYC3 and MYC4, which sets in motion defense gene expression programmes. JAs signaling also induces all known JAs biosynthesis genes in what is considered a positive feedback loop. Overexpression of the AP2/ERF-domain transcription factor ORA47 leads to elevated expression of all JAs biosynthesis genes and to elevated levels of JAs, indicating that ORA47 controls the positive feedback loop. *ORA47* is itself a JAs-responsive gene. The aim of the work described in this chapter was to identify the transcription factor(s) responsible for JAs-responsive expression of the *ORA47* gene. Based on literature data we explored the hypothesis that *ORA47* is regulated by the functionally redundant JAs-responsive transcription factors MYC2, MYC3 and MYC4. The results show that the MYC proteins can bind to a single G-box in the *ORA47* promoter. Triple knockout of the *MYC* genes or overexpression of a stable JAZ1 derivative abolished JA-responsive *ORA47* expression, demonstrating the crucial role of the MYC-JAZ module in regulation of *ORA47* expression

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 (ET) 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 (Pieterse et al., 2009). In response to damage or pathogen attack JAs are synthesized via conversion of α-linolenic acid to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). OPDA is reduced by OPDA reductase (OPR3),

followed by three rounds of beta-oxidation to yield JA (Wasternack, 2007). Subsequently JA is conjugated to the amino acid isoleucine by JA amino acid synthase (JAR1) yielding the biologically active jasmonoyl-isoleucine (JA-Ile) (Fonseca et al., 2009). This signaling molecule stimulates the interaction between the receptor COI1 and members of a family of repressor proteins called JAZ (Thines et al., 2007; Sheard et al., 2010). COI1 is an F-box protein that forms part of an SCF complex (Xu et al., 2002; Devoto et al., 2002) with putative E3 ubiquitin ligase activity. Several members of the JAZ family were shown to interact with the JA-responsive basic Helix-Loop-Helix transcription factor MYC2 (Chini et al., 2007; Chini et al., 2009; Chung et al., 2009) and the related proteins MYC3 and MYC4 (Fernandez-Calvo et al., 2011; Niu et al., 2011). JAZ1 can repress the activity of MYC2 (Hou et al., 2010) and JAZ can bind to the co-repressors TOPLESS (TPL) and TPL-related proteins either directly (Shyu et al., 2012) or via the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). More recently a variety of transcription factors were shown to interact with members of the JAZ family (Pauwels and Goossens, 2011). In response to JA-Ile JAZ proteins are rapidly degraded by the 26S proteasome (Thines et al., 2007; Chini et al., 2007) presumably via SCF^{COI1}-mediated ubiquitination, which is proposed to lead to de-repression of the activity of interacting transcription factors resulting in expression of the corresponding sets of target genes. Wounding and herbivory activate JAs signaling leading to the expression of a set of defense genes controlled by MYC2, MYC3 and MYC4 (Lorenzo et al., 2004; Fernandez-Calvo et al., 2011).

The expression of all JA biosynthesis genes, including *LOX2*, *AOS*, *AOC* and *OPR3*, is induced by wounding or treatment with exogenous JA or MeJA (Sasaki et al., 2001; Sasaki-Sekimoto et al., 2005; Wasternack, 2007; Pauwels et al., 2008) in what is considered to be a positive feedback loop. Overexpression of the AP2/ERF-domain transcription factor ORA47 leads to elevated expression levels of all JAs biosynthesis genes (Chapter 2), indicating that ORA47 controls the positive feedback loop. *ORA47* is itself a JAs-responsive gene (Pauwels et al., 2008) and shows a very fast (30 min) and transient response which is COI1-dependent (Wang et al., 2008).

The aim of the work described in this chapter was to identify the transcription factor(s) responsible for JA-responsive expression of the *ORA47* gene. Based on the facts that it is a very fast JA-responsive gene and that several reports link its expression to *MYC2* expression (Pauwels et al., 2008; Wang et al., 2008), we explored the hypothesis that *ORA47* is regulated by MYC2, MYC3 and MYC4. We discovered that the MYC proteins can bind to a single G-box in the *ORA47* promoter. Triple knockout of the *MYC* genes or overexpression of a stable JAZ1 derivative abolished JA-responsive *ORA47* expression, demonstrating the crucial role of the MYC-JAZ module in the regulation of *ORA47* expression.

RESULTS

MYC proteins bind to the *ORA47* promoter in vitro

As a first step to test the hypothesis that JA-responsive *ORA47* expression is regulated by MYC2, MYC3 and MYC4, we tested binding of recombinant MYC proteins to the *ORA47* promoter in vitro. The 1.4 kb *ORA47* promoter contains 2 G-boxes (CACGTG) and one G-box-like sequence (AACGTG). These sequences have been described as high affinity binding sites for MYC proteins (Chini et al., 2007; Dombrecht et al., 2007; Godoy et al., 2011). Mutations were introduced in each of the three sequences (Fig. 1) and single and triple mutant versions of the *ORA47* promoter fragment were generated. Analysis of recombinant MYC2 and MYC3 proteins produced in *Escherichia coli* and purified by His tag affinity chromatography by denaturing gel electrophoresis and Coomassie Brilliant Blue staining showed the presence of bands of the expected sizes, as well as smaller bands presumably representing degradation products (Fig. 2). Further purification of MYC4 by Strep tag affinity chromatography resulted in a preparation showing a single band of the expected size (Fig. 2).

Electrophoretic mobility shift assays (EMSAs) with the radio-labeled wildtype and mutant versions of the *ORA47* promoter fragment and the MYC protein preparations showed identical patterns of binding (Fig. 3). In EMSAs with versions with a single mutated G-box, only mutation of sequence 2 had a

strong negative effect on binding of the MYC proteins, indicating that it was the main binding site. Whereas the single m2 mutant showed a low level of residual binding especially with MYC2 and MYC4, the triple mutant version showed no binding at all to the MYC proteins.

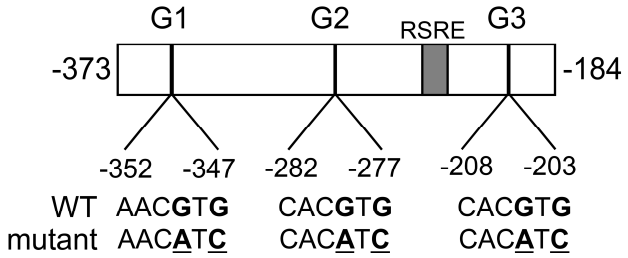

Figure 1. Positions and sequences of wild-type and mutated versions of G-boxes in the *ORA47* promoter fragment used for EMSA. Bold and underlined nucleotides indicate point mutations in the G-boxes. Numbers indicate positions relative to the ATG start codon. The Rapid Stress Response Element (RSRE) located at positions -234 to -229 (Walley et al., 2007) is also indicated.

