Functional analysis of jasmonate-responsive transcription factors in Arabidopsis thaliana

Zarei, A.

Chapter 1

General introduction

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

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues (Turner et al., 2002; Devoto and Turner, 2003). These signaling molecules affect a variety of plant processes, including wounding and abiotic stresses, and defense against insects (McConn et al., 1997), and necrotrophic pathogens (Thomma et al., 1999). Among developmental processes which are known to be influenced by JAs are root growth, pollen maturation and dehiscence, ovule development and senescence as evidenced by various Arabidopsis mutants in JA biosynthesis and JA signaling (Turner et al., 2002; Wasternack 2006).

Stress-induced JA biosynthesis

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

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

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

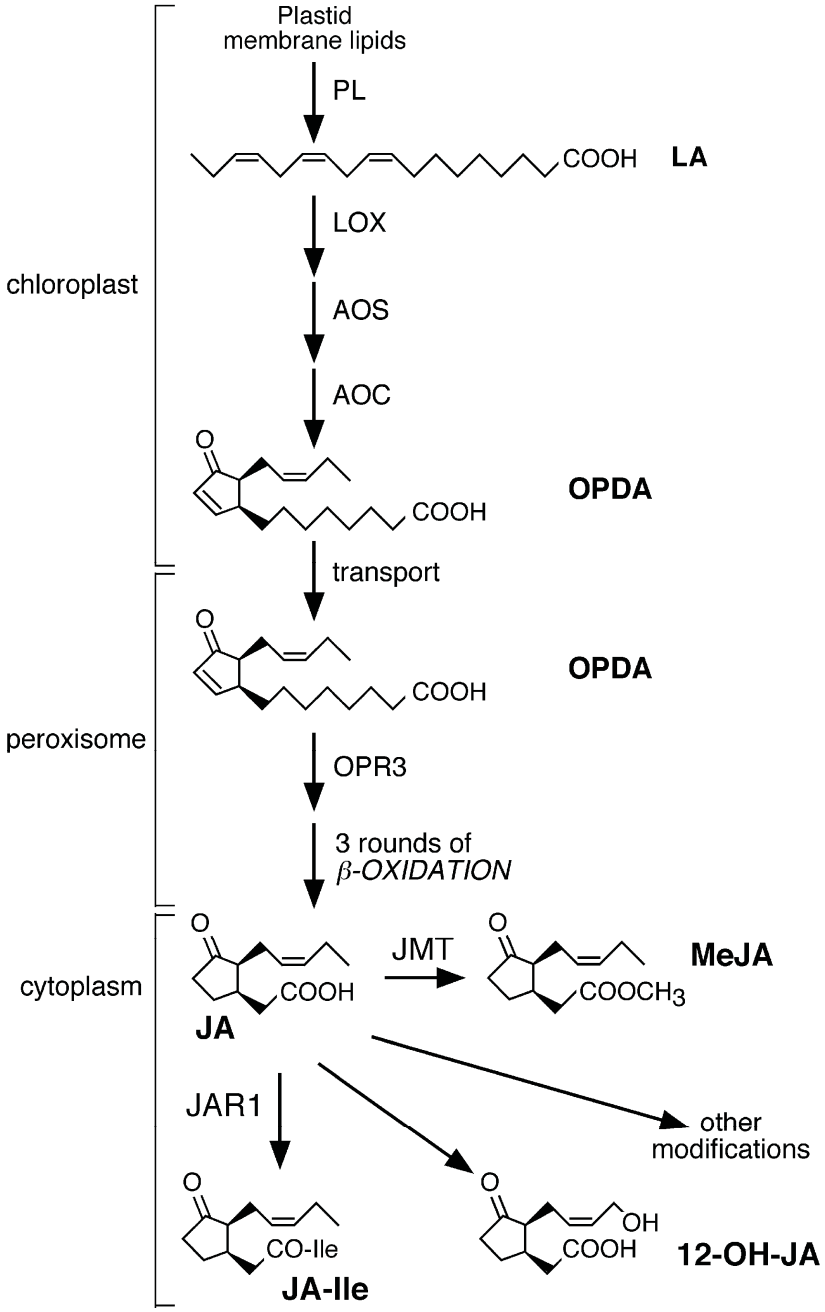

Figure 1. Schematic representation of the octadecanoid pathway leading to jasmonic acid biosynthesis. 12-OH-JA, 12-hydroxy-jasmonic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; JAR1, enzyme responsible for the conjugation of JA with isoleucine (JA-Ile); JMT, *S*-adenosyl-L-methionine:jasmonic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

