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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, plant fitness and survival are dependent on 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; Memelink et al., 2001; Turner et al., 2002), ethylene (ET; Wang et al.,. 2002; Guo and Ecker, 2004) and salicylic acid (SA; Shah et al., 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).

Stress-induced JA biosynthesis

JA has been shown to protect plants against mechanical or herbivorous insect-inflicted wounding (McConn et al., 1997), pathogens (Dong, 1998; Penninckx et al., 1998; Thomma et al., 1998; Vijayan et al., 1998), osmotic stress (Kramell et al., 1995) and ozone (Rao et al., 2000). Endogenous JA levels increase in response to these external 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 occurs after root colonization of the host plant by certain strains of non-pathogenic *Pseudomonas* species prior to infection with a pathogen (Pieterse et al., 1998; 2000).

In addition to its role in plant defense, JA is also involved in several aspects of plant development, including tendril coiling and pollen and seed development (Creelman and Mulpuri, 2002). Involvement of JA in pollen development was discovered by the observation that the JA-deficient *fad* and *coi1* mutants are male sterile (Feys et al., 1994; McConn and Browse, 1996). Although these mutants are not affected in root development, exogenous application of JA to wild-type Arabidopsis plants results in reduced root growth (Staswick et al., 1992).

JA and related compounds, collectively called jasmonates, are linolenic acid (18:3)-derived cyclopentanone based-compounds of wide distribution in the plant kingdom, which 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 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).

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;

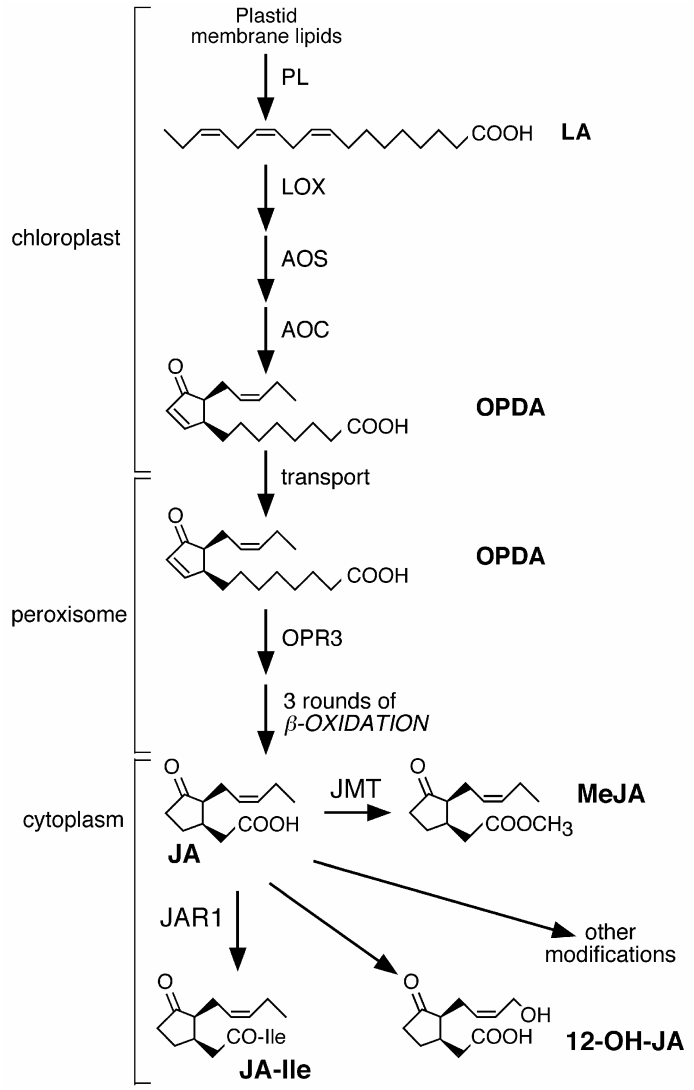

Figure 1. Schematic representation of the octadecanoid pathway leading to jasmonic acid biosynthesis. 12-OH-JA, 12-hydroxy-jasmonic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; JAR1, enzyme responsible for the conjugation of JA with isoleucine (JA-Ile); JMT, *S*-adenosyl-L-methionine:jasmonic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

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).

At present, only WIPK, a mitogen-activated protein kinase from tobacco, and CEV1, a cellulose synthetase protein from Arabidopsis, have been characterized as putative upstream regulatory components of JA production (Figure 2). JA accumulates in wounded tobacco plants, but does not accumulate in wounded *WIPK*-impaired transgenic plants (Seo et al., 1995), indicating that WIPK is a positive regulator of wound-induced JA biosynthesis. The Arabidopsis *cev1* mutant shows constitutive production of JA and ethylene and constitutive expression of JA-responsive defense-related genes (Ellis and Turner, 2001; Ellis et al., 2002). The cell wall-related CEV1 protein is thought to act as a negative regulator of stress perception or signal transduction, upstream of JA production. The *cet1* mutant also exhibits constitutively elevated levels of JA and constitutive expression of the defense-related gene *THIONIN* (Hilpert et al., 2001), indicating that the protein encoded by the *CET1* gene is likely to function as a negative regulator of JA biosynthesis. The gene affected by the *cet1* mutation remains to be cloned. In *Catharanthus roseus* cell suspensions, elicitor-induced JA biosynthesis depends on an increase in cytoplasmic Ca^{2+} concentration and protein phosphorylation (Memelink et al., 2001).

JA perception

Transduction of the JA signal is thought to occur via activation of receptors that bind JA; however, receptors have thus far not been identified, nor is it known where in the cell they would be localized. In order to identify molecular components of JA signal transduction, a large number of mutant screens for insensitivity to (Me)JA and to coronatine (a bacterial toxin which is a structural analog of MeJA) and for constitutive JA responses have been performed (Lorenzo and Solano, 2005). Several mutants were characterized but none of the cloned genes affected by the mutation encodes a protein which is an obvious candidate for a JA receptor. It is assumed, therefore, that there is redundancy among multiple functionally similar JA receptors. The *coi1* mutant was isolated in a screen for Arabidopsis mutants insensitive to growth inhibition by coronatine and was also shown to be insensitive to MeJA (Feys et al., 1994). The JA-insensitive *coi1* mutant is defective in resistance to certain insects and pathogens and fails to express JA-regulated genes (McConn et al., 1997; Thomma et al., 1998; Benedetti et al., 1995). The *COI1* gene encodes an F-box protein (Xie et al., 1998). F-box proteins associate with cullin and Skp1 proteins to form an E3 ubiquitin ligase known as the SCF complex (Bai et al., 1996), where the F-box subunit functions as a specific receptor targeting proteins for ubiquitin-mediated proteolysis by the 26S proteasome.

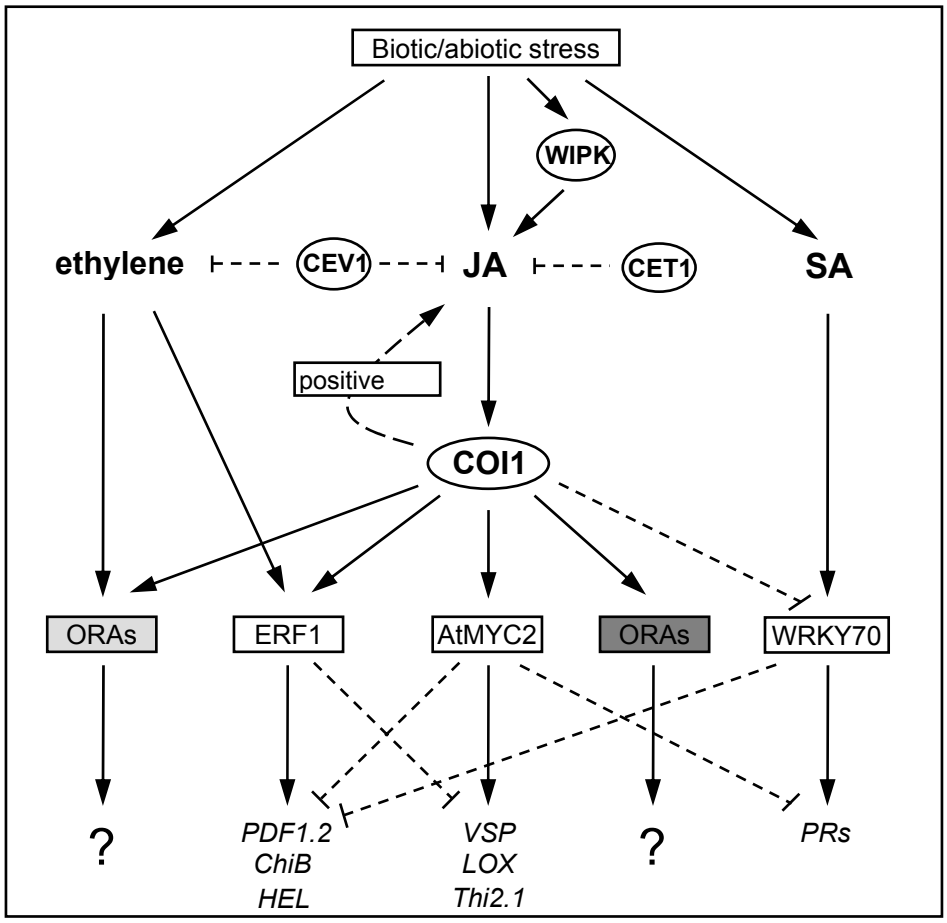

Figure 2. Stress-responsive network connecting the jasmonic acid (JA), ethylene and salicylic acid (SA) signaling pathways in Arabidopsis. Regulatory proteins are circled. Transcription factors are boxed. Arrows indicate induction of gene expression or positive interaction. Dashed lines indicate repression of gene expression or negative interaction. Dashed arrow represents the auto-stimulatory loop in JA production.

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 of 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 (Tiryaki and Staswick, 2002; Feng et al., 2003; Lorenzo and Solano, 2005), further supporting the requirement for protein degradation in JA signal transduction. Given the fact that the *coi1* mutation is recessive, the widely accepted model is that COI1 targets one or more repressors of JA responses for degradation. The histone deacetylase

RPD3b has been identified as a potential substrate for COI1-mediated ubiquitination (Devoto et al., 2002). Histone deacetylation represses transcription by decreasing the accessibility of chromatin to the transcription machinery (Alberts et al., 2002). Therefore, it is possible that the COI1-interacting RPD3b suppresses the transcription of JA-responsive genes under normal conditions. Upon JA signaling, RPD3b may be degraded via recruitment by the SCF^{COI1} complex, allowing the expression of the JA-responsive genes. However, this hypothesis remains to be proven, and the current status is that the mechanisms underlying the role of COI1 in the regulation of JA responses remain to be elucidated (Pauw and Memelink, 2005).

JA responses

JA is the physiological signal for several wound- and pathogen-induced responses in plants (Turner et al., 2002). Stress-induced biosynthesis of JA is a signal for a cascade of complex responses leading to the production of defense proteins and compounds. The role of JA in defense was first shown by Farmer et al. (Farmer and Ryan, 1990; Farmer et al., 1992) who demonstrated that JA and MeJA induce proteinase inhibitors, which form part of the defense response against herbivorous insects. Exogenous application of (Me)JA results in major reprogramming of gene expression including induction of genes that are known to be involved in plant stress responses, as revealed by macro- and micro-array analyses (Schenk et al., 2000; Sasaki et al., 2001; Reymond et al., 2004). In Arabidopsis, JA increases among others the transcript levels of genes encoding the vacuolar vegetative storage proteins (VSPs) with anti-insect phosphatase activity (Benedetti et al., 1995; Liu et al., 2005), the antimicrobial proteins plant defensin 1.2 (PDF1.2; Penninckx et al., 1996) and thionin 2.1 (Thi2.1; Epple et al., 1995; Figure 2). Furthermore, JA induces the expression of biosynthesis genes leading to the accumulation of antimicrobial secondary metabolites, including alkaloids, terpenoids, flavonoids, anthraquinones and glucosinolates in different plant species (Memelink et al., 2001; Blechert et al., 1995). How JA activates the expression of specific genes is largely unknown.

The expression of a gene is determined by the sequence-specific binding of a *trans*-acting factor (commonly called transcription factor) to a *cis*-acting DNA element located in the vicinity of the gene. 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. Several *cis*-acting elements responsible for JA-induced expression have been identified in a number of JA-responsive genes (Pauw and Memelink, 2005).

AP2/ERF transcription factors and JA responses

In *Catharanthus roseus*, expression of the terpenoid indole alkaloid (TIA) biosynthesis gene *STR* by MeJA is controlled by a jasmonate- and elicitor-responsive element (JERE) located in the *STR* promoter region (Menke et al., 1999). Two transcription factors, called ORCA2 and ORCA3, positively regulate the expression of the *STR* gene via specific binding to a GCC-box-like core sequence in the JERE (Menke et al., 1999; van der Fits and Memelink, 2001). Proteins that specifically bind to the GCC-box were initially discovered in tobacco and were called ethylene-responsive element binding factors (EREBPs; now known as ethylene-responsive factors or ERFs), because the GCC-box was previously identified as an ethylene-responsive element (Ohme-Takagi and Shinshi, 1995). ERF transcription factors are characterized by a highly conserved 58- to 60-amino acid DNA-binding domain called AP2/ERF-domain (Atallah, 2005; Hao et al., 1998; Riechmann et al., 2000). However, it turned out that the role of transcription factors from the ERF family is not limited to ethylene signaling. Nonetheless, proteins from the ERF family are still referred to as AP2/ERF-domain proteins for their homology to the firstly identified tobacco ERF factors rather than for their role in regulating ethylene responses. AP2/ERF-domain proteins have been studied in several plant species, where they were found to play important roles in plant responses to various hormones and environmental cues, including dehydration, salt and cold stress (Stockinger et al., 1997; Liu et al., 1998; Fujimoto et al., 2000; Park et al., 2001), abscisic acid (Finkelstein et al., 1998), ethylene (Büttner and Singh, 1997; Solano et al., 1998; Fujimoto et al., 2000) and pathogen infection (Zhou et al., 1997; Solano et al., 1998; Maleck et al., 2000; Schenk et al., 2000; Park et al., 2001). Several other members of the AP2/ERF-domain family, including TINY (Wilson et al., 1996) and LEAFY PETIOLE (LEP; van der Graaff et al., 2000) are involved in development.

The *C. roseus* ORCA2 and ORCA3 proteins belong to the AP2/ERF-domain family of transcription factors and expression of the *ORCA* genes is rapidly induced by MeJA (Menke et al., 1999; van der Fits and Memelink, 2001). Overexpression of *ORCA3* in transgenic *C. roseus* suspension cells induced several genes encoding enzymes involved in primary and secondary metabolism leading to TIA biosynthesis, including *STR* (van der Fits and Memelink, 2000). This demonstrates that the jasmonate-induced expression of the *STR* gene is controlled by the JA-responsive AP2/ERF-domain transcription factors ORCA2 and ORCA3. This was the first evidence for a link between JA signaling and members of the AP2/ERF-domain family.