A combination of MYC2, MYC3 and MYC4 trans-activates the *ORA47* promoter

Next we tested whether MYCs were able to trans-activate the *ORA47* promoter in protoplasts. As shown in Figure 4, a combination of MYC2, MYC3 and MYC4 trans-activated the 1.4 kb *ORA47* promoter around two-fold compared to the empty vector control.

JA-responsive expression of *ORA47* is controlled by MYC2, MYC3 and MYC4

To obtain more proof for regulation of *ORA47* expression by MYC transcription factors, we determined the expression of *ORA47* and one of its target genes, *AOC2*, in *myc* mutants. The *myc2/jin1-2* mutant has a point mutation introducing a stop codon early in the gene, and therefore lacks most of the MYC2 protein including the DNA-binding domain (Lorenzo et al., 2004). The *myc3* and *myc4* mutants carry a T-DNA insertion in the genes and do not express the corresponding full-length transcripts (Niu et al., 2011). Plant lines were treated for 15 min and 6 hrs with JA to be able to observe induction of *ORA47* and *AOC2*, respectively. In addition we determined the expression of

VSP1, which is probably a direct target gene of MYCs. As shown in Figure 5A, mutation of a single *MYC* gene had no effect on JA-responsive gene expression. Simultaneous mutation of two *MYC* genes resulted in a lower expression of JA-responsive genes (Fig. 5B). The observed effect was stronger for *ORA47* than for its target gene *AOC2*. Simultaneous mutation of all three *MYC* genes strongly reduced JA-responsive gene expression (Figs. 5A and 5B). However still some induction of *ORA47* (clearly visible in Fig. 5A) and *AOC2* and *VSP1* occurred, indicating that although the three MYC proteins are the main regulators there must be additional transcription factors controlling the residual JA-responsive expression.

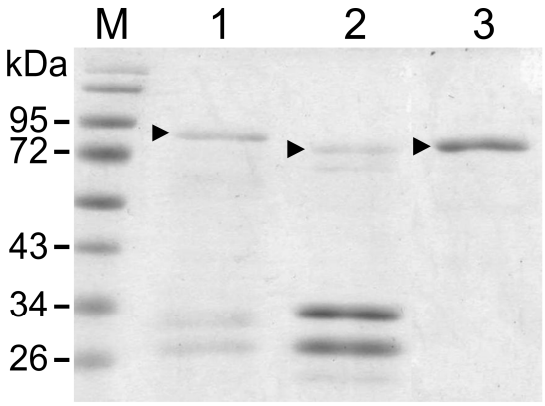

Figure 2. Analysis of recombinant MYC proteins. The proteins were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Sizes of relevant marker (M) bands are indicated in kD. MYC2 (lane 1) and MYC3 (lane 2) were purified by His tag affinity chromatography while MYC4 (lane 3) was purified by sequential His and Strep tag affinity chromatography. The arrowheads indicate the full-length proteins.

Overexpression of *JAZ1ΔC* represses JA-responsive expression of *ORA47*

If MYC transcription factors are controlling JA-responsive expression of *ORA47*, the current widely accepted model of JA signaling predicts that *ORA47* expression should also be regulated by JAZ proteins. To test whether this is correct, we made transgenic plants overexpressing JAZ1 or JAZ3 variants lacking the C-terminal part which contains the conserved Jas domain. The Jas domain acts as a degron by interaction with the SCF^{COI1} complex in the

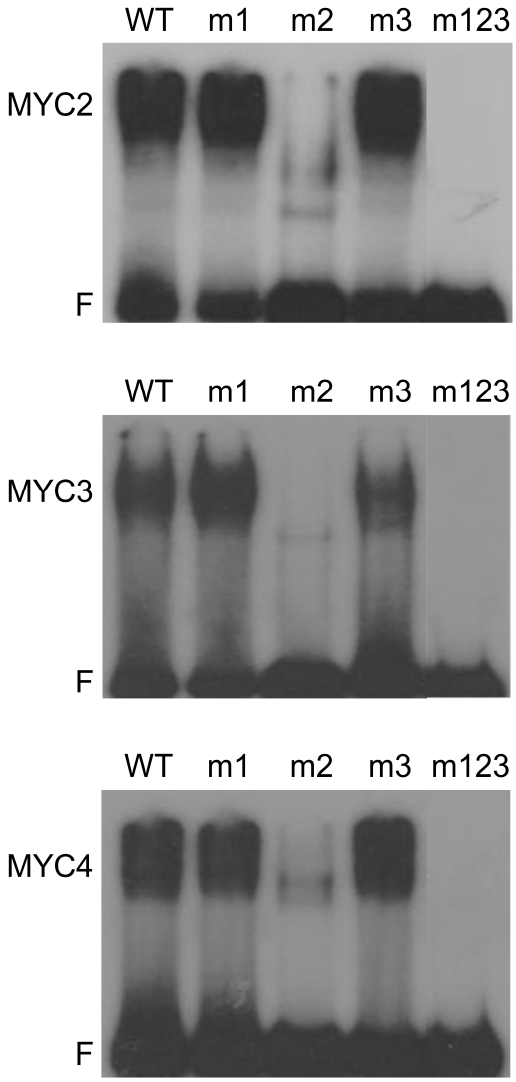

Figure 3. In vitro binding of recombinant MYC proteins to the wild-type *ORA47* promoter fragment or mutated derivatives containing one or more mutated G-boxes as indicated at the top. Radio-labeled fragments were used as probes in in vitro binding. F indicates free probes. DNA-MYC complexes are indicated

presence of JA-Ile (Thines et al., 2007). Variants of JAZ1 (Thines et al., 2007) and JAZ3 (Chini et al., 2007) lacking the Jas domain are stable and repress JA

responses. Based on expression analysis (Fig. 6), we selected three lines for each JAZΔC with the highest expression levels for further analysis.