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

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

JA-responsive promoter elements

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

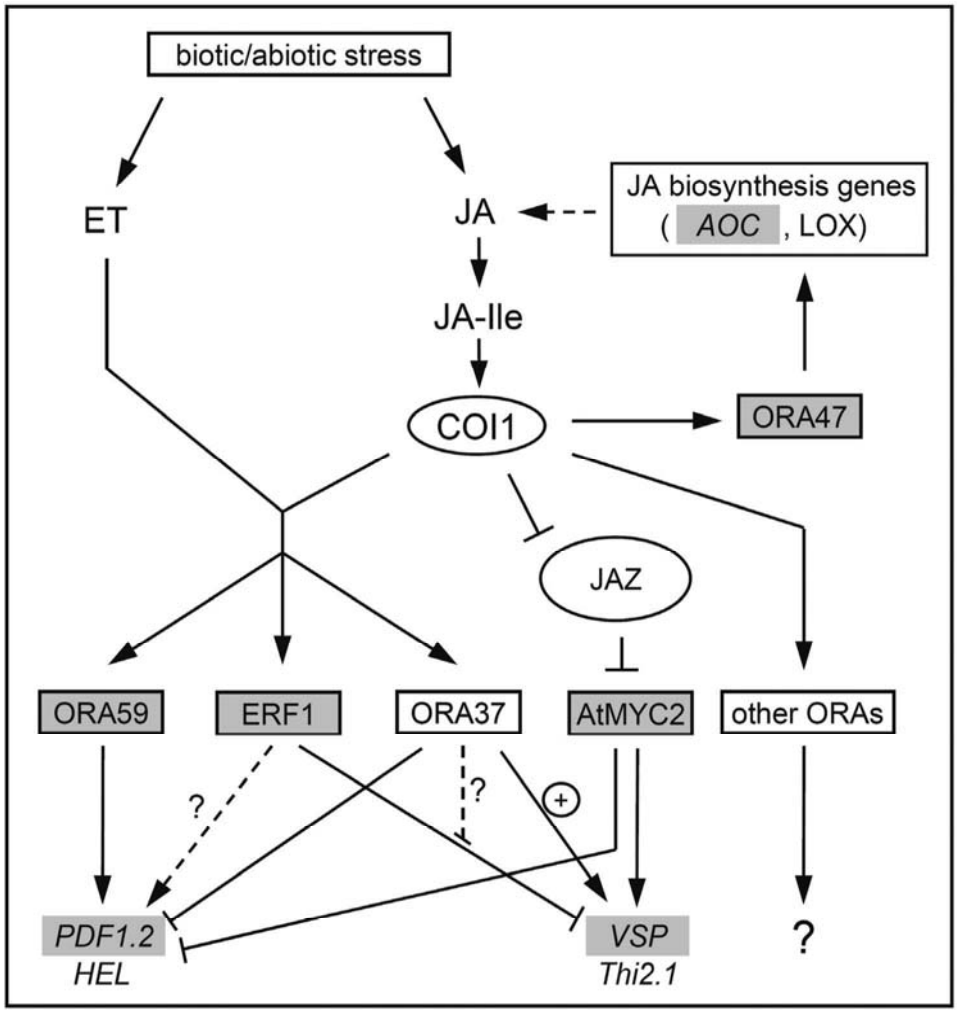

Figure 2. Role of transcription factors in the stress-responsive network involving the JA and ET signaling pathways. Different types of biotic or abiotic stress, including wounding, herbivore attack and infection with necrotrophic pathogens, induce the synthesis of JA and related oxylipins such as the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

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

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

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

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

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

Transcription factors and JA responses

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

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

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

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

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

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

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

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

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

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

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

JA perception and signaling

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

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

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

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

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

A particularly effective screen for JA signaling mutants is described by Lorenzo et al. (2004). Screening for mutants affected in JA-induced root growth inhibition in an *ethylene-insensitive*3 (*ein3*) background resulted in the identification of five complementation groups identifying 5 loci called *JA-insensitive* (*JAI*) 1-5. The *JAI1* locus corresponds to the *AtMYC2* gene (Lorenzo et al., 2004). The *JAI2* locus corresponds to the previously characterized (Staswick et al., 1992; Staswick and Tiryaki, 2004) *JAR1* gene. The *JAI4* locus corresponds to the *SGT1b* gene (Lorenzo and Solano, 2005). The *JAI5* locus corresponds to the *COI1* gene (Lorenzo et al., 2004).