Based on the observation that the *C. roseus* AP2/ERF-domain transcription factors involved in regulating JA-responsive genes are themselves transcriptionally regulated by JA, Atallah (2005) identified 14 genes, encoding AP2/ERF-domain transcription factors from Arabidopsis,

whose expression was induced by JA. These so-called *Octadecanoid-Responsive Arabidopsis AP2/ERF-domain* (*ORA*) genes were rapidly induced in young seedlings exposed to JA in a COI1-dependent manner (Atallah, 2005). It was therefore speculated that, as for the ORCAs in *C. roseus*, ORAs play a major role in JA-responsive gene expression in Arabidopsis. Differences in expression kinetics in response to JA between certain *ORA* genes suggested that the JA signal triggers different mechanisms for regulating expression of the *ORA* genes (Atallah, 2005). This also suggested that ORA proteins might have different functions in JA signaling. However, at the start of the studies described in this thesis the functions and target genes of the ORAs were unknown.

Recently, expression of the AP2/ERF-domain transcription factor *ERF1* was shown to be induced by JA or ethylene and to be synergistically induced by both hormones (Figure 2; Lorenzo et al., 2003). Overexpression of *ERF1* results in increased expression of several genes that are induced synergistically by JA and ethylene, including the defense genes *PDF1.2* and *basic chitinase* (Figure 2; Lorenzo et al., 2003). In addition, the expression levels of five other Arabidopsis genes encoding AP2/ERF-domain transcription factors, *AtERF2* (also referred to as *ORA2*; Atallah, 2005), *AtERF3*, *AtERF4*, *AtERF13* and *RAP2.10*, were also increased by MeJA treatment (Oñate-Sánchez and Singh, 2002; Brown et al., 2003). Overexpression of *AtERF1* (Atallah, 2005; also referred to as *ORA1*) and *AtERF2* (Atallah, 2005; Brown et al., 2003) upregulates *PDF1.2* and *basic chitinase* gene expression. Therefore, it appears that JA controls the expression of defense genes by regulating transcription factor abundance via adjustment of the production of the encoding mRNA. Additionally, it can be envisaged that JA can modulate gene expression by controlling transcription factor activity, stability or sub-cellular localization (Vom Endt et al., 2002; Pauw and Memelink, 2005).

JA and related oxylipins

The vigorous production of oxygenated fatty acids (called oxylipins) is a characteristic response to pathogenesis and herbivory (Creelman and Mulpuri, 2002; Howe and Schilmiller, 2002). Plant oxylipins constitute a group of bioactive fatty acid derivatives that perform several important roles in growth and development. Oxylipins from the jasmonate family are generated via the octadecanoid pathway (Figure 1) and are characterized by a pentacyclic ring structure. Until recently, jasmonic acid and its volatile methyl ester (MeJA) were considered as the main important signaling molecules in plant development and adaptation to environmental stress that involve oxylipin-dependent responses. However, biological activity is not limited to JA and MeJA, but extends to many JA metabolites and conjugates as well as

JA precursors (Kramell et al., 1997; Stintzi et al., 2001). The intrinsic biological function of these JA-related compounds may even differ between related molecules.

Studies with the Arabidopsis *opr3* mutant, in which JA synthesis is blocked downstream of OPDA formation (Figure 1), indicate that OPDA is active as a defense signal against insect and fungal attack without conversion to JA (Stintzi et al., 2001). The JA-deficient *fad* triple mutant and the JA-insensitive *coi1* mutant are both susceptible to challenge with the insect *Bradysia impatiens* (McConn et al., 1997; Stintzi et al., 2001) and with the fungus *Alternaria brassicicola* (Stintzi et al., 2001; Thomma et al., 1998). In contrast, the *opr3* mutant was fully resistant to *B. impatiens* and *A. brassicicola* (Stintzi et al., 2001), demonstrating that, in the absence of JA, OPDA acts as a bioactive signal molecule in the resistance response against insects and fungi. Conversely, as for the *fad* triple mutant, the *opr3* mutant (also referred as *dde1*) is male sterile, and male fertility can be restored by application of JA (Sanders et al., 2000; Stintzi and Browse, 2000), demonstrating the critical requirement of jasmonic acid for pollen development. Structure-activity studies have shown that exogenous OPDA is more potent than JA in its ability to activate the tendril coiling response of *Bryonia dioica* to mechanical stimulation (Weiler et al., 1993; Blechert et al., 1999). OPDA is also more effective than JA in eliciting the synthesis of certain diterpenoid volatiles in lima bean (*Phaseolus lunatus*) (Koch et al., 1999) as well as the accumulation of glyceollin phytoalexins in soybean (*Glycine max*) (Fliegmann et al., 2003). This suggests that different processes may be controlled by different oxylipins *in vivo*.

The *jar1* mutant exhibited decreased sensitivity to exogenous JA and reduced resistance against several pathogens (Staswick et al., 1992, 1998, 2002; van Loon et al., 1998; Clarke et al., 2000). In contrast to the male sterile JA-insensitive *coi1* mutant, *jar1* plants are fully fertile, indicating that some but not all of the JA responses are affected in this particular mutant (Staswick et al., 2002). *JAR1* encodes an enzyme responsible for the synthesis of JA-amino acid conjugates, preferentially JA-Isoleucine (Figure 1). Thus, JAR1-mediated conjugation of JA is likely to be needed for some, but not all, JA responses. Overexpression of the *JMT* gene, which encodes the enzyme that methylates JA to methyl jasmonate (MeJA; Figure 1), increases resistance to *Botrytis cinerea* (Seo et al., 2001), suggesting that MeJA induces pathogen defense responses more efficiently than JA.

The activity of JA and JA-related oxylipins as signals for defense suggests that host responses to attackers may be regulated by a complex mix of signals, which has been termed the “oxylipin signature” (Weber et al., 1997). Such a modular action of a mix of different signaling molecules is thought to allow the plants to fine-tune the response to diverse environmental factors in a specific manner.

Cross-talk with other signaling molecules

In addition to the production of different JA-related signals, plants mount an appropriately adapted defense response against a specific stress by producing other signaling molecules, including ethylene, salicylic acid (SA) and abscisic acid. Recent evidence indicates that the corresponding signal transduction pathways are not separate linear pathways, but that they are integrated through a network of cross-talk connections that appear to co-ordinate the response output. Depending on the nature of the external stimuli, the JA, ethylene and salicylic acid pathways can act synergistically or antagonistically (Kunkel and Brooks, 2002).

SA plays a central role in both local defense responses, including hypersensitive cell death, and distant responses, including systemic acquired resistance (SAR; Ryals et al., 1996). SA levels increase in plant tissue following pathogen infection, and exogenous application of SA results in enhanced resistance to a broad range of pathogens (Ryals et al., 1996). SA induces the expression of a large number of defense genes, including *pathogenesis-related* (*PR*) genes. SA and JA signaling pathways can act synergistically or antagonistically during the activation of gene expression. However, the primary mode of interaction between these pathways appears to be mutual antagonism (Rojo et al., 2003; Kunkel and Brooks, 2002). The WRKY70 transcription factor was shown to be an important node of divergence between the JA and SA signaling pathways during plant defense responses (Li et al., 2004). Expression of *WRKY70* is induced by SA, and repressed by JA. Constitutive overexpression of *WRKY70* increases resistance to virulent pathogens and results in constitutive expression of SA-induced *pathogenesis-related* (*PR*) genes (Figure 2; Li et al., 2004). Conversely, expression of several JA-responsive genes was enhanced in transgenic plants with antisense suppression of *WRKY70*, suggesting that WRKY70 acts as an activator of SA-induced genes and as a repressor of JA-responsive genes (Figure 2).

The gaseous molecule ethylene affects many aspects of the plant life cycle, including seed germination, abscission and fruit ripening (Guo and Ecker, 2004). The role of ethylene in plant defense seems to depend on the type of pathogen and plant species. In some cases, ethylene seems to inhibit symptom development (Norman-Setterblad et al., 2000; Thomma et al., 1999; Knoester et al., 1998; Verberne et al., 2003), whereas it enhances disease progression in others (Lund et al., 1998; Hoffman et al., 1999). Several studies provide evidence for positive interactions between the JA and ethylene signaling pathways (Kunkel and Brooks, 2002). Arabidopsis plants impaired in JA (i.e. the *coi1* mutant) or ethylene (i.e. the *ein2* mutant; Guzman and Ecker, 1990) signaling pathways show enhanced susceptibility to the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* (Thomma et al., 1998 and 1999; Penninckx et al., 1996), demonstrating that JA and ethylene are important signal

molecules for resistance towards these pathogens. Expression of several defense genes, including the *PDF1.2*, *Hevein-like* (*HEL*) and *basic chitinase* (*ChiB*) genes, is induced by JA and ethylene and a combination of both hormones has a synergistic effect on gene expression (Figure 2; Norman-Setterblad et al., 2000; Penninckx et al., 1998). Both JA and ethylene signaling pathways are required for *PDF1.2* gene expression in response to any of the two hormones (Penninckx et al., 1998), indicating that JA and ethylene coordinately regulate defense-related gene expression. This demonstrates that signal transduction initiated by each hormone depends on components that are crucial for both pathways. In Arabidopsis, expression of the AP2/ERF-domain transcription factor *ERF1* is induced by JA or ethylene and a combination of both hormones has a synergistic effect on *ERF1* expression (Solano et al., 1998; Lorenzo et al., 2003). Constitutive overexpression of *ERF1* activates the expression of several defense-related genes, including *PDF1.2* and *ChiB* (Figure 2; Solano et al., 1998; Lorenzo et al., 2003), and was shown to confer resistance to several fungi, including *B. cinerea* (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). Therefore, ERF1 was suggested to act as an integrator of JA and ethylene signaling pathways in the activation of plant defenses (Lorenzo et al., 2003). Similarly, expression of *ORA31*, *ORA37*, *ORA44*, *ORA59* and *ORA68* genes were super-induced by a combined treatment with JA and ethylene (Atallah, 2005), indicating that positive cross-talk between the JA and ethylene signaling pathways occurs at the level of multiple AP2/ERF-domain transcription factors. These results suggest that these 5 ORAs integrate inputs from the JA and ethylene signaling pathways, together with the previously identified ERF1 transcription factor (Lorenzo et al., 2003).

In addition to positive interactions between the JA and ethylene signaling pathways, several studies provide evidence for negative cross-talk between JA and ethylene. The ethylene 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). The JA-induced expression of the *VSP1* and *CYP79B2* genes was reduced in plants treated with a combination of ethephon and JA compared to plants treated with JA alone. Moreover, *VSP1* expression is increased in ethylene-insensitive mutants (Rojo et al., 1999; Norman-Setterblad et al., 2000). This indicates that the ethylene signal pathway has a negative effect on a branch of the JA signaling pathway involved in the wound response. Expression of a group of JA-responsive genes, including *VSP2*, was suggested to be controlled by AtMYC2, a transcription factor from the basic helix-loop-helix-leucine zipper family (Figure 2; Lorenzo et al., 2004). In a*tmyc2* mutant plants, application of JA fails to induce *VSP2* expression. In contrast, expression of a distinct group of JA-responsive genes, including *PDF1.2*, was increased in JA-treated *atmyc2* mutant plants compared to JA-treated

wild-type plants (Lorenzo et al., 2004), indicating that AtMYC2 plays a dual role in differentially regulating two branches in the JA pathway (Figure 2). Interestingly, *VSP2* expression in response to JA was largely prevented in plants overexpressing the *ERF1* gene (Lorenzo et al., 2004). These results indicate the existence of mutual antagonism between AtMYC2 and ERF1 and suggest that the negative effect of ethylene on a branch of the JA signaling pathway is executed through ERF1 (Figure 2).

Outline of the thesis

Jasmonic acid is a plant signaling molecule that plays an important role in defense against certain pathogens and insects. JA induces the expression of a battery of genes encoding defense-related proteins and enzymes involved in biosynthesis of protective secondary metabolites. Little is known about the mode of action of JA on the regulation of gene expression. The characterization of the transcription factors regulating JA-responsive genes is of major importance to understand the mechanisms whereby JA signaling occurs. Moreover, it appears that cross-talk between signaling molecules converges at the level of transcription factors, which subsequently control the final expression output of a subset of defense genes. Therefore, identification and characterization of the transcription factors involved in JA signaling pathway contributes to unraveling the complex network of signal transduction leading to fine-tuning of the defense response.

In *C. roseus*, JA-responsive gene expression is regulated by ORCA transcription factors, which belong to the class of AP2/ERF-domain transcription factors (Menke et al., 1999b; van der Fits and Memelink, 2000; 2001). The expression of the *ORCA* genes themselves is JA-responsive. Based on these observations, Atallah (2005) postulated the following hypothesis: JA-responsive gene expression in Arabidopsis is also mediated by members of the AP2/ERF-domain transcription factor family, and the corresponding genes are also expressed in a JA-responsive manner. Atallah (2005) identified early on 10 JA-responsive genes encoding AP2/ERF-domain transcription factors (named ORA transcription factors), followed later on by the discovery of 4 additional members.

At the start of the work described in this thesis, it was completely unclear (i) which genes in Arabidopsis are regulated by which ORA transcription factors (Figure 2), (ii) what the function is of the different JA-responsive ORAs, (iii) whether there is functional redundancy among ORAs, and (iv) which roles, if any, the ORA proteins play in positive and negative crosstalk between JA and ethylene signaling. The goal of this thesis work was to characterize the function of these first 10 ORAs by identifying ORA-regulated genes and to establish their possible roles as nodal points in the JA signal transduction pathway.

In **Chapter 2**, the role of the AP2/ERF-domain transcription factor ORA59 is described. ORA59 is shown to regulate the expression of a large number of JA- and ethylene-responsive genes. ORA59 overexpression confers resistance against *Botrytis cinerea*, whereas ORA59 loss-of-function enhances susceptibility. The loss-of-function approach also demonstrates that ORA59, rather than the previously suggested ERF1, acts as the molecular integrator of the concomitant action of JA and ethylene in defense responses.

The role of the transcription factor ORA47 is described in **Chapter 3**. Inducible overexpression of the *ORA47* gene in Arabidopsis plants resulted in induced expression of multiple JA biosynthesis genes and in an increased level of the JA-precursor OPDA. The results show that ORA47 controls OPDA biosynthesis via regulation of the JA biosynthesis genes. As a result of OPDA biosynthesis, several JA-responsive genes are upregulated in *ORA47*-overexpressing plants. ORA47 appears to act as the regulator of the auto-stimulatory loop in oxylipin biosynthesis.