Figures 7A and 7B show that in the JAZ1ΔC lines the JA-responsive expression of *ORA47* and its target gene *AOC2* was strongly reduced. Also the expression of the putative MYC target gene *VSP1* was strongly reduced. In the JAZ3ΔC lines JA-responsive expression of *ORA47*, *AOC2* and *VSP1* was not different from wild-type. As shown in Figure 6 the *JAZ3ΔC* gene was expressed in these lines at a high level similar to the *JAZ1ΔC* expression level. JAZ3ΔC corresponds to the mutant *jai3-1* described in Chini et al. (2007). Consistent with our results in that report *VSP1* did not emerge as a repressed gene in a micro-array analysis of the *jaz3/jai3-1* mutant line. To get an indication that JAZ3ΔC is functional, we determined the expression of a *PR1* gene (At2g14610) which was among the strongest repressed genes in the micro-array analysis. As shown in Figures 7A and 7B the relatively weak induction of the *PR1* gene in response to JA was completely repressed in all JAZΔC lines, demonstrating that JAZ3ΔC is functional.

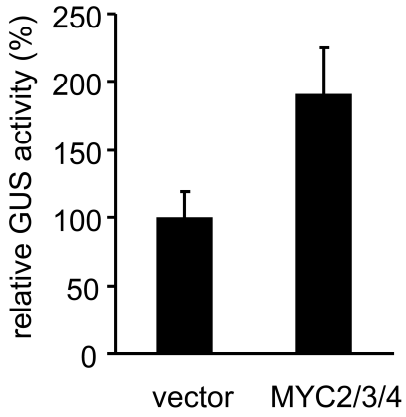

Figure 4. A combination of MYC2, MYC3 and MYC4 trans-activates the *ORA47* promoter. Arabidopsis cell suspension protoplasts were co-transformed with plasmids carrying a 1.4 kb derivative of the *ORA47* promoter fused to GUS and overexpression vectors without or with the *MYC* genes driven by the CaMV 35S promoter. GUS activities represent means ± SE of triplicate experiments and are expressed relative to the vector control set at 100%.

In conclusion, consistent with the widely accepted MYC-JAZ model, JA-responsive *ORA47* expression is positively regulated by MYC transcription factors and repressed by a stable variant of JAZ1.

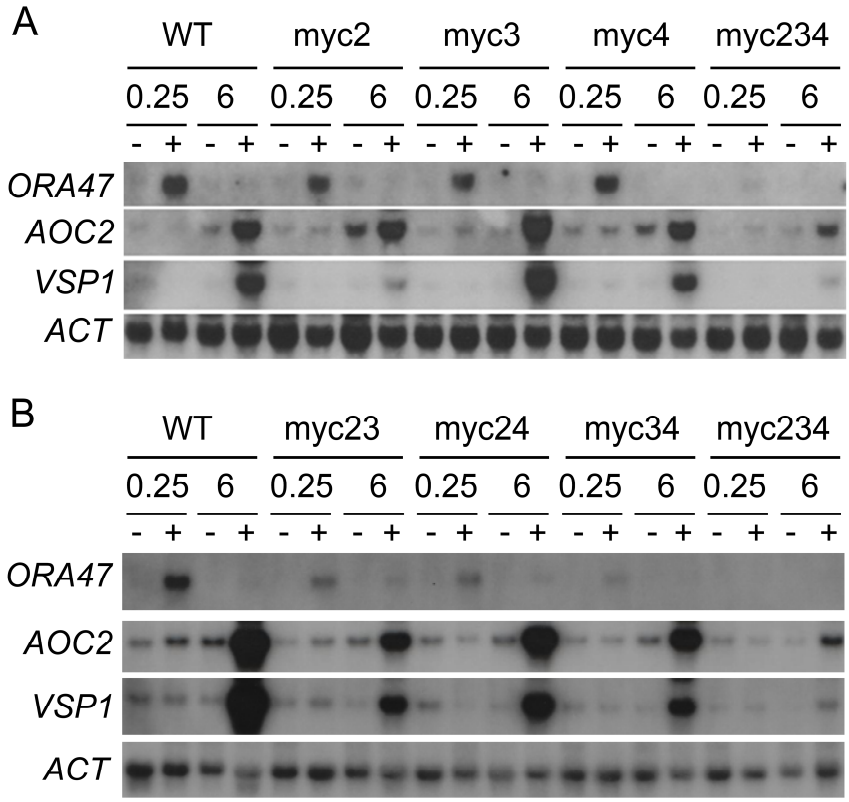

Figure 5. JA-responsive expression of *ORA47* is controlled by MYC2, MYC3 and MYC4. RNA was extracted from 2-week-old wildtype or single, double or triple mutant Arabidopsis seedlings treated with 50 µM JA (+) or the solvent DMSO (-) for the number of hours indicated. The RNA gel blot was hybridized with the indicated probes. The *ACTIN* probe was used to verify RNA loading.

DISCUSSION

Here we have shown that the JA-responsive expression of *ORA47* is controlled by the central MYC-JAZ module as depicted in Figure 8. The genetic evidence shows that functional MYC2, MYC3, MYC4 and JAZ1 proteins are required for

JA-responsive expression of the *ORA47* gene. Binding of the MYC proteins to a G-box in the *ORA47* promoter and their ability to trans-activate the promoter in protoplasts indicated that *ORA47* is a direct target gene of the MYCs. In addition to this regulation of *ORA47* gene expression at the transcriptional level, ORA47 activity is regulated at the protein level via the JA-Ile receptor COI1. As shown in Chapter 2, analysis of a mutant containing a T-DNA insertion in the ORA47 promoter which expresses the *ORA47* gene at a basal level but is unable to induce its expression in response to JA demonstrates that JA-responsive expression of the *ORA47* gene is not necessary for JA-responsive expression of *AOC2*, indicating that regulation at the protein level occurs. In addition the JA-responsive expression of *AOC2* is not negatively affected by the protein synthesis inhibitor cycloheximide (Zarei, 2007), also pointing to regulation of ORA47 protein activity at the post-transcriptional level.

The *ORA47* gene was previously identified as a wounding-responsive gene (Walley et al., 2007). These authors reported a Rapid Stress Response Element (RSRE) present in promoters of rapid wound-responsive genes, which also occurs in the *ORA47* promoter between G-boxes G2 and G3 (Fig. 1). A tetramer of the RSRE conferred a transcriptional response to wounding and a variety of other stress signals, but it was not reported whether it responds to JAs or any other defense hormone, or whether its response depends on COI1 or any other receptor or signaling protein. Based on our results it is unlikely that the RSRE contributes to JAs-responsive expression since this depends on the three redundant MYC transcription factors. These proteins bind mainly to G-box G2 in the *ORA47* promoter and a triple G-box mutant promoter showed no MYC binding at all demonstrating that MYCs do not bind to the RSRE.