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

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

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

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

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

Outline of the thesis

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

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

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

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

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

Chapter 4 describes studies on the role of the transcription factor ORA47 in the regulation of the *AOC* genes encoding the JA biosynthesis enzyme allene oxide cyclase with emphasis on the highest expressed member *AOC2*. The expression of all four members of the *AOC* gene family was induced by overexpression of ORA47. A GCC-like box in the *AOC2* promoter interacted specifically with ORA47 in vitro and in vivo, and this GCC box is important for ORA47-mediated activity of the *AOC2* promoter in a transient assay. In addition

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

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

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

References

Atallah, M. (2005) Jasmonate-responsive AP2-domain transcription factors in Arabidopsis. PhD thesis. Leiden University, Leiden, The Netherlands.

Austin, M.J., Muskett, P., Kahn, K., Feys, B.J., Jones, J.D.G., and Parker, J.E. (2002). Regulatory role of SGT1 in early R gene-mediated plant defenses. Science **295**, 2077-2080.

Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K., and Schulze-Lefert, P. (2002). The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. Science **295**, 2073-2076.

Bell, E., and Mullet, J.E. (1993). Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol. **103**, 1133-1137.

Benedetti, C.E., Xie, D., and Turner, J.G. (1995). *COI1*-dependent expression of an Arabidopsis vegetative storage protein in flowers and siliques and in response to coronatine or methyl jasmonate. Plant Physiol. **109**, 567-572.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J. **29**, 23-32.

Boter, M., Ruiz-Rivero, O., Abdeen, A., and Prat, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev. **18**, 1577-1591.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J., and Manners, J.M. (2003). A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of Arabidopsis. Plant Physiol. **132**, 1020-1032.

Cheong, Y.H., Chang, H.-S., Gupta, R., Wang, X., Zhu, T., and Luan, S. (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol. **129**, 661-677.

Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J.M., Lorenzo, O., García-Casado, G., López-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature. **448**, 666-671.

Creelman, R.A. and Mullet, J.E. (1997). Biosynthesis and action of jasmonates in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**, 355-381.

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

del Pozo, J.C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W.M., and Estelle, M. (2002). AXR1-ECR1-dependent conjugation of RUB1 to the Arabidopsis cullin AtCUL1 is required for auxin response. Plant Cell **14**, 421-433.

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J.G. (2002). COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. Plant J. **32**, 457-466.

Devoto, A. and Turner, J.G. (2003). Regulation of jasmonate-mediated plant responses in Arabidopsis. Ann. Bot. (Lond) **92**, 329-337.

Dharmasiri N, Dharmasiri S, Estelle M. (2005). The F-box protein TIR1 is an auxin receptor. Nature **435**, 441-445.

Ellis, C., Karafyllidis, I., and Turner, J.G. (2002). Constitutive activation of jasmonate signaling in an Arabidopsis mutant correlates with enhanced resistance to *Erysiphe cichoracearum, Pseudomonas syringae,* and *Myzus persicae*. Mol. Plant Microbe Interact. **15**, 1025-1030.

Feng, S., Ma,L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N., and Deng, X.W. (2003). The COP9 signalosome interacts physically with SCF^{COI1} and modulates jasmonate responses. Plant Cell **15**, 1083-1094.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12**, 393-404.

Gray, W.M., Muskett, P.R., Chuang, H.W., and Parker, J.E. (2003). Arabidopsis SGT1b is required for SCF^{TIR1}-mediated auxin response. Plant Cell **15**, 1310-1319.

Guerineau, F., Benjdia, M., and Zhou, D.X. (2003). A jasmonate-responsive element within the *A. thaliana vsp1* promoter. J. Exp. Bot. **54**, 1153-1162.