Chapter 4 describes the function of ORA37, which is distinct from the other ORAs in that it has the EAR motif, which is a well characterized transcriptional repressor domain (Otha et al., 2001). ORA37 is therefore predicted to be a transcriptional repressor. Atallah (2005) demonstrated that the expression of the *ORA37* gene is synergistically induced by a combination of JA and ethylene. The results in this chapter show that ORA37 negatively regulates a subset of JA- and ethylene-responsive genes. In addition, overexpression of the *ORA37* gene enhances the JA-induced expression of another set of genes, indicating that ORA37 acts differentially on separate branches of the JA response.

In **Chapter 5**, the utility of the estradiol-inducible XVE system for functional analysis of the ORA proteins is explored. The results for ORA59, ORA47 and ORA37 are presented in Chapters 2-4, respectively, and the results for the other ORAs are presented in this chapter. Inducible overexpression of several *ORA* genes did not result in increased expression of the JA-responsive genes tested. Therefore, a possible function of these ORAs in JA-responsive gene expression could not be demonstrated (yet). The results also show that certain ORAs, such as ORA33 and ORA59, regulate a similar subset of JA-responsive genes, whereas other subsets of JA-responsive genes are exclusively regulated by a specific ORA transcription factor. This indicates that some ORA transcription factors have distinct, but some others have overlapping functions in JA signaling. Finally, in **Chapter 6** a summarizing general discussion is presented.

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Chapter 2

The Arabidopsis AP2/ERF-domain transcription factor ORA59, and not ERF1, integrates jasmonate and ethylene signal inputs in plant defense

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Submitted

Abstract

Plant defense against pathogen attack depends on the action of several endogenously produced secondary signaling molecules, including jasmonic acid (JA), ethylene and salicylic acid. In certain defense responses ethylene and jasmonate signaling pathways synergize to activate specific sets of defense genes. Here we describe the role of the Arabidopsis AP2/ERF-domain transcription factor ORA59 in ethylene and jasmonate signaling. *ORA59* (*At1g06160*) gene expression was induced by JA or ethylene, and synergistically induced by a combination of both hormones. Such induced expression required both JA and ethylene signaling pathways simultaneously. Overexpression of *ORA59* activated the expression of several JA- and ethylene-responsive defense-related genes, including the plant defensin gene *PDF1.2*, and caused increased resistance against the necrotrophic fungus *Botrytis cinerea.* In *ORA59*-silenced plants, expression of *PDF1.2* and other defense-related genes was blocked in response to JA and/or ethylene, or after infection with *B. cinerea* or *Alternaria brassicicola*. Moreover, these plants were also more susceptible to *B. cinerea* infection. Several AP2/ERF-domain transcription factors have been suggested to be positive regulators of *PDF1.2* gene expression. Here, we found that only ORA59 and ERF1 were able to function as transcriptional activators of *PDF1.2* gene expression, whereas AtERF2 and the related AtERF1 were not. Our results demonstrate that ORA59 is an essential integrator of the JA and ethylene signal transduction pathways, and thereby provide new insight in the nature of the molecular components involved in the crosstalk between these two hormones.

Introduction

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

Jasmonic acid belongs to a family of signaling molecules including certain precursors and derivatives, which are collectively indicated as jasmonates (JAs). Besides their roles in certain aspects of plant growth and development, such as anther dehiscence, fruit ripening and root growth, JAs are major intermediate signaling molecules involved in defense against wounding, attack by herbivores and infection by certain pathogens (Creelman and Mullet, 1997; Turner et al., 2002). Several studies revealed complex cross-talk relationships between

JA, ethylene and SA, which can act synergistically or antagonistically, in order to fine-tune the defense response (Kunkel and Brooks, 2002). Arabidopsis plants impaired in JA or ethylene signaling pathways showed enhanced susceptibility to the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* (Thomma et al., 1998 and 1999a; Penninckx et al., 1996), demonstrating that JA and ethylene are important signal molecules for resistance against these pathogens.

A crucial step in the JA-dependent defense response is the rapid transcription of genes coding for antimicrobial proteins or enzymes involved in the biosynthesis of secondary metabolites. Studying the mechanisms whereby the expression of these defense-related genes is regulated is therefore of major importance to understand signal transduction pathways and plant responses to environmental stress.

In the past several years, a number of transcription factors regulating defense-related genes have been functionally characterized. Several of these regulatory proteins belong to a subgroup of the plant-specific APETALA2 (AP2)-domain protein family known as the ethylene response factor (ERF) subfamily. Proteins from this AP2/ERF subfamily are characterized by a single AP2-type DNA-binding domain with a conserved amino acid sequence. Several *AP2/ERF* genes have been shown to be regulated by a variety of stress-related stimuli, such as wounding, JA, ethylene, SA, or infection by different types of pathogens (Liu et al., 1998; Chen et al*.,* 2002; Suzuki et al., 1998; Gu et al., 2000; Menke et al*.,* 1999; van der Fits and Memelink, 2001; Fujimoto et al., 2000; Solano et al., 1998). The transcription factor ERF1 was suggested to act as an integrator of JA and ethylene signaling pathways in Arabidopsis (Lorenzo et al., 2003). Constitutive overexpression of *ERF1* activates the expression of several defense-related genes including *plant defensin1.2* (*PDF1.2*) and *basic-chitinase* (*ChiB*; Solano et al., 1998; Lorenzo et al., 2003) and was shown to confer resistance to several fungi (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). Constitutive overexpression of another AP2/ERF-domain transcription factor, AtERF2, was also shown to cause high levels of *PDF1.*2 and *ChiB* gene expression in transgenic Arabidopsis plants (Brown et al., 2003).

Atallah (2005) previously characterized 14 genes encoding AP2/ERF-domain proteins, which were rapidly induced by JA treatment in 10-days-old Arabidopsis seedlings. The JA-induced expression of these genes, named *Octadecanoid-Responsive Arabidopsis AP2/ERF* (*ORA*) genes, was severely reduced in the JA-insensitive *coi1* mutant. In addition, expression of the *ORA59* gene was also induced by ethylene, and a combination of both JA and ethylene had a synergistic positive effect on *ORA59* mRNA accumulation (Atallah, 2005). Analysis of *ORA59* gene expression in Arabidopsis mutants revealed the necessity of intact JA and ethylene signaling pathways for JA-responsive expression of *ORA59* (Atallah, 2005). Similar

observations have been made for *ERF1* gene expression (Lorenzo et al., 2003). This suggests that multiple AP2/ERF-domain transcription factors are involved in JA-dependent transcriptional events, as well as in synergism between the JA and ethylene signaling pathways. These transcription factors might regulate distinct subsets of JA- and ethylene-responsive genes in certain cell types, or alternatively, they might be functionally redundant.

In this study, we show that ORA59 is a major component of the jasmonate- and ethylene-controlled regulatory network. Overexpression of *ORA59* induced the expression of a large number of defense-related genes including the *PDF1.2*, *ChiB* and *hevein-like* (*HEL*) genes. Expression of these genes in response to JA and/or ethylene, or after pathogen infection was dramatically reduced in plants showing post-transcriptional silencing of the *ORA59* gene. In addition, infection experiments with *B. cinerea* showed that plant resistance or susceptibility was directly linked to the presence or absence of ORA59, respectively. In transient transactivation assays as well as in transgenic plants inducibly overexpressing various AP2/ERF-domain transcription factors, ORA59 and ERF1, but not AtERF2 and AtERF1, were able to activate the *PDF1.2* promoter. Our findings show that ORA59, and not ERF1 or AtERF2, responds to JA and integrates JA and ethylene signals to regulate the expression of defense genes such as *PDF1.2* and *ChiB*, altering the current view of the molecular components involved in JA-responsive gene expression and in the crosstalk between JA and ethylene.

Results

ORA59 gene expression is controlled by the JA and ethylene signal transduction pathways

Previous analysis of *ORA59* gene expression in Arabidopsis mutants revealed the necessity of intact JA and ethylene signaling pathways for JA-responsive expression of *ORA59* (Atallah, 2005). In this respect, *ORA59* expression is similar to *ERF1* expression (Lorenzo et al., 2003). For *ERF1*, it was shown that ethylene-responsive expression also required intact JA and ethylene signaling pathways (Lorenzo et al., 2003). To establish whether *ORA59* expression was also similar to *ERF1* expression in this respect, we analyzed the induction of *ORA59* gene expression after treatment with JA, ethephon (an ethylene-releasing agent), or a combination of both, in wild-type plants and mutants impaired in jasmonate or ethylene signaling (*coi1-1* and *ein2-1,* respectively; Feys et al., 1994; Guzman and Ecker, 1990). In accordance with previously reported data (Atallah, 2005), the results shown in Figure 1 indicate that the induction of *ORA59* gene expression by JA is transient and requires both

intact JA and ethylene signaling pathways. Moreover, as described before (Atallah, 2005), a combined treatment with JA and ethephon led to a prolonged super-induction of *ORA59* gene expression. In response to ethephon, *ORA59* gene expression was strongly reduced in both mutants compared to wild-type (Figure 1).

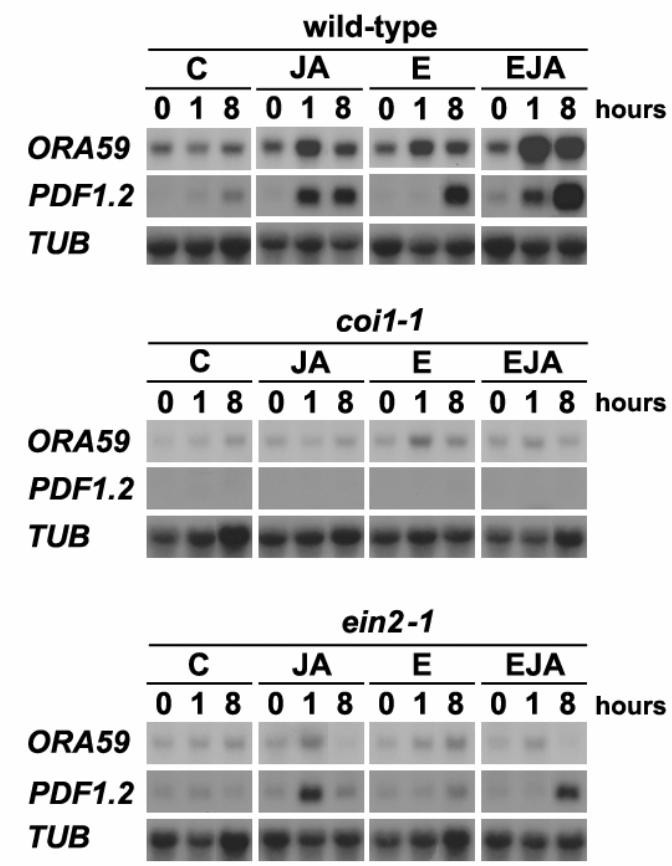

Figure 1. *ORA59* gene expression is controlled by the JA and ethylene signal transduction pathways. RNA was extracted from 14-days-old wild-type or mutant Arabidopsis seedlings treated with 50 µM jasmonic acid (JA), 1 mM ethephon (E; an ethylene releaser), a combination of both (EJA) or with the solvents (C), for the number of hours indicated. The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify the 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.

This indicates that the induction of *ORA59* gene expression by either JA, ethylene or a combination of both requires intact JA as well as ethylene signaling pathways for full responsiveness. Therefore, the responsiveness of the *ORA59* gene to JA and ethylene is

similar to *ERF1* expression in all aspects studied in this experiment. The *PDF1.2* defense-related gene is a well-characterized marker of the JA and ethylene signaling pathways. Expression of *PDF1.2* in response to JA and/or ethylene was similar to *ORA59* gene expression, except that it responded more slowly and less transiently in wild-type plants. Equal amounts of RNA were loaded on the gel as shown by the *β-tubulin* (*TUB*) mRNA level.

Genome-wide identification of putative ORA59 target genes

To characterize the genes regulated by ORA59, a genome-wide transcriptome analysis of *ORA59*-overexpressing plants using the Agilent Arabidopsis 3 Oligo microarray platform, which covers the full Arabidopsis genome, was performed. Two transgenic Arabidopsis lines expressing *ORA59* in an estradiol-inducible manner (XVE-*ORA59*) and two transgenic control lines expressing the *GUS* gene in an inducible manner (XVE-*GUS*) were grown for two weeks in liquid culture. Expression of the transgenes was induced by treating the samples with 2 µM estradiol and RNA was collected after 16 hours. Control samples were treated with the solvent DMSO for the same period of time. Microarray data analyses revealed that 405 genes had increased expression levels of at least 2-fold after induction of *ORA59* gene expression in both lines. From these 405 genes upregulated in XVE-*ORA59* lines, those genes that were also upregulated in one or both of the XVE-*GUS* lines were subtracted, resulting in 140 genes which were specifically upregulated in plants overexpressing the *ORA59* gene. As shown in Table 1, many of these genes are involved in defense against biotic or abiotic stress, signaling, primary and secondary metabolism or coding for other transcription factors. Several defense-related genes, such as *PDF1.2* (*a*, *b* and *c* genes), *HEL* and *ChiB* were highly expressed in plants overexpressing the *ORA59* gene. The expression of these genes is induced by JA or ethylene, and superinduced by a combination of both (Figure 1; Penninckx et al., 1998; Potter et al., 1993; Thomma et al., 1998). To get an indication of the role of ORA59 in the regulation of JA and/or ethylene-responsive genes, expression profiles of XVE-*ORA59* plants were compared with those of wild-type plants treated with JA or JA in combination with ethephon for 8 or 24 hours (Figure 2). From the 140 genes upregulated in XVE-*ORA59* plants, 45% were upregulated in wild-type plants treated with JA for 8 hours (63 genes) while 69% were upregulated in wild-type plants treated with JA for 24 hours (96 genes). For plants treated simultaneously with JA and ethylene, proportions were even higher with 86% and 69% of common genes after 8 hours (121 genes) and 24 hours of treatment (96 genes), respectively (Figure 2).