The three G-box or G-box-like sequences in the *ORA47* promoter all perfectly fit the sequence requirements for high-affinity MYC binding sites (Chini et al., 2007; Dombrecht et al., 2007; Godoy et al., 2011). Surprisingly, only G-box 2 turned out to be a high-affinity in vitro binding site for MYC proteins. This indicates that besides the G-box flanking nucleotides contribute to MYC binding. The *JAZ2* promoter contains 2 G-boxes and 2 G-box-like elements, but only one of them is involved in JA- and MYC-responsive expression (Figueroa

and Browse, 2012). These authors show evidence indicating that 4 thymidine nucleotides flanking the G-box at the 3′ side are essential for JA-responsive activity. However in that study no EMSAs were performed, therefore it remains unclear whether G-box activity correlates with MYC binding. G-box 2 in the *ORA47* promoter is flanked at the 3′ side by an adenine followed by three thymidine nucleotides. Further experiments are needed to establish whether these nucleotides contribute to binding of MYCs to G-box 2 and to JA-responsive activity of the *ORA47* promoter.

Figure 6. Selection of *JAZ1ΔC* and *JAZ3ΔC* overexpression lines. A, Amino acid sequences of the C-terminal ends of JAZ1 and JAZ3. The conserved Jas domain is shown in a box. The arrow indicates the C-terminal end of the JAZΔC derivatives. B, Equal amounts of RNA from 2-week-old seedlings of independent *JAZΔC* overexpression lines were hybridized with the indicated *JAZ* probes. The lines with the highest expression indicated with asterisks were selected for further analysis. The *ROC* probe was used to verify RNA loading.

The three MYC proteins were redundant in regulating JA-responsive gene expression. Single and double mutants showed only slightly lower expression levels than the wild-type, whereas in the triple mutant gene expression was strongly reduced consistent with a previous report (Fernandez-

Calvo et al., 2011). However, the triple mutant still showed readily detectable expression of *AOC2* and *VSP1* in response to JA treatment, indicating that

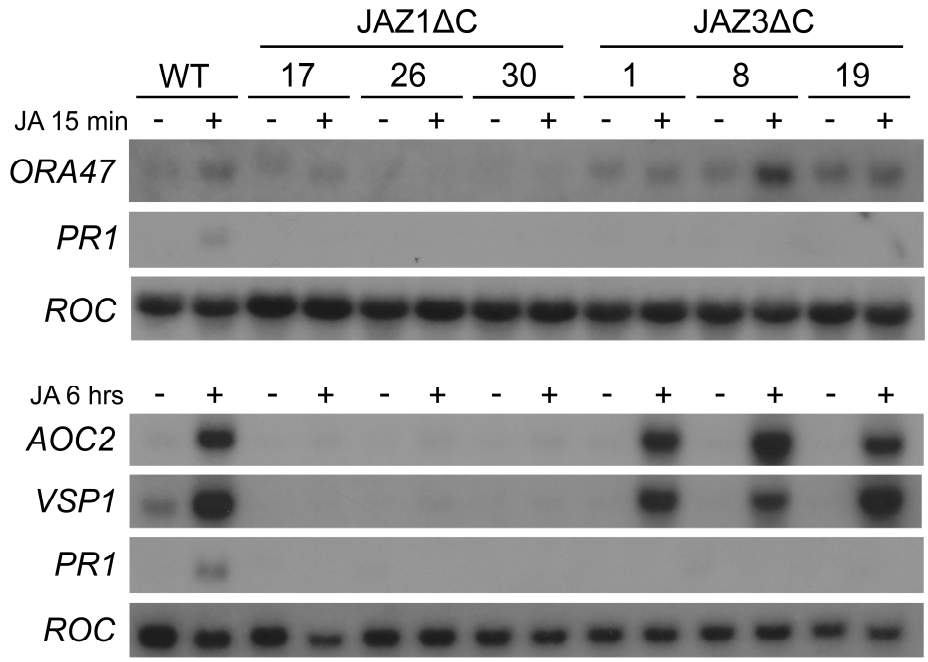

Figure 7. Overexpression of *JAZ1ΔC* represses JA-responsive expression of *ORA47* and its target gene *AOC2*. Two-week-old wildtype seedlings or seedlings from the indicated *JAZΔC* overexpression lines treated for the indicated times with 50 µM JA (+) or the solvent DMSO (-). The RNA gel blots were hybridized with the indicated probes. The *ROC* probe was used to verify RNA loading.

additional transcription factors are involved in JA-responsive gene expression. A candidate is MYC5 (bHLH28) which belongs to the same subgroup IIIe of the Arabidopsis bHLH family as MY2, MYC3 and MYC4 (Heim et al., 2003). MYC5 was ruled out as a JA-responsive transcription factor because it did not interact with JAZ in yeast two-hybrid assays or in in vitro pull-down assays (Niu et al., 2011; Fernandez-Calvo et al., 2011) and it was not captured in TAP tagging

screens for in vivo complexes using JAZ3 or JAZ5 as baits (Fernandez-Calvo et al., 2011). However it is possible that MYC5 interacts with other JAZ proteins via an adaptor protein or it may heterodimerize with another bHLH protein that directly interacts with JAZ. MYC2, MYC3 and MYC4 can form hetero- and homodimers (Fernandez-Calvo et al., 2011), therefore it is likely that MYC5 can do so too.