Guilfoyle, T. (2007). Sticking with auxin. Nature **446**, 621 - 622.

Guo, H., and Ecker, J.R. (2004). The ethylene signaling pathway: new insights. Curr. Opin. Plant Biol. **7**, 40-49.

He, Y. and Gan, S. (2001). Identical promoter elements are involved in regulation of the *OPR1* gene by senescence and jasmonic acid in Arabidopsis. Plant Mol. Biol. **47**, 595-605.

Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., and Okada, K. (2001). The *DEFECTIVE IN ANTHER DEHISCENCE1* gene encodes a novel phospholipase A1 catalyzing the initial step of

jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell **13**, 2191-2209.

Kepinski, S. and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature **435**, 446-451.

Kim, S.R., Choi, J.L., Costa, M.A., and An, G. (1992). Identification of G-box sequence as an essential element for methyl jasmonate response of potato *proteinase inhibitor II* promoter. Plant Physiol. **99**, 627-631.

Kim, S.R., Kim, Y., and An, G. (1993). Identification of methyl jasmonate and salicylic acid response elements from the *nopaline synthase (nos)* promoter. Plant Physiol. **103**, 97-103.

Kim, Y., Buckley, K., Costa, M.A., and An, G. (1994). A 20 nucleotide upstream element is essential for the *nopaline synthase (nos)* promoter activity. Plant Mol.Biol. **24**, 105-117.

Kunkel, B.N. and Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. **5**, 325-331.

Laudert, D. and Weiler, E.W. (1998). Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signalling. Plant J. **15**, 675-684.

Li, L., Zhao, Y., McCaig, B.C., Wingerd, B.A., Wang, J., Whalon, M.E., Pichersky, E., and Howe, G.A. (2004b). The tomato homolog of *CORONATINE-INSENSITIVE1* is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16**, 126-143.

Liu, Y., Schiff, M., Serino, G., Deng, X.W., and Dinesh-Kumar, S.P. (2002). Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to tobacco mosaic virus. Plant Cell **14**, 1483-1496.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell **16**, 1938-1950.

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

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

Lusser, A., Kolle, D., and Loidl, P. (2001). Histone acetylation: lessons from the plant kingdom. Trends in Plant Science **6**, 59-65.

Mahalingam, R., Gomez-Buitrago, A., Eckardt, N., Shah, N., Guevara-Garcia, A., Day, P., Raina, R., and Fedoroff, N.V. (2003). Characterizing the stress/defense transcriptome of Arabidopsis. Genome Biol. **4**, R20.

Mandaokar, A., Thines, B., Shin, B., Lange, B.M., Choi, G., Koo, Y.J., Yoo, Y.J., Choi, Y.D., Choi, G., and Browse, J. (2006). Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. Plant J. **46**, 984-1008.

Mason, H.S., DeWald, D.B., and Mullet, J.E. (1993). Identification of a methyl jasmonate-responsive domain in the soybean *vspB* promoter. Plant Cell **5**, 241-251.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., and Browse, J. (1997). Jasmonate is essential for insect defense in Arabidopsis. Proc. Natl. Acad. Sci. USA **94**, 5473-5477.

McGrath, K.C., Dombrecht, B., Manners, J.M., Schenk, P.M., Edgar, C.I., Maclean, D.J., Scheible, W.R., Udvardi, M.K., and Kazan, K. (2005). Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. Plant Physiol. **139**, 949-959.

Menke, F.L.H., Champion, A., Kijne, J.W., and Memelink, J. (1999). A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J. **18**, 4455-4463.

Mikkelsen, M.D., Hansen, C.H., Wittstock, U., and Halkier, B.A. (2000). Cytochrome P450CYP79B2 from Arabidopsis catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. J. Biol. Chem. **275**, 33712-33717.

Mussig, C., Biesgen, C., Lisso, J., Uwer, U., Weiler, E.W., and Altmann, T. (2000). A novel stress-inducible 12-oxophytodienoate reductase from *Arabidopsis thaliana* provides a potential link between brassinosteroid action and jasmonic acid synthesis. J. Plant Physiol. **157**, 143-152.