Table 1. Analysis of microarray data for genes up-regulated in XVE-*ORA59* lines

Gene annotation and putative function [1]	Fold-change: XVE-*ORA59*	wild-type: JA 8 hours	wild-type: JA 24 hours	wild-type: E+JA 8 hours	wild-type: E+JA 24 hours	AGI code
Defense						
chitinase B (ChiB)	34.0	3.3	4.8	7.0	18.7	At2g43580
chitinase	28.4	2.2	3.7	5.6	16.0	At2g43590
chitinase	25.6	2.4	3.7	5.9	13.8	At3g47540
legume lectin	21.2	2.1	5.5	11.8	10.2	At3g15356
legume lectin	16.6	— [2]	5.1	9.1	7.8	At3g16530
patatin	14.5	—	6.0	28.8	12.5	At2g26560
plant defensin PDF1.3	11.6	—	—	16.9	22.8	At2g26010
plant defensin PDF1.2b	11.1	2.5	—	34.2	25.7	At2g26020
avrRpt2-induced AIG2 protein (AIG2)	11.1	3.3	6.4	9.7	9.2	At3g28930
plant defensin PDF1.1	10.8	—	4.4	—	-2.2	At1g75830
plant defensin PDF1.2c	10.6	2.1	6.6	21.1	21.7	At5g44430
pathogenesis-related protein	9.0	—	3.1	2.7	5.4	At4g25780
osmotin-like protein (OSM34)	8.5	—	2.8	3.4	8.2	At4g11650
plant defensin PDF1.2a	7.9	2.2	7.4	31.7	16.9	At5g44420
pathogenesis-related protein	7.7	—	—	—	—	At4g33710
GCN5-related N-acetyltransferase (GNAT)	6.4	—	—	3.3	—	At4g37670
stress-responsive protein	6.0	—	—	2.6	2.8	At5g01410
jacalin lectin	5.8	—	3.4	3.3	—	At1g52130
basic endochitinase	5.5	—	—	—	6.8	At3g12500
jacalin lectin	5.3	—	2.5	3.2	—	At5g49860
hevein-like protein (HEL)	5.0	—	2.8	3.5	5.1	At3g04720
curculin-like (mannose-binding) lectin	4.0	—	2.7	4.0	3.1	At5g18470
disease resistance protein (TIR-NBS class)	3.9	2.5	3.3	4.3	4.5	At1g72910
disease resistance protein (TIR-NBS class)	3.9	4.0	6.2	4.8	4.9	At1g72900
disease resistance protein (TIR-NBS-LRR class)	3.7	—	—	4.3	—	At1g56540
jacalin lectin	3.3	—	—	—	—	At5g38550
avirulence-responsive protein	3.2	2.1	2.0	2.5	2.1	At1g33930
glycine-rich protein	3.1	2.3	2.8	2.7	2.7	At1g02710
legume lectin	3.1	—	—	—	—	At5g03350
Transcription						
ORA59	15.6	—	2.2	5.5	4.4	At1g06160
AP2 domain-containing transcription factor	11.5	3.0	5.0	10.8	7.7	At4g06746
AP2 domain-containing transcription factor	9.9	—	—	4.8	3.7	At2g31230
IAA20	6.7	—	—	—	—	At2g46990
floral homeotic protein APETALA1 (AP1)	5.1	—	4.8	3.0	—	At1g69120
Dof-type zinc finger domain-containing protein	4.8	—	—	—	—	At3g50410
WRKY family transcription factor	4.2	2.7	3.1	3.1	—	At3g56400
no apical meristem (NAM) family protein	3.9	9.8	10.2	14.1	7.8	At2g43000
zinc finger (C3HC4-type RING finger)	3.8	—	—	—	—	At2g26130
zinc finger (C3HC4-type RING finger) family protein	3.4	—	—	—	—	At5g27420
zinc finger (HIT type) family protein	3.0	3.0	3.1	3.1	2.8	At5g63830
Shikimate/Tryptophan metabolism						
tryptophan synthase, beta subunit 2 (TSB2)	4.7	3.0	4.3	5.0	6.0	At4g27070
tryptophan synthase, beta subunit 1 (TSB1)	4.2	2.2	3.5	4.0	4.8	At5g54810
anthranilate synthase beta subunit (ASB1)	3.9	4.3	5.1	8.2	7.5	At1g25220
3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 1 (DHS1)	3.7	6.1	5.5	4.8	5.9	At4g39980
anthranilate synthase beta subunit	3.6	—	5.9	8.6	7.5	At1g24807
indole-3-glycerol phosphate synthase (IGPS)	3.0	2.4	2.6	3.7	4.3	At2g04400

List of genes upregulated in XVE-*ORA59* transgenic lines overexpressing the *ORA59* gene and their fold-change ratios in wild-type plants after hormone treatment.

All genes listed had a fold-change ≥ 2 (P-values ≤ 0.001) in both XVE-*ORA59* transgenic lines overexpressing *ORA59* compared to non-induced XVE-*ORA59* lines. For each gene, the fold-change in wild-type plants treated with jasmonic acid (JA) or with a combination of ethephon and JA (E+JA) compared to control-treated plants, is also indicated. In bold are the genes whose expression was confirmed by RNA blot analyses.

[1]Annotations are as given by the MIPS Arabidopsis thaliana Genome Database (MAtDB; http://mips.gsf.de/proj/thal/db/index.html) except for *ORA59*.

[2]Fold-change values with P-values ≥ 0.001 were considered statistically non-reliable and therefore are represented by a bar (-).

Table 1 (continued). Analysis of microarray data for genes up-regulated in XVE-*ORA59* lines

Gene annotation and putative function [1]	Fold-change XVE-*ORA59*	wild-type JA 8 hours	wild-type JA 24 hours	wild-type E+JA 8 hours	wild-type E+JA 24 hours	AGI code
Signaling						
transducin family protein / WD-40 repeat family protein	3.9	3.2	3.5	3.4	3.2	At5g58760
mitogen-activated protein kinase kinase (MAPKK7)	3.9	4.1	5.9	16.3	9.5	At1g18350
mitogen-activated protein kinase kinase (MAPKK9)	3.4	4.4	5.5	13.7	9.4	At1g73500
Protein modification / synthesis / degradation / transport						
phosphorylase	17.6	13.3	23.0	20.6	17.9	At4g24350
phosphorylase	12.9	12.9	24.0	17.0	14.3	At4g24340
protein kinase	10.7	—	2.7	5.7	6.1	At5g10520
expressed protein similar to phosphatase	9.5	—	—	—	—	At1g73010
protein kinase	5.0	—	—	—	—	At2g23770
proteasel (pfpI)-like protein (YLS5)	4.9	—	2.5	3.5	5.0	At2g38860
cysteine proteinase	4.4	—	—	—	—	At4g36880
protein disulfide isomerase	3.3	2.4	2.9	2.7	3.2	At1g21750
mitochondrial import inner membrane translocase subunit	3.2	2.2	2.5	3.1	—	At1g17530
serine/threonine protein kinase	3.1	3.0	3.0	3.4	3.2	At1g66880
polyubiquitin (UBQ4)	3.1	2.1	2.6	3.0	2.5	At5g20620
Ran-binding protein 1a (RanBP1a)	3.0	2.4	2.4	2.6	2.6	At1g07140
Primary Metabolism/ Secondary metabolism						
anthocyanin 5-aromatic acyltransferase (AN5-AT)	26.8	2.2	7.7	17.7	14.8	At5g61160
UDP-glucoronosyl/UDP-glucosyl transferase family protein	23.6	2.8	3.4	15.3	12.9	At1g07260
2-oxoacid-dependent oxidase, putative (DIN11)	12.5	14.9	56.1	36.5	35.3	At3g49620
aldo/keto reductase	11.2	—	—	8.2	—	At1g59950
glycosyl hydrolase	10.4	—	2.9	5.2	7.0	At4g16260
pyruvate decarboxylase	7.9	—	—	4.4	4.4	At5g54960
S-adenosyl-L-methionine:carboxyl methyltransferase family protein	7.6	—	—	4.6	—	At1g15125
S-adenosyl-L-methionine:carboxyl methyltransferase family protein	6.5	—	4.0	15.5	7.6	At1g66700
lipase	5.8	—	—	—	—	At1g30370
O-methyltransferase	5.8	2.4	4.6	6.7	3.0	At1g21100
O-methyltransferase	5.7	—	3.5	4.4	2.2	At1g21130
alcohol dehydrogenase	5.3	—	2.0	6.4	3.8	At1g64710
terpene synthase/cyclase	5.1	—	—	2.4	—	At3g29110
malate oxidoreductase	4.9	—	—	3.0	2.8	At5g25880
UDP-glucoronosyl and UDP-glucosyl transferase	4.9	11.2	8.7	12.6	19.9	At2g15490
bifunctional dihydrofolate reductase-thymidylate synthase	4.7	—	—	—	—	At2g21550
5'-adenylylsulfate reductase (APR3)	4.5	4.0	3.6	5.5	4.7	At4g21990
phosphofructokinase	4.3	2.7	3.5	2.8	—	At5g47810
sulfotransferase	4.1	11.8	19.3	38.2	30.2	At5g07010
Fe-S metabolism associated domain-containing protein	4.1	—	2.7	9.2	5.5	At1g67810
UDP-glucoronosyl/UDP-glucosyl transferase family protein	4.0	5.7	8.5	14.8	9.7	At4g27570
sulfotransferase	4.0	8.5	12.5	27.9	16.1	At5g07000
glycerophosphoryl diester phosphodiesterase family protein	3.7	—	3.4	3.8	—	At5g43300
D-3-phosphoglycerate dehydrogenase	3.6	4.6	4.3	9.3	7.3	At4g34200
D-3-phosphoglycerate dehydrogenase	3.5	4.5	4.6	7.6	7.4	At1g17745
2-oxoacid-dependent oxidase	3.5	—	4.6	23.0	22.4	At3g49630
guanylate kinase 1 (GK-1)	3.5	—	—	—	—	At2g41880
glutamate-cysteine ligase	3.5	5.9	7.6	9.2	8.6	At4g23100
(S)-2-hydroxy-acid oxidase	3.4	2.7	3.1	3.4	—	At3g14130
sulfate adenylyltransferase 1	3.3	5.2	3.9	6.0	5.6	At3g22890
tropinone reductase	3.2	—	6.8	9.4	8.5	At2g29350
Cytochrome P450s						
cytochrome P450 (CYP1)	9.3	2.1	4.1	8.0	6.0	At4g22710
cytochrome P450 71B22	5.6	—	—	3.2	3.2	At3g26200
cytochrome P450	5.0	—	9.7	5.6	—	At1g64930
cytochrome P450	4.6	—	—	2.2	—	At4g00360
Cell wall						
pectinesterase	5.7	—	2.4	3.1	2.6	At3g14310
invertase/pectin methylesterase inhibitor	4.4	4.3	3.3	3.8	—	At3g47380

Table 1 (continued). Analysis of microarray data for genes up-regulated in XVE-*ORA59* lines

Gene annotation and putative function [1]	Fold-change XVE-*ORA59*	wild-type JA 8 hours	wild-type JA 24 hours	wild-type E+JA 8 hours	wild-type E+JA 24 hours	AGI code
Cell wall						
pectinesterase	5.7	—	2.4	3.1	2.6	At3g14310
invertase/pectin methylesterase inhibitor	4.4	4.3	3.3	3.8	—	At3g47380
Redox						
peroxidase	24.8	—	3.5	6.5	14.1	At4g08780
peroxidase	14.1	—	3.4	5.2	10.0	At4g08770
peroxidase	11.2	—	—	2.3	13.3	At3g03670
glutathione S-transferase	6.6	2.1	2.3	4.9	3.7	At1g27130
glutathione S-transferase	4.8	—	2.1	3.1	3.1	At4g02520
glutathione S-transferase	4.8	3.1	3.4	2.6	3.2	At2g30870
peroxidase	4.6	—	—	2.2	2.8	At4g11290
glutathione S-transferase	4.6	—	—	3.6	—	At1g69920
glutathione S-transferase	4.5	—	4.8	4.0	2.9	At1g02930
glutathione S-transferase	4.5	—	—	2.9	3.3	At2g02930
glutathione S-transferase	4.5	—	—	—	3.1	At1g10370
peroxidase	4.1	—	2.9	5.8	6.8	At4g37520
thioredoxin family protein	3.6	2.9	3.7	3.5	—	At4g32580
glutathione S-transferase	3.5	—	4.2	3.8	2.4	At1g02920
respiratory burst oxidase protein D (RbohD)	3.3	—	—	3.1	3.9	At5g47910
RNA processing						
DEAD box RNA helicase (DRH1)	3.7	2.7	3.0	3.3	3.1	At3g01540
U2 snRNP auxiliary factor small subunit	3.3	2.1	3.2	3.8	—	At1g27650
U2 snRNP auxiliary factor small subunit	3.1	3.2	3.3	3.2	3.0	At5g42820
Hormone metabolism						
ACC synthase 6 (ACCS6)	6.8	—	—	2.8	—	At4g11280
nitrilase 4 (NIT4)	4.8	—	5.5	18.7	14.5	At5g22300
Transport						
MATE efflux family protein	14.2	4.0	12.9	17.9	14.5	At3g23550
uclacyanin II	7.9	—	—	4.2	3.9	At2g44790
sigA-binding protein identical to SigA binding protein	5.6	—	—	2.7	—	At3g56710
proton-dependent oligopeptide transport (POT) family protein	5.2	4.8	8.4	14.4	10.3	At4g21680
ubiquinol-cytochrome C reductase iron-sulfur subunit	3.3	2.9	2.9	3.3	3.2	At5g13430
glycerol-3-phosphate transporter	3.1	—	—	4.7	6.0	At3g47420
Unclassified proteins						
heavy-metal-associated domain-containing protein	17.9	—	—	2.4	—	At5g52720
hypothetical protein	16.9	—	6.5	5.8	10.0	At1g13490
expressed protein	15.3	—	2.1	6.0	3.2	At5g25250
expressed protein	5.5	—	—	2.5	—	At1g52270
FAD-binding domain-containing protein	5.2	—	4.0	14.2	—	At1g26380
sigA-binding protein-related low similarity to SigA binding protein	5.1	—	—	3.1	—	At2g41180
hypothetical protein	5.0	—	—	—	—	At5g44565
expressed protein	3.6	2.3	—	—	—	At2g16485
expressed protein	3.5	-2.6	-2.8	—	2.1	At5g42530
expressed protein	3.5	—	—	3.4	—	At3g57400
expressed protein	3.3	4.6	3.9	6.9	7.2	At2g27260
unknown protein	3.2	3.0	2.7	3.0	3.7	At1g05130
expressed protein	3.2	2.1	3.3	4.2	3.2	At5g35525
extracellular dermal glycoprotein	3.2	—	3.1	2.9	—	At1g03230
expressed protein	3.2	2.9	2.4	2.7	2.2	At1g68120
expressed protein	3.1	2.7	3.1	3.1	—	At4g31080

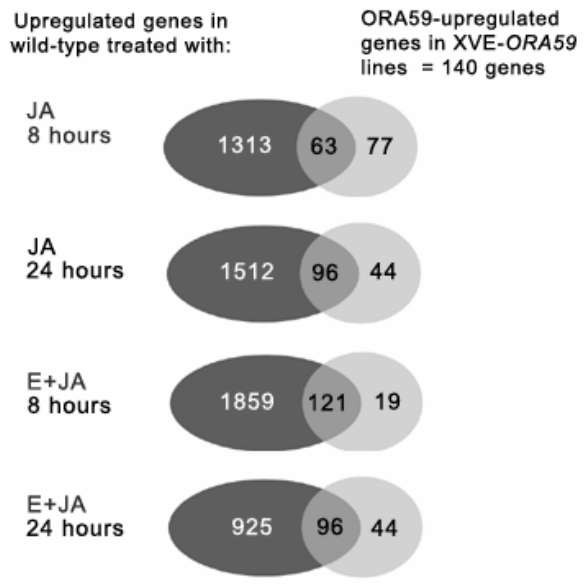

Figure 2. Venn diagrams presenting the results of the genome-wide transcriptome analyses of the putative ORA59 target genes. Genes that were significantly upregulated in two-weeks-old XVE-*ORA59* lines overexpressing *ORA59* (140 genes) were compared with genes induced in two-weeks-old wild-type (Col-0) plants treated with 50 µM jasmonic acid (JA) or 50 µM JA and 1 mM of the ethylene-releaser ethephon (E+JA) for 8 and 24 hours. In each ellips is the number of genes upregulated in response to the particular treatment indicated. All genes had a fold-change ≥ 2 (*P*-values ≤ 0.001). Indicated in the overlapping areas are the number of genes commonly upregulated in XVE-*ORA59* lines and in wild-type plants after the specified treatment. Numbers in the nonoverlapping areas of the ellipses represent genes specifically upregulated under the given conditions.