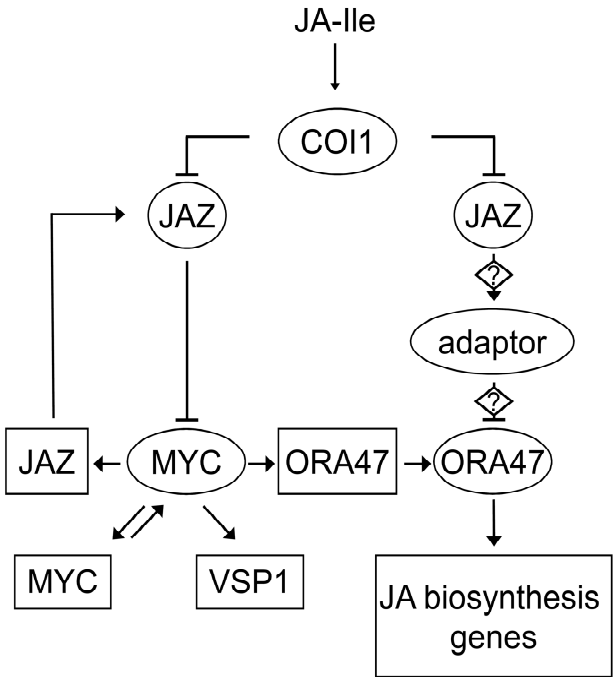

Figure 8. Model for jasmonate signal transduction leading to the expression of JA biosynthesis genes in Arabidopsis. JA-Ile enhances the interaction between COI1 and JAZ, leading to degradation of the latter proteins. MYC2, MYC3 and MYC4 then activate transcription of the gene encoding the AP2/ERF-domain transcription factor ORA47, which in turn activates the expression of JA biosynthesis genes. MYC2, MYC3 and MYC4 also activate transcription of *JAZ* genes as part of a negative feedback loop and their own expression in a positive feedback loop. In addition they activate the expression of defense genes including *VSP1*. ORA47 protein activity is also regulated by JA in a COI1-dependent manner, presumably by members of the JAZ family binding to ORA47 via an adaptor protein. Round symbols indicate proteins and square symbols indicate genes. Questions marks indicate hypothetical interactions.

Overexpression of a stable JAZ1 derivative abolished JA-responsive gene expression. Overexpression of a similar C-terminal deletion derivative of JAZ3 had no effect on JA-responsive *AOC2* or *VSP1* expression, whereas it did abolish JA-responsive *PR1* gene expression. Apparently JAZ1 and JAZ3 are not functionally equivalent. It also indicates that different transcription factor-JAZ complexes operate at different JA-responsive promoters. Apparently JAZ3 does not assemble in complexes on the *VSP1* promoter.

MATERIALS AND METHODS

Growth conditions and treatments

Arabidopsis thaliana ecotype Columbia (Col 0) is the genetic background for all wild type, mutant and transgenic plants. The *myc2/jin1-2* mutant (Lorenzo et al, 2004) and *myc3* (GK445B11) and *myc4* (GK491E10) (Fernandez-Calvo et al., 2011; Niu et al., 2011) and double and triple mutants (Fernandez-Calvo et al., 2011) have been described before. Following stratification for 3 days at 4ºC, the surface-sterilized seeds were germinated for 10 days at 21ºC 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. Seedlings were treated with 50 µM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO; 0.1% v/v final concentration). As control, seedlings were treated with 0.1% DMSO.

Binary constructs and plant transformation

The *JAZ1ΔC* and *JAZ3ΔC* sequences were amplified by PCR using the primer sets 5′-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC-3′ and 5′-GGG ATC CGT CGA CTC AAA GTT CTG TCA ATG GTG TTG G-3′ for *JAZ1ΔC* and 5′-CGG AAT TCA CCA TGG AGA GAG ATT TTC TCG GG-3′ and 5′-CCG CTC GAG CTA CAC GTT GGA GCC ATT ACA TTG-3′ for *JAZ3ΔC*,

respectively. *JAZ1ΔC* and *JAZ3ΔC* were cloned as BamHI and EcoRI fragments respectively into pRT101 (Töpfer et al., 1987). The 35S expression cassettes were excised with PstI and cloned in pCAMBIA1300. The 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.

Transient expression assay

A 1.4 kb *ORA47* promoter fragment was amplified with the primer set 5′-CCA TGG GCT GAC ACC AAC CAG ATC AA-3′ and 5′- CCA TGG ATA AAA ACT CCG GTT CG-3′, digested with NcoI and cloned in GusXX (Pasquali et al., 1994). The *MYC2* (At1g32640) gene was excised from the *Rap-1* cDNA in pBluescript SK (GenBank acc. No. X99548; de Pater et al., 1997) with XmaI and cloned in pRT101. The *MYC3* (At5g46760) gene was PCR amplified from a cDNA library using the primer set 5′-CCT CGA GAA TGA ACG GCA CAA CAT CAT C-3′ and 5′- CGG ATT CTC AAT AGT TTT CTC CGA CTT TC-3′, digested with XhoI/BamHI and cloned in pRT101. The *MYC4* (At4g17880) gene was PCR amplified from a cDNA library using the primer set 5′-GAT CGA ATT CAT GTC TCC GAC GAA TGT TCA AG-3′ and 5′-CAG TGG ATC CTC ATG GAC ATT CTC CAA CTT-3′, digested with EcoRI/BamHI and cloned in pRT101. Protoplasts prepared from *Arabidopsis thaliana* cell suspension ecotype Col-0 were co-transformed with plasmids carrying the *ORA47* promoter-*GUS* fusion and effector plasmids carrying *MYC* fused to the CaMV 35S promoter. As a control, co-transformation of *ORA47* promoter-*GUS* with the empty pRT101 expression vector was carried out. Protoplasts were transformed using polyethylene glycol as described previously (Schirawski et al., 2000) with the constructs in a ratio of 2:2:2:2 (µg GUS:MYC2:MYC3:MYC4). The protoplasts were harvested 18 hrs after transformation and were frozen in liquid nitrogen. GUS activity assays were performed as described (van der Fits and Memelink, 1997). GUS activities were related to protein concentrations to correct for differences protein extraction efficiencies.