Norman-Setterblad, C., Vidal, S., and Palva, E.T. (2000). Interacting signal pathways control defense gene expression in Arabidopsis in response to cell wall-degrading enzymes from *Erwinia carotovora*. Mol.Plant Microbe Interact. **13**, 430-438.

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

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

Penninckx, I.A.M.A., Thomma, B.P.H.J., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10**, 2103-2113.

Pieterse, C. M. J., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P. J., and van Loon, L. C. (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell **10**, 1571-1580.

Pieterse, C. M. J., van Pelt, J. A., Ton, J., Parchmann, S., Mueller, M.J., Buchala, A.J., Métraux, J.-P., and van Loon, L.C. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol. Mol. Plant Pathol. **57**, 123-134.

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

Rojo, E., Leon, J., and Sanchez-Serrano, J.J. (1999). Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. Plant J. **20**, 135-142.

Rouster, J., Leah, R., Mundy, J., and Cameron-Mills, V. (1997). Identification of a methyl jasmonate-responsive region in the promoter of a *lipoxygenase 1* gene expressed in barley grain. Plant J. **11**, 513-523.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, K.-I., Ohta, H., and Tabata, S. (2001). Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: self-

activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. DNA Res. **8**, 153-161.

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

Schaller, F., Schaller, A., and Stintzi, A. (2005). Biosynthesis and metabolism of jasmonates. J. Plant Growth Regul. **23**, 179-199.

Schwechheimer, C., Serino, G., and Deng, X.W. (2002). Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. Plant Cell **14**, 2553-2563.

Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., and Choi, Y.D. (2001). Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. USA **98**, 4788-4793.

Shah, J. (2003). The salicylic acid loop in plant defense. Curr. Opin. Plant Biol. **6**, 365-371.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. **12**, 3703-3714.

Staswick, P.E., and Tiryaki, I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell **16**, 2117-2127.

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

Takeda, S., Sugimoto, K., Otsuki, H., and Hirochika, H. (1998). A 13-bp *cis*-regulatory element in the LTR promoter of the tobacco retrotransposon *Tto*1 is involved in responsiveness to tissue culture, wounding, methyl jasmonate and fungal elicitors. Plant J. **18**, 383-393.

Tan, X., Calderon-Villalobos, L.I., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M., and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature **446**, 640-645.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature. **448**, 661-665.

Thomma, B.P.H.J., Nelissen, I., Eggermont, K., and Broekaert, W.F. (1999). Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J. **19**, 163-171.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA **95**, 15107-15111.

Tiryaki,I. and Staswick,P.E. (2002). An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signaling mutant *axr1*. Plant Physiol. **130**, 887-894.

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

Vanholme, B., Grunewald, W., Bateman, A., Kohchi, T., Gheysen, G. (2007). The tify family previously known as ZIM. Trends Plant Sci. **12**, 239-244.

van der Fits, L. and Memelink, J. (2000). ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science **289**, 295-297.

Vick, B., and Zimmerman, D.C. (1984). Biosynthesis of jasmonic acid by several plant species. Plant Physiol. **75,** 458-461.

Vijayan, P., Shockey, J., Lévesque, C.A., Cook, R.J., and Browse, J. (1998). A role for jasmonate in pathogen defense of *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95**, 7209-7214.

Vom Endt, D., Soares e Silva, M., Kijne, J.W., Pasquali, G., and Memelink, J. (2007). Identification of a bipartite jasmonate-responsive promoter element in the *Catharanthus roseus ORCA3* transcription factor gene that interacts specifically with AT-hook DNA-binding proteins. Plant Physiol. **144**, 1680-1689.

Wang, K.L., Li, H., and Ecker, J.R. (2002). Ethylene biosynthesis and signaling networks. Plant Cell **14** (suppl.), S131-S151.

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

Xiang, C., Miao, Z.H., and Lam, E. (1996). Coordinated activation of *as-1*-type elements and a tobacco *glutathione S-transferase* gene by auxins, salicylic acid, methyl-jasmonate and hydrogen peroxide. Plant Mol.Biol. **32**, 415-426.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). *COI1*: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280**, 1091-1094.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF^{COI1} ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell **14**, 1919-1935.

Zhou, C., Zhang, L., Duan, J., Miki, B., and Wu, K. (2005). HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. Plant Cell **17**, 1196-1204.