These results show that a large number of ORA59-upregulated genes are responsive to JA alone or in combination with ethephon. The microarray data were confirmed by RNA blot analyses for a selected set of genes (Figure 3). Estradiol-induced activation of *ORA59* gene expression in XVE-*ORA59* line #10 resulted in high expression levels of all genes tested, including *PDF1.2, ChiB*, *HEL, AN5-AT* and *AIG2.* Expression of these genes was not induced in the estradiol-treated XVE-*GUS* control line #7. Consistent with the microarray data (Table 1), the expression of several genes encoding enzymes of the shikimate/tryptophan biosynthesis pathway, such as *IGPS*, *ASB1* and *TSB1*, was increased in the XVE-*ORA59* line overexpressing *ORA59* (Figure 3). Similar results were obtained with RNA samples originating from another independent XVE-*ORA59* line treated with estradiol (line #6; data not shown).

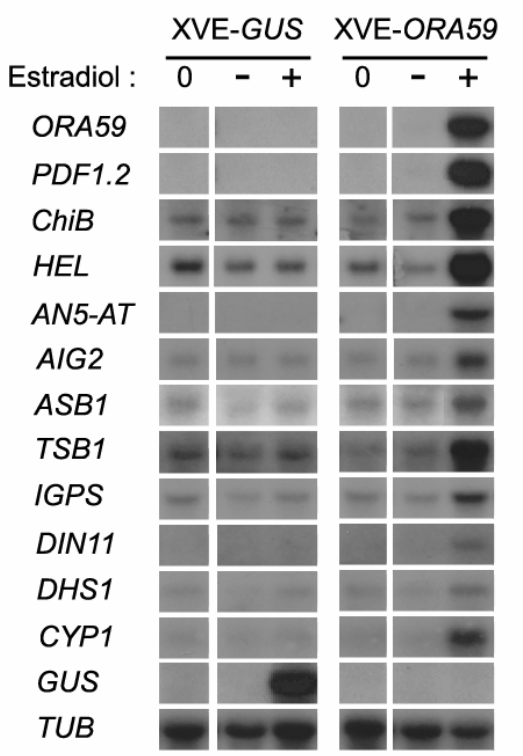

Figure 3. Verification of putative ORA59 target genes obtained from the microarray data by RNA blot analyses. Expression levels of a selected set of genes upregulated in plants overexpressing *ORA59* identified from the microarray data were analyzed in 2-weeks-old plantlets from XVE-*ORA59* transgenic line #10 grown in liquid medium. XVE-*GUS* transgenic line #7 was used as a control. RNA samples were collected from plants which were untreated (0), or treated for 16 hours with 2 µM estradiol (+) or with the solvent DMSO at 0.1% final concentration (-).The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

ORA59 functions downstream from COI1

The expression studies indicate that ORA59 regulates several JA- and ethylene-responsive genes (Table 1). Figure 2 shows that only a small proportion of genes induced by JA or JA and ethylene (10 % at most) are regulated by ORA59. We hypothesized that for these genes ORA59 forms the terminal component of the ethylene and jasmonate signal transduction pathways, and that it serves as the integrator of the JA and ethylene signal inputs thereby determining the final expression output for this defense gene set. To test whether ORA59 functions downstream of the JA signal transduction component COI1, we investigated whether *ORA59* overexpression would lead to target gene expression without requiring COI1. We introduced the XVE-*ORA59* expression module in the *coi1-1* mutant background and analyzed the expression of *PDF1.2*, *HEL* and *ChiB* after treatment with the inducer estradiol.

As shown in Figure 4, estradiol-induced expression of *ORA59* resulted in high expression levels of the target genes *PDF1.2* and *HEL* in a COI1-independent manner. This result demonstrates that ORA59 functions downstream from COI1, which is compatible with the hypothesis that ORA59 is the terminal integrator of the JA and ethylene signal inputs for a subset of JA- and ethylene-responsive genes including *PDF1.2* and *HEL*. In contrast, estradiol-induced expression of *ORA59* in the *coi1-1* mutant background did not lead to high expression of the *ChiB* gene (Figure 4), indicating that regulation of the *ChiB* gene by ORA59 is controlled by COI1-dependent molecular mechanisms.

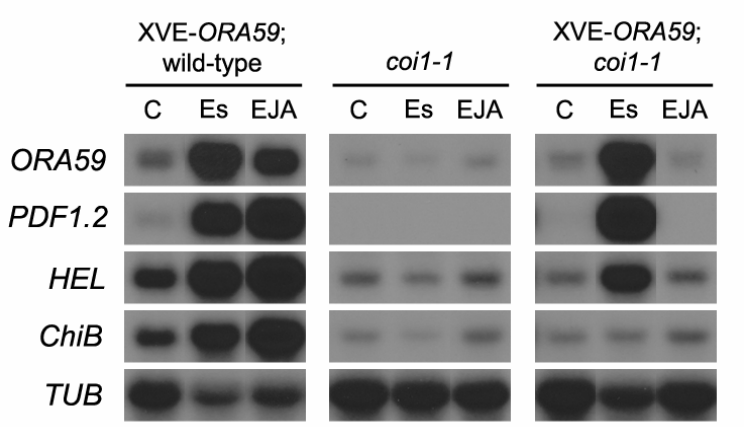

Figure 4. *ORA59* overexpression activates target gene expression without requiring COI1. Two-weeks-old *coi1-1* mutant plants, and plants containing the XVE-*ORA59* expression module in the wild-type and in the *coi1-1* backgrounds were treated for 8 hours with 50 µM jasmonic acid and 1mM ethephon (EJA), 5 µM estradiol (Es) or the solvents DMSO and Na-phosphate (C). The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

Silencing of the *ORA59* gene compromises JA- and ethylene-induced expression of several defense-related genes

To assess the role of ORA59 in a loss-of-function approach, *ORA59* gene expression was silenced using the RNA interference (RNAi) technique. Two copies of the *ORA59* full-length open reading frame were cloned in an inverted repeat orientation in the pHANNIBAL vector (Wesley et al., 2001). In plants this construct directs the expression of an RNA consisting from 5' to 3' of the anti-sense *ORA59* ORF, the *Pdk* intron and the sense *ORA59* ORF, which can fold in a hairpin structure, generating after splicing a double-stranded *ORA59* RNA. Transgenic *ORA59*-silenced plants growing under normal conditions displayed no visible aberrant phenotype compared to wild-type plants. RNA blot analyses using a probe specific

for *ORA59* revealed a smeary signal in five out of seven independent lines (Figure 5A). A similar autoradiographic pattern was obtained using the *Pdk* intron as a probe (*PDKi*; Figure 5A), suggesting that the smear corresponds to the complete inverted repeat-intron RNA and its degradation products. In these transgenic lines, *ORA59* mRNA was undetectable after treatment with JA. In contrast, transgenic line #5 showed wild-type *ORA59* gene expression in response to JA, whereas line #13 showed reduced JA-induced expression. Induction of the *PDF1.2* and *HEL* genes in response to JA treatment was severely compromised in those *ORA59*-silenced lines with undetectable *ORA59* mRNA (Figure 5B). Furthermore, the reduced *PDF1.2* and *HEL* transcript abundance correlated with the *ORA59* expression level in individual lines, indicating that the JA-induced expression of these defense genes is dependent on ORA59.

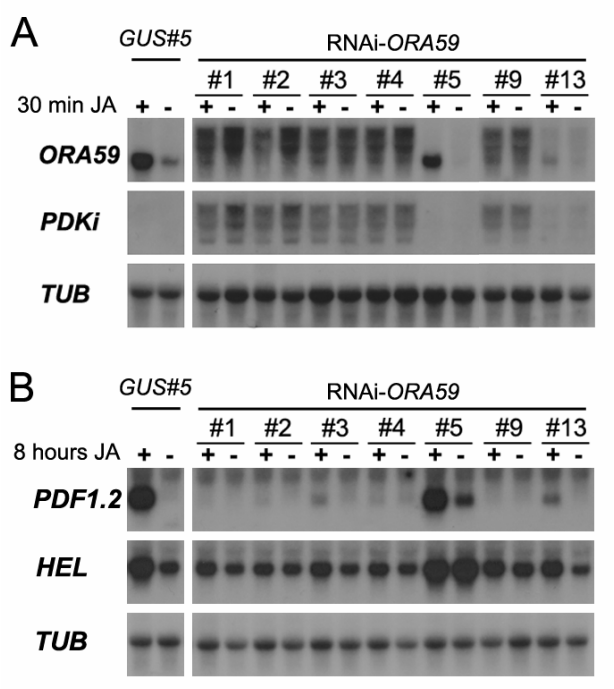

Figure 5. The *ORA59* gene is silenced in the majority of independent transgenic RNAi-*ORA59* lines. Ten-days-old seedlings from control line (*GUS* #5) or seedlings from several independent RNAi-*ORA59* transgenic lines (indicated by numbers) were treated for 30 minutes (min) **(A)** or for 8 hours **(B)** with 50 µM jasmonic acid (+) or with 0.1% of the solvent DMSO (-).The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

Transgenic line RNAi-*ORA59* #9 was used for further analyses. As shown in Figure 6A, the JA- and ethephon-induced expression of several genes identified by the microarray analysis, including *PDF1.2*, *AN5-AT* and *HEL* genes, was dramatically reduced in *ORA59*-silenced

plants, compared to the control line. To verify that the silencing effects of the RNAi construct on defense gene expression were specific for ORA59, the mRNA levels of ERF1 and AtERF2, two possible functionally equivalent transcription factors, were determined (Figure 6A). The JA- and ethephon-induced gene expression levels of the two putative functional homologues *ERF1* and *AtERF2* in the *ORA59*-silenced line were similar to those observed in the control line, indicating that ERF1 and AtERF2 protein levels are likely to remain unchanged in the *ORA59*-silenced line. Equal loading of RNA on gel was checked by hybridization with the *TUB* gene (Figure 6A and B).

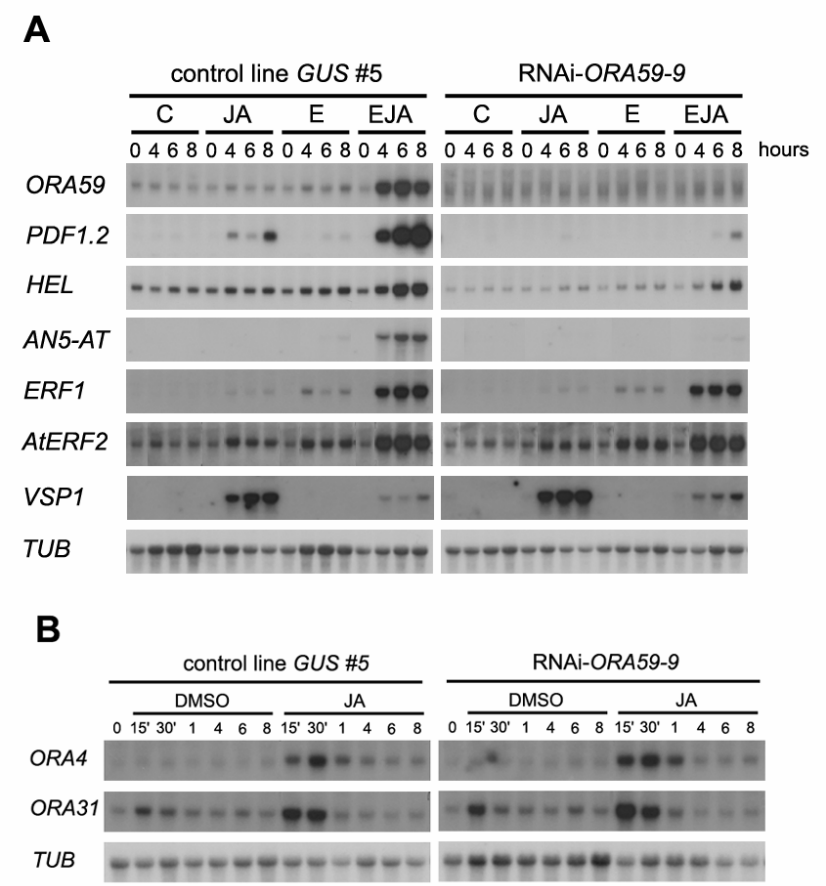

Figure 6. Specific silencing of the *ORA59* gene severely compromises the JA- and/or ethylene-responsive expression of several defense genes. **(A).** Two-weeks-old seedlings from the representative RNAi-*ORA59* transgenic line #9 or from the control line *GUS* #5 were treated for the number of hours indicated with 50 µM jasmonic acid (JA), 1 mM ethephon (E), a combination of both (EJA), or the solvents DMSO and Na-phosphate (C). **(B).** Two-weeks-old seedlings from the same lines as in **(A)** were treated for 15 and 30 minutes or for the number of hours indicated with 50 µM jasmonic acid (JA) or 0.1 % of the solvent DMSO. The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

RNAi-mediated silencing is based on targeted degradation of the endogenous mRNA (Hamilton and Baulcombe, 1999). mRNA degradation is triggered by sequence-specific short 21 to 25 nucleotide (nt) oligomers derived from the double-stranded RNA molecule (Hamilton and Baulcombe, 1999). Two JA-responsive AP2/ERF genes, *ORA4* and *ORA31* (Atallah, 2005), have 21 to 23 nt-long perfect sequence homologies to the *ORA59* gene in the highly conserved region encoding the AP2 domain, and are therefore putative targets for mRNA degradation. Expression levels of the *ORA4* and *ORA31* genes in response to JA treatment were similar to those observed in control plants, indicating that in *ORA59*-silenced plants, silencing was specifically directed at *ORA59* mRNA (Figure 6B). These results demonstrate that ORA59 is responsible for the activation of a subset of JA- and ethylene-responsive genes including *PDF1.2*, *AN5-AT,* and *HEL,* and that ORA59 is an essential node of convergence of the concomitant activation of the JA and ethylene signal transduction pathways, which is absolutely required for expression of these genes.