RNA extraction and Northern blot analyses

Total RNA was extracted from frozen tissue by phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% v/v ethanol, and resuspended in water. Ten 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, http://www.perkinelmer.com). Probes were ^{32}P-labeled by random priming. (Pre-) hybridization and subsequent washings of blots were performed as described (Memelink et al., 1994) with minor modifications. The following sets of primers were used: 5′-GAA GAT CTC AAT GGA AGA AGA ATC GGG TTT AGT A-3′ and 5′-GAA GAT CTC ATC AAA AAT CCC AAA GAA TCA-3′ for *ORA47* (*At1g74930*), 5′-GTC GAC TTC ATG AAA TTA AAA TGT TTC TC-3′ and 5′-GTC GAC CCA AAA GAT TAC AAA GAC TTT TC-3′ for *AOC2* (*At3g25770*), 5′-CGG GAT CCA TGA AAA TCC TCT CAC TTT-3′ and 5′-CCC TCG AGT TAA GAA GGT ACG TAG TAG G-3′ for *VSP1* (*At5g24780*), 5′-CGG GAT CCG TCG ACG AAT GTC GAG TTC TAT GGA ATG TTC-3′ and 5′-GGG ATC CGT CGA CTC AAA GTT CTG TCA ATG GTG TTG G-3′ for *JAZ1* (*At1g19180*), 5′-CGG AAT TCA CCA TGG AGA GAG ATT TTC TCG GG-3′ and 5′-CCG CTC GAG CTA CAC GTT GGA GCC ATT ACA TTG-3′ for *JAZ3* (*At3g17860*), 5′-ATG AAT TTT ACT GGC TAT TCT CG-3′ and 5′- TCA GTA TGG CTT CTC GTT C-3′ for *PR1*(At2g14610), 5′-CTG TGC CAA TCT ACG AGG GTT-3′ and 5′-GGA AAC CTC AAA GAC CAG CTC-3′ for ACT2 (*At1g18780*) and 5′-CGG GAA GGA TCG TGA TGG A-3′ and 5′- CCA ACC TTC TCG ATG GC C T-3′ for *ROC1* (*At4g38740*).

Isolation of recombinant MYC proteins and EMSA

Plasmid pASK-IBA45 (IBA Biotechnology, Göttingen, Germany) containing *MYC2* was described before (Montiel et al., 2011). *MYC3* (*At5g46760*) was amplified with primer set 5′-CGA GCT CGA TGA ACG GCA CAA CAT CAT C-3′ and 5′- CCC ATG GAT TAG TTT TCT CC GAC TTT CGT C-3′, digested with SacI/NcoI and cloned in pASK-IBA45plus. *MYC4* (*At4g17880*) was

amplified with the primers 5′- GGA ATT CGA TGT CTC CGA CGA ATG TTC AAG-3′ and 5′- CCC ATG GAT GGA CAT TCT CCA ACT TTC TC-3′, digested with EcoRI/NcoI and cloned in pASK-IBA45plus. Double Strep/His-tagged MYC proteins were expressed in *E. coli* strain BL21 (DE3) pLysS and purified by Ni-NTA agarose (Qiagen, http://www.qiagen.com) chromatography followed in the case of MYC4 by Strep-Tactin sepharose (IBA Biotagnology) chromatography. The wild-type *ORA47* promoter fragment was amplified with the primers 5′- GAT CCT CGA GAA AAT CTC AGT ATT TAA AAC A-3′ and 5′- CAG TCT CGA GTG GCG CGT GAA GAT GGG A-3′ and cloned in pJET1.2 (Fermentas). Mutations were generated according to the QuickChange Site-Directed Mutagenesis protocol (Stratagene) using the primers 5′-CTC AGT ATT TAA AAC AAA CAT CCC TAA ACA AAT AGA G-3′ (G1), 5′- GAG AGT TGA ATT AAA TCA CAT CGA AAA CAA GGA ACA CG-3′ (G2) or 5′- CTC AAT ACA ATC CGC CAC ATC TCC CAT CTT CAC GCG CCA G-3′ (G3) and their respective reverse complementary primers. Fragments were isolated with XhoI and labelled by filling in the overhangs with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. DNA-binding reactions contained 0.1 ng of end-labeled DNA fragment, 500 ng of poly(dAdT)-poly(dAdT), binding buffer (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% v/v glycerol) and protein extract in a 10 µl volume. Following the addition of protein extract, reactions were incubated for 30 min at room temperature before loading on a 5% w/v acrylamide/bisacrylamide (37:1) gel in half-strength Tris-Borate-EDTA buffer under tension. After electrophoresis at 125 V for 30 min, the gel was dried on Whatman DE81 paper and autoradiographed.

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SUMMARY

Jasmonic acid (JA) and related oxylipins, collectively known as jasmonates (JAs), are key regulators of plant development and plant responses to abiotic and biotic challenges. The major bioactive JAs is the amino acid conjugate jasmonoyl-isoleucine (JA-Ile). JA-Ile is perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1) which is part of an SCF complex with putative E3 ubiquitin ligase activity. This leads to degradation of JAZ repressor proteins, which sets in motion gene expression programmes.

Genes involved in defense of Arabidopsis plants against wounding and insect herbivory are controlled by the transcription factors MYC2, MYC3 and MYC4. JAZ can bind to MYC and are thought to repress their activity. JAZ degradation is thought to release these transcription factors from repression leading to expression of defense genes.

The majority of the enzymes acting in the octadecanoid pathway for biosynthesis of JAs have been identified and the corresponding genes are known. The expression of all these biosynthesis genes including *LOX* and *AOC* is induced by treatment with exogenous JA or methyl-JA (MeJA), indicating that JAs signaling is amplified by a positive feedback loop initiated by JAs. How this feedback loop is controlled at the transcriptional level is not well understood. Candidate transcription factors were reported in the literature. In a transient assay to screen for activators MYC2 and the AP2/ERF-domain transcription factor ORA47 were able to activate the *LOX3* promoter in tobacco protoplasts. Whereas MYC2 has been extensively studied and is one of the major JAs-responsive transcription factors, little is known about the function of ORA47. The expression of the *ORA47* gene was reported to be induced by JA in a COI1-dependent manner.

The studies described in this thesis were focused on the functional analysis of ORA47 in Arabidopsis. The aim of the research was (1) to investigate whether ORA47 regulates the positive feedback loop in JAs biosynthesis, (2) to determine its target genes, (3) to establish how ORA47 is regulated at the protein level, and (4) to understand the regulation of JAs-responsive *ORA47* gene expression.

The current knowledge of the octadecanoid pathway for biosynthesis of JAs and of the different components of the JAs signaling pathway are reviewed in **Chapter 1**.

Chapter 2 describes the role of ORA47 in JAs biosynthesis. Overexpression of the *ORA47* gene conferred JAs-related phenotypes, such as inhibition of growth and anthocyanin production, and induced the expression of all JAs biosynthesis genes tested. JAs measurements in *ORA47*-overexpressing plants showed an increase in the amounts of the JA precursor 12-oxophytodienoic acid (OPDA), JA, the bioactive JA-Ile and the inactive derivative 12-hydroxy-JA. Probably as a consequence of JAs production several JAs-responsive defense genes were upregulated in *ORA47*-overexpressing plants**.** The results indicate that ORA47 acts as the key regulator in the positive feedback loop by controlling the expression of the JAs biosynthesis genes.

