Functional analysis of ORA47, a key regulator of jasmonate biosynthesis in arabidopsis

Khurshid, M.

CHAPTER 1

GENERAL INTRODUCTION

Muhammad Khurshid and Johan Memelink

Institute of Biology, Leiden University, P.O. Box 9505, 2300 RA Leiden, the Netherlands

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).

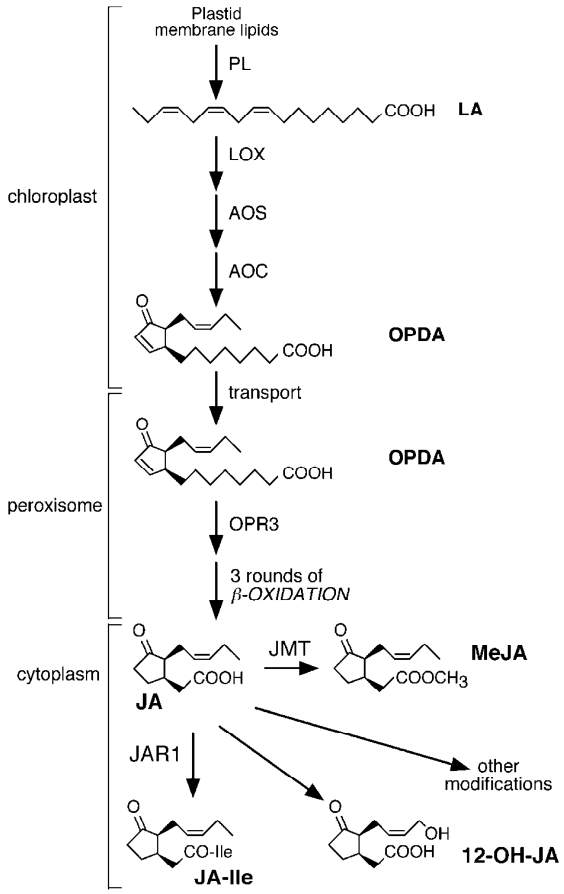

Figure 1. Schematic representation of the octadecanoid pathway leading to jasmonate biosynthesis. 12-OH-JA, 12-hydroxy-jasmonic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; JAR1, enzyme responsible for the conjugation of JA with isoleucine (JA-Ile); JMT, *S*-adenosyl-L-methionine:jasmonic 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

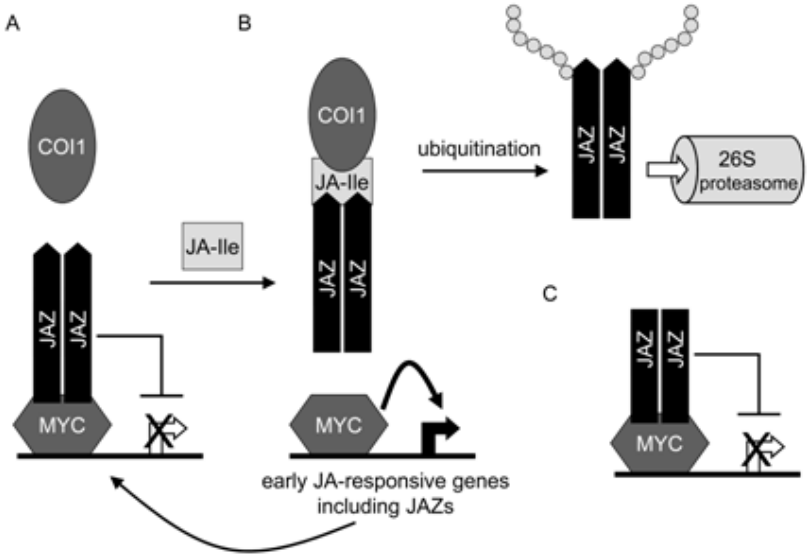

Figure 2. Model for regulation of jasmonate-responsive gene expression by MYC and JAZ proteins. Although depicted as a single protein, COI1 forms part of the putative E3 ubiquitin ligase SCF^{COI1}. (A) In the absence of JA-Ile, a (hetero) dimer of JAZ proteins interacts with MYC maintaining these transcription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

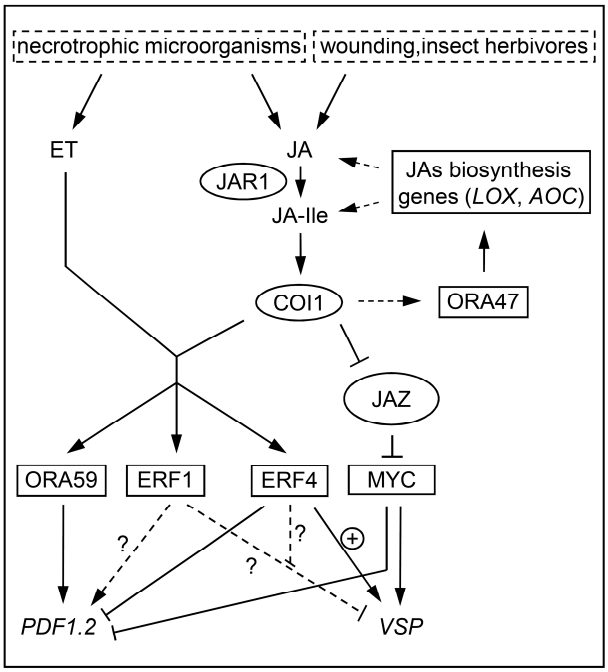

Figure 3. Model for the role of transcription factors in the stress-responsive network involving the JA and ET signaling pathways. Different types of biotic or abiotic stress, including wounding, attack by herbivorous insects and infection with necrotrophic pathogens, induce the synthesis of JA and related 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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