ORA59 controls resistance against the necrotrophic fungus *Botrytis cinerea*

In Arabidopsis, the JA and ethylene signal transduction pathways are involved in resistance against the necrotrophic fungi *B. cinerea* and *A. brassicicola* (Thomma et al., 1998 and 1999a; Ferrari et al., 2003; Penninckx et al., 1996). The regulation by ORA59 of JA- and ethylene-responsive defense-related genes prompted us to test whether constitutive expression or silencing of *ORA59* would affect resistance to these pathogens.

Several independent transgenic lines constitutively overexpressing the *ORA59* gene were constructed. As shown by RNA gel blot analyses, the ORA59-regulated genes *PDF1.*2, *HEL* and *ChiB* were highly expressed in the 35S:*ORA59* lines compared to control lines (Figure 7).

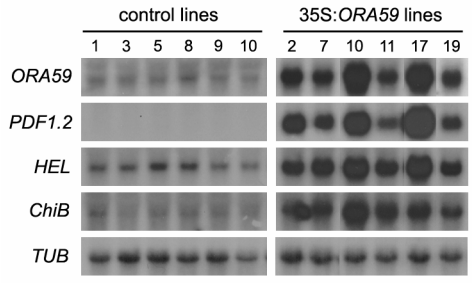

Figure 7. ORA59 target gene expression in constitutive *ORA59*-overexpressing plants. RNA was extracted from ten-days-old seedlings from several (indicated by numbers) independent 35S:*ORA59* lines and control lines (1301). The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

The level of defense gene expression correlated with the level of *ORA59* gene expression in each independent line. In general, independent 35S:*ORA59* lines showed a severe dwarf phenotype under normal growth conditions, similar to that observed with transgenic plants

overexpressing *ERF1* (Solano et al., 1998). As shown in Figure 8, 35S:ORA59 plants from line #10 and #17 were smaller than wild-type plants when grown on normal medium.

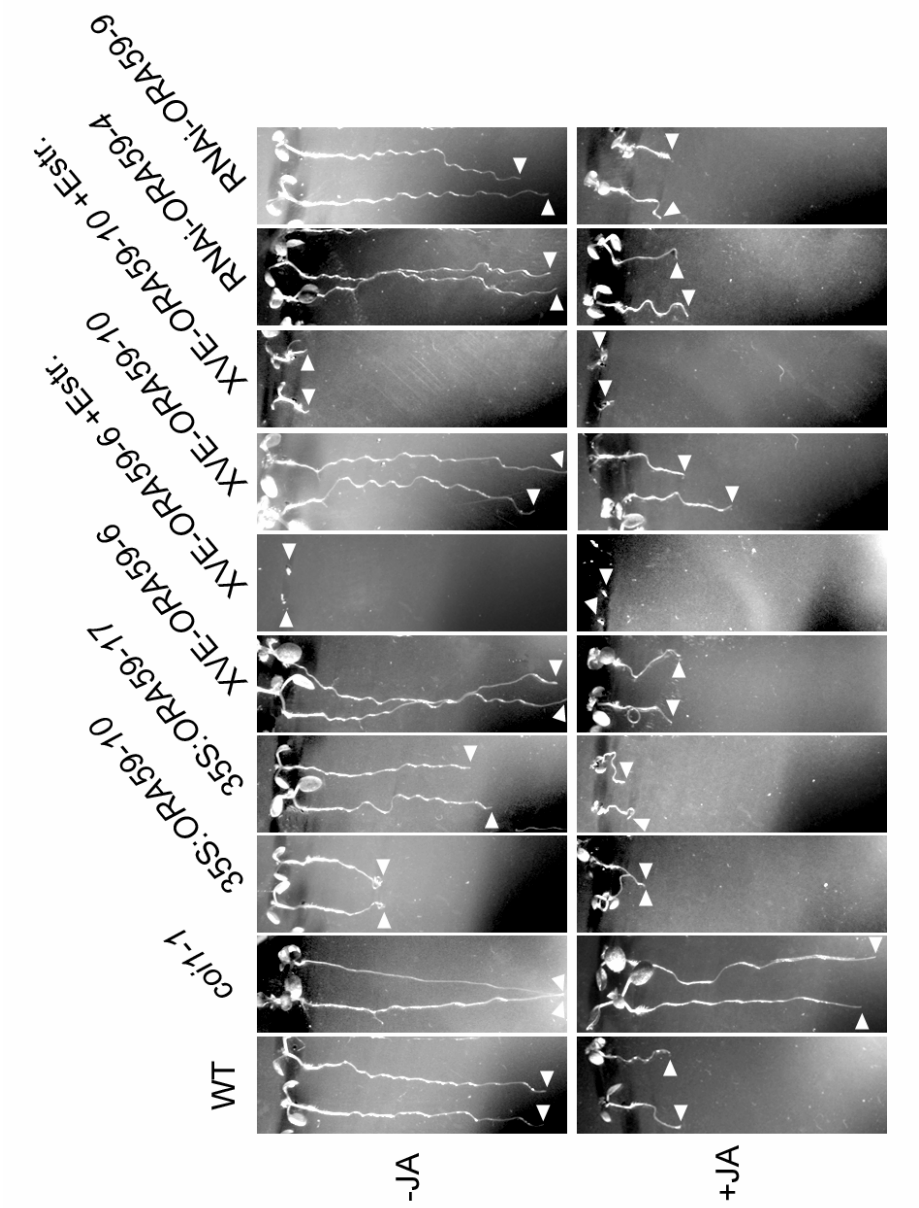

Figure 8. *ORA59* overexpression results in dwarf plants but ORA59 does not participate in root growth inhibition by JA. Two-weeks-old Arabidopsis wild-type, *coi1* mutant, and transgenic plants constitutively (35S:*ORA59*) and inducibly (XVE-*ORA59*) overexpressing or silencing (RNAi-*ORA59*) the *ORA59* gene were grown on plates containing (+JA) or lacking (-JA) 50 µM jasmonic acid. In addition, XVE-*ORA59* plants were grown with or without 4 µM estradiol (Estr.). The position of the root tip is indicated with an arrowhead.

In the presence of JA, wild-type and overexpressing plants were equally sensitive to JA with respect to root growth inhibition. In contrast, the JA-insensitive *coi1-1* mutant did not show reduced root growth in response to JA. The relationship between reduction of plant size and *ORA59* overexpression was further analyzed with the inducible XVE-*ORA59* plants. In the absence of estradiol, XVE-*ORA59* plants from independent lines #6 and #10 showed wild-type phenotypes when cultured either on normal medium or on medium containing JA. However, plant growth was dramatically impaired in the XVE-*ORA59 #10* line on medium inducing the *ORA59* transgene, and JA treatment had additional negative effect on growth. Seeds from the line XVE-*ORA59 #6* did not even germinate on medium containing estradiol or JA/estradiol. This indicates that overexpression of *ORA59* has a negative effect on plant growth. Transgenic *ORA59*-silenced plants grown on medium containing JA showed reduced root growth similar to wild-type plants, demonstrating that ORA59 is not involved in root growth inhibition by JA. These results suggest that a high and constant expression of the ORA59-regulated genes, including the defense related genes *PDF1.2, HEL* and *ChiB*, generates a stress condition that compromises plant growth. However, ORA59 does not control the branch of the JA response responsible for root growth inhibition.

Because of its mild dwarf phenotype (Figure 8) and its constitutive overexpression of *ORA59* and of the ORA59 target genes (Figure 7), the 35S:*ORA59* line #17 was chosen for subsequent disease resistance tests. In order to reach a plant size similar to the other genotypes, the 35S:*ORA59* plants were allowed to grow for seven weeks before inoculation, as compared to five weeks for all other genotypes. Disease resistance tests were performed at the same time for all genotypes. Mature leaves of five- to seven-weeks-old wild-type plants, JA-insensitive *coi1-1* mutant plants, and transgenic 35S:*ORA59* line #17 and RNAi-*ORA59* line #9 were inoculated with *B. cinerea* ($5x10^5$ spores/mL). Figure 9A shows the disease progression in three representative leaves of each genotype scored 5 days post inoculation. Infected wild-type leaves were relatively tolerant to this inoculum density of *B. cinerea*, while *coi1-1* plants showed increased susceptibility with a large percentage of leaves with spreading necrotic lesions (Figure 9B), confirming previous findings (Thomma et al., 1998). Interestingly, 35S:*ORA59 #17* plants showed enhanced resistance to *B. cinerea* with a majority of leaves with no or mild symptoms, whereas infected leaves from *ORA59*-silenced plants mainly developed spreading necrotic lesions. These results demonstrate that ORA59 plays an important role in resistance to *B. cinerea,* and that overexpression of *ORA59* increases resistance to this fungus. For gene expression analyses, RNA was extracted from primary infected (local) and distal (systemic) leaves that were collected 2 and 4 days after inoculation. Inoculation of wild-type plants resulted in an increase in the mRNA level of *ORA59* both locally and systemically (Figure 9C). In the severely diseased JA-insensitive

coi1-1 mutant, *B. cinerea* infection did not result in the induction of *ORA59*. Also, the expression of the ORA59 target genes was either not induced (*PDF1.2* and *ChiB*) or strongly reduced (*HEL*) in *coi1-1* plants. In the *ORA59*-silenced lines, which showed enhanced susceptibility to *B. cinerea*, expression of these defense genes was strongly reduced in response to *B. cinerea*, whereas in 35S:*ORA59* plants, *PDF1.2, HEL* and *ChiB* mRNA levels were constitutively high.

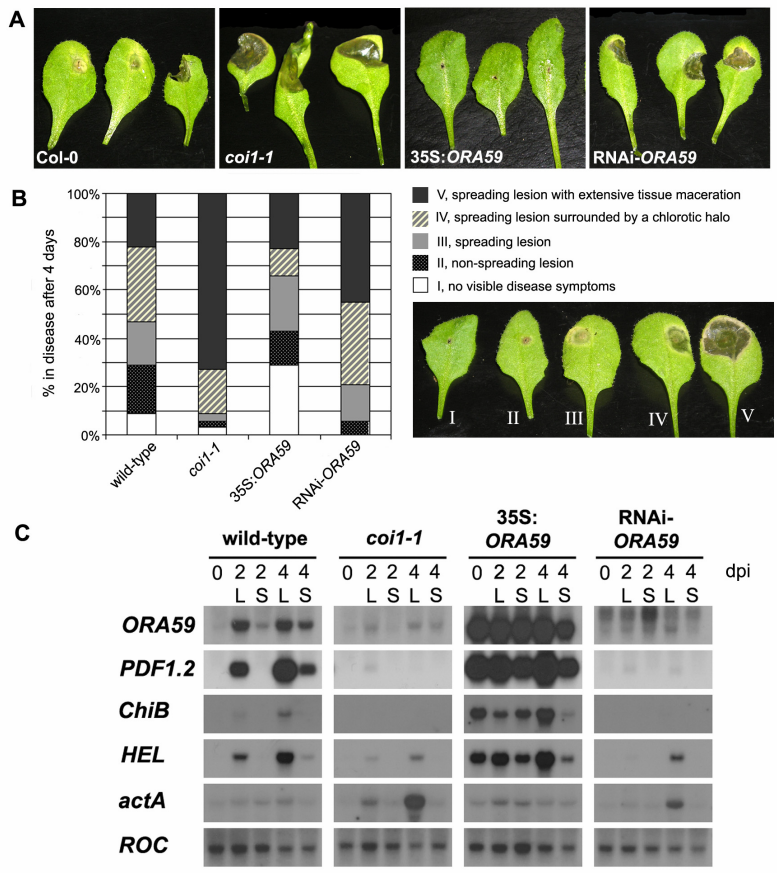

Figure 9. ORA59 is involved in resistance against *Botrytis cinerea*. Disease severity was scored in Arabidopsis wild-type plants, *coi1-1* mutants and transgenic plants overexpressing (35S:*ORA59-17*) or silencing (RNAi-*ORA59-9*) the *ORA59* gene. Disease ratings were performed 4 days after inoculation. **(A).** Representative disease symptoms at 4 days after inoculation. (**B).** Distribution of disease severity classes. Disease severity is expressed as the percentage of leaves falling in disease severity classes: I, no visible disease symptoms; II, non-spreading lesion; III, spreading lesion; IV, spreading lesion surrounded by a chlorotic halo; and V, spreading lesion with extensive tissue maceration and sporulation by the pathogen. Data represent 60 to 100 leaves of 15 to 20 plants per genotype. The experiment was repeated three times with similar results. (**C).** Infected local (L) and non-infected systemic (S) leaves from several inoculated plants of each genotype were collected at day 0, day 2 and day 4 after inoculation (dpi) with *B. cinerea* and RNA was extracted. The RNA gel blot was hybridized with the indicated probes. The *ROC* probe was used to verify RNA loading.

The *actin* (*actA*) gene from *B. cinerea* (Benito et al., 1998) was used as a molecular marker of disease progression. As shown in Figure 9C, the *actA* gene was expressed in local leaves of the susceptible *coi1-1* and RNAi-*ORA59* plants four days after infection, indicating growth of the pathogen *B. cinerea* in these leaves. Hybridization with the *ROC* gene, encoding a cytosolic cyclophilin, showed equal loading of RNA. These results show a strong correlation between the effects of *ORA59* expression levels on defense gene expression and on resistance against *B. cinerea.*

To test whether ORA59 also controls resistance against *A. brassicicola*, five-weeks-old wild-type plants, *ORA59*-silenced plants, *pad3-1* mutant plants impaired in camalexin biosynthesis (Glazebrook and Ausubel, 1994) and JA-insensitive *coi1-1* mutant plants were inoculated with *A. brassicicola*. Disease ratings were assessed 7 days after inoculation. In comparison to wild-type Col-0 plants, the *pad3-1* and *coi1-1* mutant plants developed severe symptoms upon *A. brassicicola* inoculation (data not shown), confirming previous findings showing that these mutants have enhanced susceptibility towards this pathogen (Thomma et al., 1998 and 1999b). In contrast to *coi1-1*, the level of resistance against *A. brassicicola* in *ORA59*-silenced plants did not differ from that of wild-type plants (data not shown). RNA blot analyses of infected and systemic leaves revealed that *A. brassicicola* induced *ORA59* gene expression in wild-type plants, both locally and systemically (Figure 10). Similar to *coi1-1* mutant plants, *ORA59*-silenced plants were strongly impaired in *ORA59* and ORA59 target gene expression in response to *A. brassicicola* (Figure 10), consistent with the results from *B. cinerea* infection.