Chapter 3 describes the identification of candidate target genes of ORA47. To distinguish between direct target genes and secondary genes responding to JAs an inducible *ORA47* overexpression system was used in the wildtype background and in *aos* mutant plants, which are unable to synthesize JAs. Changes in transcript abundance of 24,000 genes in response to switching on *ORA47* expression in the two genetic backgrounds were determined using Arabidopsis whole genome Affymetrix (ATH1) gene chips. Unexpectedly, most JAs biosynthesis genes responded to ORA47 overexpression to a much lesser degree in the mutant background. The JAs biosynthesis gene *JASMONATE RESISTANT 1* (*JAR1*) responded equally strong in the wildtype and mutant background, making it a strong candidate for an ORA47 target gene. It is hypothesized that the other JAs biosynthesis genes are also direct target genes of ORA47, but that they are subject to a second layer of JAs-responsive regulation.

Chapter 4 describes the characterization of proteins that interact with ORA47 and may regulate its activity. The ORA47 target gene *AOC2* is a primary JAs-responsive gene, indicating that the activity of ORA47 is regulated by a repressor protein that is degraded in a JAs-responsive and COI1-dependent manner. Via yeast two-hybrid screening and pull-down assays all 5 members of

the Arabidopsis BTB-TAZ protein family except BT2 were identified as ORA47 interactors. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-activation assay, whereas JAZ1 had no effect. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of *AOC2*. From these results it was concluded that BT proteins are not the hypothetical repressors or adaptor proteins that were the target of the research described in this chapter.

Chapter 5 describes the identification of the promoter element(s) and the transcription factor(s) responsible for JAs-responsive expression of the *ORA47* gene. Based on literature data the hypothesis that *ORA47* is regulated by the functionally redundant JAs-responsive transcription factors MYC2, MYC3 and MYC4 was explored. The results show that the MYC proteins can bind to a single G-box in the *ORA47* promoter. Triple knockout of the *MYC* genes or overexpression of a stable JAZ1 derivative abolished JA-responsive *ORA47* expression, demonstrating the crucial role of the MYC-JAZ module in the regulation of *ORA47* expression.

The aim of the studies described in this thesis was to determine the role of the AP2/ERF-domain transcription factor ORA47 in the transcriptional regulation of the JAs-responsive positive feedback loop in JAs biosynthesis.

In short, the following new results were obtained. Overexpression of ORA47 resulted in increased expression of all JAs biosynthesis genes tested and in elevated levels of several JAs including JA and JA-Ile. The JAs biosynthesis genes are probably direct target genes of ORA47. ORA47 does not directly interact with JAZ repressors in yeast two-hybrid assays. The JA-responsive expression of the *ORA47* gene is controlled by the transcription factors MYC2, MYC3 and MYC4 which interact with a G-box in the ORA47 promoter.

One important aim of the thesis research was to isolate the hypothetic repressor protein which is thought to control ORA47 activity in a manner

similar to the MYC-JAZ interaction. Unfortunately the research failed to identify this repressor. Assuming that JAZ are the only proteins which are degraded via SCF^{COI1} activity, the preferred hypothesis is that ORA47 is regulated via interaction of its C-terminal domain with an adaptor protein that recruits certain members of the JAZ repressor family.

SAMENVATTING

Jasmonzuur (JA) en verwante oxylipines, gezamenlijk bekend als jasmonaten (JAs), zijn belangrijke regulatoren van de ontwikkeling van planten en van de reacties van planten op suboptimale omstandigheden veroorzaakt door (a)biotische factoren. Het belangrijkste bioactieve JAs is het aminozuurconjugaat jasmonoyl-isoleucine (JA-Ile). JA-Ile wordt waargenomen door het F-box eiwit CORONATINE INSENSITIVE1 (COI1) dat deel uitmaakt van een SCF complex met vermeende E3 ubiquitine ligase activiteit. Dit leidt tot afbraak van JAZ repressoreiwitten, wat genexpressieprogramma's in gang zet.

Genen die betrokken zijn bij de verdediging van Arabidopsis planten tegen verwonding en herbivorie door insecten worden gereguleerd door de transcriptiefactoren MYC2, MYC3 en MYC4. JAZ eiwitten kunnen binden aan MYC en worden gedacht hun activiteit te onderdrukken. Verondersteld wordt dat JAZ afbraak deze transcriptiefactoren vrij maakt wat leidt tot expressie van verdedigingsgenen.

De meerderheid van de enzymen actief in de octadecanoïde route voor biosynthese van JAs zijn geïdentificeerd en de overeenkomstige genen zijn bekend. De expressie van al deze biosynthesegenen, met inbegrip van *LOX* en *AOC*, wordt geïnduceerd door behandeling met exogeen JA of methyl-JA (MeJA), wat aangeeft dat JAs signalering wordt versterkt door een positieve terugkoppeling geïnitieerd door JAs. Hoe deze terugkoppeling wordt gereguleerd op transcriptioneel niveau is niet bekend. Kandidaat transcriptiefactoren werden vermeld in de literatuur. In een transiënte test om te screenen voor activators bleken MYC2 en de AP2/ERF-domein transcriptiefactor ORA47 in staat om de *LOX3* promoter te activeren in tabaksprotoplasten. Terwijl MYC2 uitgebreid is bestudeerd en één van de belangrijkste JAs-responsieve transcriptiefactoren is, is er maar weinig bekend over de functie van ORA47. De expressie van het *ORA47* gen wordt naar verluidt door JA op een COI1-afhankelijke wijze geïnduceerd.

De studies beschreven in dit proefschrift zijn gericht op de functionele analyse van ORA47 in Arabidopsis. Het doel van het onderzoek was (1) om vast te stellen of ORA47 de positieve terugkoppeling in de JAs biosynthese

reguleert, (2) om de doelwitgenen van ORA47 te bepalen, (3) om vast te stellen hoe ORA47 wordt gereguleerd op eiwitniveau, en (4) om de regulatie van JAs-responsieve *ORA47* genexpressie te begrijpen.

De huidige kennis over de octadecanoïde route voor de biosynthese van JAs en over de verschillende componenten van de JAs signaleringsroute wordt beschreven in **Hoofdstuk 1**.

Hoofdstuk 2 beschrijft de rol van ORA47 in JAs biosynthese. Overexpressie van het *ORA47* gen in transgene Arabidopsis planten veroorzaakt JAs-gerelateerde kenmerken, zoals groeiremming en anthocyaanproductie, en verhoogt de expressie van alle geteste JAs biosynthesegenen. JAs metingen in planten die *ORA47* tot overexpressie brengen toonden een toename van de hoeveelheden van de JA precursor 12-oxophytodienoic acid (OPDA), JA, het bioactieve JA-Ile en het inactieve derivaat 12-hydroxy-JA. Waarschijnlijk als gevolg van JAs productie kwamen verscheidene JAs-responsieve verdedigingsgenen verhoogd tot expressie in planten met *ORA47* overexpressie. De resultaten geven aan dat ORA47 fungeert als de centrale regulator in de positieve terugkoppeling door controle van de expressie van de JAs biosynthesegenen.

Hoofdstuk 3 beschrijft de identificatie van kandidaat doelwitgenen van ORA47. Om onderscheid te kunnen maken tussen directe doelwitgenen en secundaire genen die reageren op JAs is een induceerbare *ORA47* overexpressiemethode toegepast in de wildtype achtergrond en in *aos* mutant planten die geen JAs kunnen maken. Veranderingen in de transcripthoeveelheden van 24.000 genen in reactie op het aanschakelen van ORA47 expressie in de twee genetische achtergronden werden bepaald met de Affymetrix Arabidopsis volledige genoom (ATH1) chips. Onverwachts reageerden de meeste JAs biosynthesegenen op ORA47 overexpressie in veel mindere mate in de mutant achtergrond. Het JAs biosynthese gen JASMONATE RESISTANT 1 (JAR1) reageerde even sterk in de wildtype en de mutant achtergrond, waardoor het een goede kandidaat is voor een direct ORA47 doelwitgen. Er wordt verondersteld dat de andere JAs

biosynthesegenen ook directe doelwitgenen van ORA47 zijn, maar dat ze zijn onderworpen aan een tweede laag van JAs-responsieve regulatie.

Hoofdstuk 4 beschrijft de karakterisering van eiwitten die interactie vertonen met ORA47 en haar activiteit zouden kunnen reguleren. Het ORA47 doelwitgen *AOC2* is een primair JAs-responsief gen, wat aangeeft dat de activiteit van ORA47 wordt gereguleerd door een repressoreiwit dat afgebroken wordt op een JAs-responsieve en COI1-afhankelijke wijze. Via twee-hybride screening in gist en in vitro pull-down assays werden vier van de vijf leden van de Arabidopsis BTB-TAZ eiwitfamilie geïdentificeerd als ORA47 interactoren. ORA47 bindt niet direct aan JAZ eiwitten, maar alle BT eiwitten behalve BT2 doen dat wel, wat in overeenstemming is met een scenario waarbij BT eiwitten fungeren als adaptors tussen ORA47 en JAZ. BT4 en BT5 onderdrukten ORA47 activiteit gedeeltelijk in een transiënte trans-activatie assay, terwijl JAZ1 geen effect had. Analyse van viervoudige *bt* knockout planten, die ofwel een wildtype BT2 of BT3 gen bezaten, toonde geen effect aan op basale of JA-responsieve expressie van *AOC2*. Uit deze resultaten werd geconcludeerd dat BT eiwitten niet de hypothetische repressoren of adaptoreiwitten zijn die het doelwit waren van het onderzoek beschreven in dit hoofdstuk.

Hoofdstuk 5 beschrijft de identificatie van de promoterelement(en) en de transcriptiefactor(en) die verantwoordelijk zijn voor JAs-responsieve expressie van het *ORA47* gen. De op literatuurgegevens gebaseerde hypothese dat *ORA47* wordt gereguleerd door de functioneel redundante JAs-responsieve transcriptiefactoren MYC2, MYC3 en MYC4 is onderzocht. De resultaten toonden aan dat de MYC eiwitten binden aan een G-box in de *ORA47* promoter. Drievoudige knock-out van de *MYC* genen of overexpressie van een stabiele JAZ1 afgeleide deed JA-responsieve *ORA47* expressie volledig teniet, wat de cruciale rol van de MYC-JAZ-module in de regulatie van *ORA47* expressieaantoont.

Het doel van de studies beschreven in dit proefschrift was om de rol te bepalen van de AP2/ERF-domein transcriptiefactor ORA47 in de transcriptionele regulatie van de JAs-responsieve positieve terugkoppeling in JAs biosynthese. In het kort werden de volgende nieuwe resultaten verkregen. Overexpressie

van ORA47 resulteerde in een verhoogde expressie van alle geteste JAs biosynthesegenen en in verhoogde niveaus van verschillende JAs waaronder JA en JA-Ile. De JAs biosynthesegenen zijn waarschijnlijk directe doelwitgenen van ORA47. ORA47 bindt niet direct aan JAZ repressoren in twee-hybride assays in gist. De JA-responsieve expressie van het *ORA47* gen wordt gecontroleerd door de transcriptiefactoren MYC2, MYC3 en MYC4 die binden aan een G-box in de *ORA47* promoter.

Een belangrijk doel van het onderzoek beschreven in dit proefschrift was de identificatie van het hypothetische repressoreiwit waarvan wordt verondersteld dat het ORA47 activiteit reguleert op een wijze vergelijkbaar met de MYC-JAZ interactie. Helaas is dit onderzoek er niet in geslaagd om deze repressor te identificeren. Ervan uitgaande dat JAZ de enige eiwitten zijn die worden afgebroken via SCF^{COI1} activiteit, is de favoriete hypothese dat ORA47 wordt gereguleerd via interactie van de C-terminale helft met een adaptoreiwit dat bepaalde leden van de JAZ repressorfamilie rekruteert.

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Curriculum Vitae

Muhammad Khurshid was born on the 8th of July 1982 in Rahim Yar Khan, Pakistan. He got his early education in Rahim Yar Khan. In 2001, after passing his higher secondary school examination, he joined the faculty of Agriculture at University of Agriculture Faisalabad in Faisalabad, Pakistan. He received his Bachelor degree from University of Agriculture Faisalabad in June 2005. From January 2006 until May 2008, he followed the master program of biotechnology and received his master's degree (M.Phil) from NIBGE Quaid-e-Azam University in Faisalabad, Pakistan. In 2007 he got the overseas Scholarship offered by Higher Education Commission of Pakistan for the PhD studies. From March 2008 until October 2012, he worked on PhD research project under the supervision of Prof. Dr. Johan Memelink at the Institute of Biology, Leiden University.