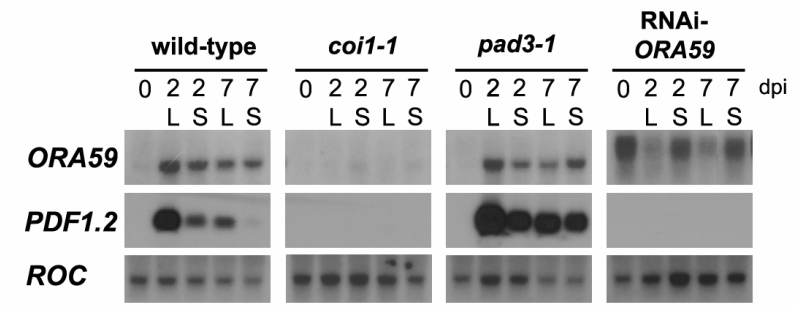

Figure 10. *PDF1.2* gene expression induced by infection with *Alternaria brassicicola* is impaired in *ORA59*-silenced plants. Four-weeks-old Arabidopsis wild-type plants, *coi1-1* and *pad3-1* mutant plants and transgenic plants silencing the *ORA59* gene (RNAi-*ORA59*) were inoculated with *A. brassicicola*. Three-µL droplets containing 10^6 spores/mL were applied on mature leaves. Infected (L) and non-infected (S) leaves of several inoculated plants of each genotype were collected at day 0, day 2 and day 7 after inoculation and RNA was extracted. The RNA gel blot was hybridized with the indicated probes. The *ROC* probe was used to verify RNA loading.

Hybridization with the *ROC* gene showed equal loading of RNA. Thus, although ORA59 is responsible for defense gene expression and the *ORA59* and *PDF1.2* mRNA levels are increased after *A. brassicicola* infection, ORA59 and its target genes do not play an important role in the resistance against this pathogen under the conditions of this experiment.

Resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was found to be affected both in plants impaired in salicylic acid (SA) signaling and in JA and ethylene response mutants (Pieterse et al., 1998), suggesting that resistance against this pathogen is controlled by a combined action of SA, JA and ethylene. To characterize the involvement of ORA59 in resistance against *P. syringae*, above-ground parts of wild-type and transgenic plants overexpressing *ORA59* were inoculated with this pathogen and infection rate was determined by scoring disease symptoms on the leaves. Transgenic plants did not show elevated resistance against *P. syringae* compared to wild-type plants, indicating that ORA59 does not control the genes essential for the JA-dependent defense response against *P. syringae* (data not shown). These results indicate that ORA59 regulates genes that are specific for defense against particular pathogens, such as *PDF1.2*, *ChiB* and *HEL* in the case of *B. cinerea*.

Which AP2/ERF-domain transcription factors regulate *PDF1.2*?

Our results show that ORA59 is the crucial regulator of several defense-related genes including *PDF1.2.* Loss-of-function studies revealed that only ORA59, and no other AP2/ERF-domain transcription factor or member of another class of transcriptional regulators, was sufficient to activate the expression of *PDF1.2* in response to JA and ethephon treatments (Figure 6A) or to infection with *B. cinerea* (Figure 9C) or *A. brassicicola* (Figure 10). These results appear to contradict a previous report showing that constitutive overexpression of the AP2/ERF-domain transcription factor *ERF1* gave rise to elevated *PDF1.2* gene expression (Solano et al., 1998). Constitutive overexpression of another AP2/ERF-domain transcription factor, *AtERF2*, was also reported to lead to an increase in *PDF1.2* gene expression (Brown et al., 2003). Similarly, constitutive overexpression of *AtERF1*, a close homologue of *AtERF2*, led to high *PDF1.2* expression levels (Figure 11A). One possible explanation for the apparent discrepancy with our results is that overexpression of *ERF1*, *AtERF*1 or *AtERF2* causes a stress condition, manifested also by the dwarf phenotype, which indirectly leads to *PDF1.2* expression. To address the question which AP2/ERF-domain proteins directly control the activity of the *PDF1.2* promoter, we used two different approaches. In one approach, we measured the expression of the *PDF1.2* gene in stably transformed Arabidopsis plants expressing *ORA59, ERF1, AtERF1* and *AtERF2* in an estradiol-inducible manner. Under non-induced conditions, the *AP2/ERF* transgene is silent.

We speculated that the relatively short period of transgene expression in response to estradiol treatment is unlikely to cause a general stress condition, and that therefore nonspecific activation of *PDF1.2* gene expression is less likely to occur in these plants. As shown in Figure 11B, estradiol treatment effectively induced the expression of the *ORA59, ERF1, AtERF1* and *AtERF2* transgenes in the different lines to essentially similar levels. The XVE-*ORA59-TAP* and XVE-*ERF1-TAP* lines carry an inducible expression module of the *ORA59* and *ERF1* genes, respectively, fused to a *TAP* tag (Puig et al., 2001). Expression of the *PDF1.2* gene was only induced by estradiol in XVE-*ORA59*, XVE-*ORA59-TAP* and XVE-*ERF1-TAP* lines. The transcript level of the *PDF1.2* gene in the estradiol-treated XVE-*ORA59-TAP* line was similar to that in the estradiol-treated XVE-*ORA59* line, indicating that ORA59 activity was not significantly affected by the fusion with a TAP tag. Similarly, ERF1 activity appears to be preserved in the TAP fusion protein. Two to three independent lines per construct were tested with essentially similar results (data not shown).

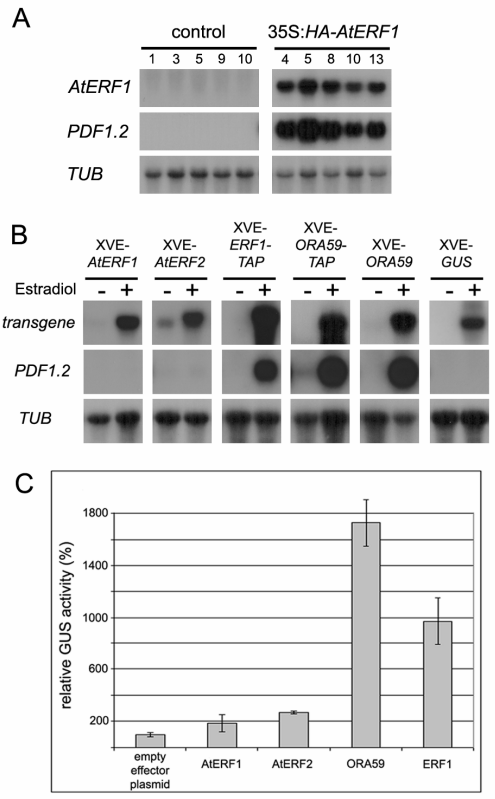

Figure 11. ORA59 and ERF1 are activators of *PDF1.2* gene expression *in planta.* **(A)** *PDF1.2* gene expression in *AtERF1*-overexpressing plants. RNA was extracted from ten-days-old seedlings from several independent 35S:*HA-AtERF1* and control lines (indicated by numbers). **(B).** Estradiol-induced *PDF1.2* gene expression in stably transformed XVE lines. Two-weeks-old Arabidopsis seedlings from transgenic lines carrying the *AtERF1*, *AtERF2*, *ORA59* or *GUS* gene under the control of the inducible XVE system (XVE-*AtERF1* #1, XVE-*AtERF2* #1, XVE-*ORA59* #10 and XVE-*GUS* #7, respectively) or carrying the *ORA59* or *ERF1* gene fused to the *TAP* tag under the control of the inducible XVE system (XVE-*ORA59-TAP* #17 and XVE-*ERF1-TAP* #16, respectively) were treated for 24 hours with estradiol (+) or the solvent DMSO (-). The top panels were hybridized with separate AP2/ERF or GUS probes and exposed for 8 - 24 hours. Expression levels cannot be directly compared but are similar within a 3-fold change. The *PDF1.2* and *TUB* panels were hybridized on the same blot and expression levels can be directly compared among individual transgenic lines. The *TUB* probe was used to verify RNA loading. **(C).** Arabidopsis protoplasts were co-transformed with a reporter plasmid carrying *PDF1.2*-promoter-*GUS* and effector plasmids carrying the *AtERF1*, *AtERF2*, *ERF1* or *ORA59* gene fused to the CaMV 35S promoter. GUS activities are shown as percentages of the empty effector plasmid value. A reference plasmid carrying the Renilla *LUC* gene fused to the 35S promoter was co-transformed to correct for transformation and protein extraction efficiencies. Values represent means ± SE of triplicate measurements.

In general, we observed that *PDF1.2* gene expression level was slightly higher in XVE-*ORA59* lines compared to XVE-*ERF1* lines. In a second approach, we analyzed the ability of ORA59, ERF1, AtERF1 and AtERF2 to transactivate the *PDF1.2* promoter in transient expression assays. Arabidopsis protoplasts were co-transformed with a reporter plasmid carrying the *PDF1.2* promoter fused to the *GUS* reporter gene, and an effector plasmid carrying *ORA59, ERF1, AtERF1* or *AtERF2* genes fused to the CaMV 35S promoter. *GUS* reporter gene activity was increased about 17- and 10-fold upon co-transformation with *ORA59* and *ERF1* effector plasmids, respectively, compared to the empty effector plasmid (Figure 11C). No significant activation of the *GUS* reporter gene was observed with effector plasmids carrying *AtERF1* or *AtERF2* genes. Both experimental approaches demonstrate that ORA59 and ERF1 are able to function as transcriptional activators of *PDF1.2* gene expression, whereas AtERF1 and AtERF2 do not activate *PDF1.2* gene expression when inducibly or transiently expressed.

***ORA59* overexpression does not repress *VSP1* expression in response to JA**

In transgenic plants overexpressing the *ERF1* gene, JA treatment failed to express efficiently the JA-responsive *VSP2* gene, encoding the vegetative storage protein 2 (Lorenzo et al., 2004), suggesting that ERF1 represses a branch of the JA signaling pathway responsible for the expression of a subset of JA-responsive genes including *VSP2*. This prompted us to test whether ORA59 could also act as a negative regulator of that part of the JA response. Two-weeks-old transgenic XVE-*ORA59* line #6 was treated with estradiol for 16 hours prior to treatment with JA for 8 hours. Transgenic XVE-*ERF1-TAP* line 16 was used as control for the negative effect of *ERF1* overexpression on *VSP* expression in response to JA. Expression of the *VSP1* gene was analyzed by RNA gel blot hybridization and *VSP1* transcript levels in the XVE-*ORA59* lines were compared to those observed in the XVE-*ERF1-TAP* line and in a XVE-*GUS* control line.

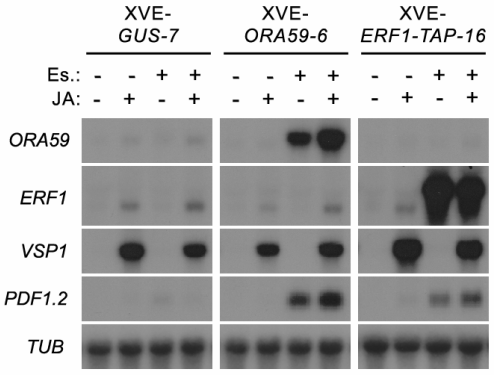

Figure 12. *ORA59* overexpression does not repress *VSP1* expression in response to JA. Two-weeks-old inducible XVE-*ORA59* (line #6), XVE-*ERF1-TAP* (line #16) and XVE-*GUS* (line #7) transgenic Arabidopsis plants were treated for 16 hours with 4 µM estradiol (Es.; +) or with DMSO (-) prior to treatment for 8 hours with 50 µM jasmonic acid (JA; +) or with DMSO (-). The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

The *VSP1* and *VSP2* nucleotide sequences share 93% identity and therefore it is likely that hybridization with the complete coding sequence of the *VSP1* gene would also detect the *VSP2* mRNA. As shown in Figure 12, JA treatment induced the expression of the *VSP1* gene in all lines. Estradiol-induced overexpression of *ORA59* prior to JA treatment did not result in reduced *VSP1* expression in the JA-treated XVE-*ORA59* line, demonstrating that *ORA59* overexpression does not lead to reduced *VSP1* gene expression in response to JA. This shows that under our experimental conditions, ORA59 does not act antagonistically on the JA signaling pathway involved in the activation of a subset of genes, including the *VSP* genes. This result is consistent with the observation that silencing of the *ORA59* gene in plants did not lead to a significant increase in *VSP1* expression in response to JA compared to control plants (Figure 6). Surprisingly, overexpression of *ERF1* prior to JA treatment failed to repress *VSP1* expression in the JA-treated XVE-*ERF1-TAP* line under our experimental conditions. This finding is in contradiction with the previous results showing that constitutive overexpression of *ERF1* inhibits *VSP* induction in response to JA (Lorenzo et al., 2004). After 16 hours of treatment with 4 µM estradiol, the transgene in XVE lines is expressed at a level that is at least, similar to that obtained in transgenic lines overexpressing a transgene under the control of the strong constitutive promoter CaMV 35S (Chapter 5). Estradiol-induced expression of *PDF1.2* indicates that both ORA59 protein and ERF1-TAP fusion protein are functional in their respective line (as demonstrated in Figure 11). Identical results were obtained with XVE-*ORA59* line 10, XVE-*ORA59-TAP* lines 4 and 17, and XVE-*ERF1-TAP* line 2 (data not shown).

Discussion

Jasmonates are key regulatory signaling molecules in plant defense. JAs interact with ethylene in the regulation of plant wound and defense responses against pests or pathogen attack. An important aspect of JA- and ethylene-dependent defense responses is their synergistic interaction in the induction of many defense-related genes.

In this study, we investigated the function of ORA59, a member of the AP2/ERF-domain transcription factor family in Arabidopsis. Here, we demonstrate that ORA59 integrates JA and ethylene signal inputs. By doing so, ORA59 controls the expression of a subset of JA- and ethylene-dependent genes, including *PDF1.2*. Expression of these genes in response to these signals, or after perception of certain pathogens, strictly depends on ORA59, and no other transcription factor, including ERF1 or AtERF2, can bypass the requirement for ORA59.

Transcriptome analysis is a powerful tool to unravel transcription factor function and to identify genes positively or negatively regulated by a given transcription factor. Most microarray analyses are set up as comparisons of transcript profiles in plants constitutively overexpressing the transcription factor of interest against a certain control genotype, such as the wild-type background. Despite the many advantages of the constitutive overexpression strategy, one needs to be cautious when interpreting phenotypes that are caused by the constitutive overexpression of transcription factors. Due to its abundance or inappropriate expression in time and space, the protein could exert new activities that it does not in the wild-type (Zhang, 2003). Constitutive overexpression of *ORA59* caused dwarfism. We speculated that this severe phenotype is due to a general stress condition caused by the constitutive activation of ORA59-regulated genes, and that this neomorphic phenotype is likely to cause the activation of a variety of genes which are not directly controlled by ORA59. Therefore we used transgenic Arabidopsis plants in which the *ORA59* gene was under the control of an inducible promoter to increase the likelihood that upregulated genes are directly controlled by ORA59. One advantage is that, in the non-induced condition, plants are similar to wild-type, as the *ORA59* transgene is silent, thereby avoiding neomorphic phenotypes. A second advantage is that the microarray experiments were performed by comparing RNA samples from induced and non-induced plants of the same genetic background, limiting changes in expression of unspecific genes due to the transgene insertion site in the genome or other differences between independent transgenic lines. Therefore, we speculate that ORA59 is likely to be a direct regulator of the majority, if not all, of the genes identified by the microarray experiment.

Genome-wide transcriptome analyses revealed that overexpression of *ORA59* positively modulated the expression of a large number of genes involved in defense (including *PDF1.2, ChiB* and *HEL*), cell signaling and protein modification (including genes encoding kinases and phosphorylases) and shikimate/ tryptophan biosynthesis (such as *ASB1*, *TSB1* and *IGPS*). The involvement of ORA59 in integration of ethylene and JA signal inputs was supported by the observation that the majority of the ORA59-upregulated genes were also induced in wild-type plants treated with JA alone or in combination with ethylene, as revealed by microarray experiments. In fact, it is entirely possible that all of the ORA59-upregulated genes are regulated by JA and/or ethylene at earlier or later time points than those selected for the microarray experiments (8 and 24 hours).

Supporting evidence for the regulatory role of ORA59 was provided by the findings that the JA- and/or ethylene-induced expression of the putative ORA59-regulated genes, including *PDF1.2, AN-5AT* and *HEL*, was severely compromised in *ORA59*-silenced plants. In contrast, expression of two other reported putative regulators of the *PDF1.2* gene, *ERF1* and *AtERF2*

(Solano et al., 1998: Brown et al., 2003) was still induced by JA and/or ethylene treatments in *ORA59*-silenced plants, indicating that ERF1 or AtERF2 are unable to induce *PDF1.2* expression in response to JA or ethylene in these plants. The transcription factor ERF1 was suggested to be a key element in the integration of JA and ethylene signals for the regulation of defense genes (Lorenzo et al., 2003). However, our results demonstrate that it is ORA59, and not ERF1 or any other transcription factor, that integrates both signals in order to activate JA- and ethylene-responsive genes, such as *PDF1.2*, leading to adapted defense responses. By means of two different approaches, only ORA59 and ERF1 were able to activate *PDF1.2* expression in Arabidopsis, whereas AtERF2, or its close homologue AtERF1, did not activate *PDF1.2* gene expression. This suggests that the observations that constitutive overexpression of *AtERF1* (this study) or *AtERF2* (Brown et al., 2003) induced the expression of *PDF1.2* in stably transformed plants, are artefacts that are likely caused by non-specific stress effects. All of our data on ERF1 are consistent with those reported by Lorenzo et al. (2003). Although these data therefore appear to be correct, additional data obtained from plants impaired in *ERF1* expression is needed to assign a proper function for ERF1. From our findings, we assume that it is not ERF1, but ORA59, that integrates JA and ethylene signal inputs to regulate defense gene expression under conditions where JA and ethylene were exogenously applied to young seedlings, or in response to infection with *B. cinerea* or *A. brassicicola* (conditions similar to those used by Lorenzo et al.). This further supports the necessity of interpreting constitutive overexpression results in conjunction with data from other approaches, such as inducible overexpression, or analysis of RNAi or knock-out mutants.

Defense against infection by the necrotrophic fungi *B. cinerea* and *A. brassicicola* is dependent on both JA and ethylene signaling, and mutations in either of these signal transduction pathways increase susceptibility (Thomma et al., 1998 and 1999a; Penninckx et al., 1996). Overexpression of *ORA59* increases resistance to *B. cinerea*, whereas plants silencing the *ORA59* gene show increased susceptibility to this pathogen, presumably by impaired expression of the ORA59-regulated defense genes. Several *B. cinerea* susceptibility loci have been identified in Arabidopsis (Mengiste et al., 2003; Veronese et al., 2004). The *bos1* (*Botrytis susceptible1*) mutant was found to be affected in resistance against *B. cinerea*. The *BOS1* gene encodes a R2R3MYB transcription factor. However, the *BOS1* gene seems to be involved in resistance against a broad range of biotic and abiotic stresses. Furthermore, *PDF1.2* transcript levels were similar in *bos1* mutants compared to wild-type in response to *B. cinerea* (Mengiste et al., 2003), indicating that the R2R3MYB transcription factor BOS1 regulates a different set of genes than ORA59 does. The *bos3* mutant shows a dramatic decrease in *PDF1.2* expression in response to *B. cinerea* infection, similar to the decrease

observed in *ORA59*-silenced plants. The *BOS3* locus maps to a region on chromosome 1, which is close to the *ORA59* gene location (Veronese et al., 2004). This suggests that the *bos3* mutation affects the *ORA59* gene. However, the different phenotypic responses to *A. brassicicola* infection observed in *bos3* mutants (susceptible; Veronese et al., 2004) and in *ORA59*-silenced plants (resistant) indicate that the *bos3* mutation does not affect the *ORA59* gene.

Surprisingly, the *ORA59*-silenced plants showed resistance to *A. brassicicola* infection, similar to wild-type Col-0 plants. ORA59 and its target genes are unlikely to play a role in defense against this pathogen, under the conditions of this disease resistance test, as their gene expression patterns are not correlated with *A. brassicicola* susceptibility. In addition to camalexin production, presumably other JA- and ethylene-dependent defense responses, regulated by another transcription factor than ORA59, are required for effective resistance against this necrotrophic pathogen.

The AP2/ERF-domain transcription factors ORA59 and ERF1 appear to regulate the same set of JA- and ethylene-responsive genes (Table 1; Lorenzo et al., 2003), suggesting functional redundancy of these two proteins. However, our loss-of-function results with *ORA59*-silenced lines clearly demonstrate that ORA59 is absolutely necessary for expression of these defense genes in response to hormone treatments and *B. cinerea* or *A. brassicicola* infection, and that ERF1 is not involved in defense gene expression in response to these signals. Our findings are summarized in the model in Figure 13.

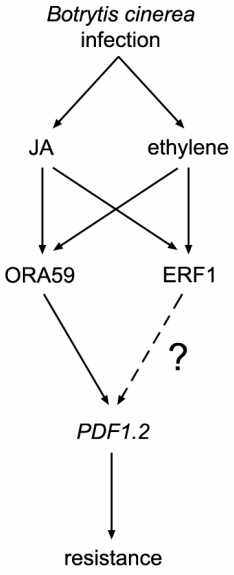

Figure 13. Model for the involvement of ORA59 and ERF1 in response to *Botrytis cinerea* in Arabidopsis. In wild-type plants, infection with *B. cinerea* activates both JA and ethylene signaling pathways and leads to the expression of *ORA59* and *ERF1* genes. In infected *ORA59*-silenced lines, the absence of ORA59 results in impaired expression of the *PDF1.2* gene, as well as increased susceptibility against the pathogen. In these lines, the JA- and ethylene-induced expression of *ERF1* does not lead to *PDF1.2* expression, demonstrating that ORA59 is the key regulator that is strictly required for the integration of the JA and ethylene signal inputs, resulting in gene activation. The exact role of ERF1 in defense awaits analyses of gene expression and pathogen susceptibility in plants impaired in *ERF1* expression.

The exact function of ERF1 in defense gene expression awaits analysis of ERF1 loss-of function plants generated via knockout or RNAi. ERF1 has been suggested to be part of a linear ethylene signal transduction cascade in which the transcription factor EIN3 was suggested to induce *ERF1* gene expression in response to ethylene (Solano et al., 1998). It is unlikely that ORA59 functions upstream or downstream from ERF1 in such a linear cascade since *ORA59* and *ERF1* gene expression was unchanged in induced XVE-*ERF1* and XVE-*ORA59* transformants, respectively (unpublished results). One possibility is that ERF1 has no essential function. Alternatively, ERF1 and ORA59 might have separate specialized functions, for example by differential expression of the corresponding genes in certain cell types or at certain developmental stages. Our results show that some discrepancy exists between ORA59 and ERF1 functions. Overexpression of *ORA59* and *ERF1* activated the expression of the basic endochitinase *ChiB* gene (*b-CHI*; Table 1; Solano et al., 1998). In the *coi1* mutant background, *ORA59* overexpression resulted in high expression levels of the target genes *PDF1.2* and *HEL* (Figure 4), demonstrating that ORA59 functions downstream from COI1 as the terminal integrator of the JA and ethylene signal inputs leading to *PDF1.2* and *HEL* gene expression. In contrast, *ORA59* overexpression failed to induce *ChiB* expression in the *coi1* mutant (Figure 4), indicating that the ORA59-mediated regulation of the *ChiB* gene is controlled by COI1-dependent signaling events. A similar experiment performed with *ERF1* showed that *ERF1* overexpression induced *ChiB* expression without the requirement for COI1 (Lorenzo et al., 2003), suggesting a distinct role between ORA59 and ERF1 in the regulation of a number of JA- and ethylene-responsive genes including *ChiB*.

Lorenzo et al. (2004) showed that the JA-induced expression of the *VSP2* gene, encoding a vegetative storage protein, was largely prevented in constitutive *ERF1*-overexpressing plants, suggesting a role for ERF1 in repressing the JA-induced expression of a subset of JA-responsive genes. In our study, a similar experimental set-up showed that *ORA59* overexpression had no effect on the JA-induced expression of the *VSP1* gene, a close homologue of *VSP2*. Similarly, JA-induced *VSP1* expression level in ORA59-silenced plants was comparable to that in control plants. These results suggest that ORA59 and ERF1 have distinct roles in JA and ethylene signaling. However, *ERF1* overexpression could not suppress the JA-induced expression of *VSP1* within a period of 8 hours of JA treatment, contradicting the findings of Lorenzo et al. (2004). Our results indicate that the negative role of ERF1 on a subset of JA-responsive genes can not be attributed to a direct effect of *ERF1* overexpression on the expression of these genes, but might be due to a late response

resulting from *ERF1* overexpression. The discrepancy of the findings from constitutive and inducible *ERF1*-overexpressing plants may also suggest that the results observed in 35S:*ERF1* transgenic plants are artifacts due to non-specific stress effects (discussed above).

Materials and Methods

Biological materials, growth conditions and treatments

Arabidopsis thaliana wild-type plants, *coi1-1*, *ein2-1* and *pad3-1* mutants, and all transgenic plants are in the genetic background of ecotype Col-0. Seeds were surface-sterilized by incubation for 1 minute in 70 % ethanol, 15 minutes in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for three hours (http://plantpath.wisc.edu/~afb/vapster.html).

Surface-sterilized seeds were transferred to 250 ml Erlenmeyer flasks containing 50 ml MA medium (Masson and Paszkowski, 1992) or grown on plates containing MA medium supplemented with 0.6% agar. Transgenic plants from T2 or T3 generations were selected on solid MA medium containing 100 mg/L timentin and either 25 mg/L kanamycin for ORA59-silenced plants or 20 mg/L hygromycin for ORA59-overexpressing plants. Following stratification for 3 days at 4ºC, seeds were incubated at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days or otherwise as indicated. Seeds in liquid medium were placed on a shaker at 120 rpm. Alternatively, seedlings were first selected on solid MA medium supplemented with the above mentioned appropriate antibiotics or 50 µM JA (for selection of *coi1-1* seedlings) for 10 days, after which 15 to 20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml MA medium and incubated on a shaker for 4 additional days before treatment. Seedlings were treated for different time periods with 50 µM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.1% final concentration), 1 mM of the ethylene-releasing compound ethephon (Sigma) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) or a combination of JA and ethephon. As controls, seedlings were treated with 0.1% DMSO, 0.5 mM sodium phosphate pH 7 or a combination of both. Transgenic plants carrying an XVE expression module containing the *ORA59*, *GUS*, *AtERF1* or *AtERF2* gene were treated with 2 µM estradiol (Sigma) dissolved in DMSO (0.2% final concentration). In order to reach similar expression levels of the transgene, transgenic plants carrying an XVE expression module containing the ORA59 or ERF1 gene fused to the TAP tag were treated with 4 µM estradiol. As control, seedlings were treated with 0.2% DMSO.

Botrytis cinerea and *Alternaria brassicicola* strain MUCL 20297 were grown on potato dextrose agar plates for 2 weeks at 22°C. Spores were harvested as described by Broekaert et al. (1990).

Binary constructs and plant transformation

The *ORA59* (*At1g06160*) open reading frame (ORF) was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-CGG GAT CCA TAT GGA ATA TCA AAC TAA CTT C-3' and 5'-CGG GAT CCT

CAA GAA CAT GAT CTC ATA AG-3'. The *ORA59* ORF digested with BamHI was inserted in pBluescript SK+ (Stratagene, La Jolla, CA) such that the 5'-end of the *ORA59* ORF was flanking the HindIII restriction site to generate pSK-ORA59.

For the construction of transgenic lines constitutively overexpressing *ORA59*, the *ORA59* ORF digested with BamHI was cloned into pRT101 (Töpfer et al., 1987) to generate pRT101-ORA59. The Cauliflower Mosaic Virus (CaMV) 35S cassette containing the *ORA59* ORF in sense orientation was excised with PstI and cloned into the binary vector pCAMBIA1300 (accession number AF234296). The binary vector pCAMBIA1301 (accession number AF234297) carrying the *GUS* gene under the control of the CaMV 35S promoter was used to generate control lines (1301 lines).

For the construction of transgenic lines showing post-transcriptional silencing of the *ORA59* gene, the *ORA59* ORF digested with BamHI was cloned into pIC-20H (Marsh et al., 1984) digested with BglII such that the 5'-end of the *ORA59* ORF flanked the EcoRI restriction site. The *ORA59* insert was excised from pIC-20H with EcoRI/XhoI and cloned into the pHANNIBAL vector (accession number AJ311872) to generate pHAN-ORA59as. To create an inverted repeat, the *ORA59* ORF was excised from pSK-ORA59 with XbaI/HindIII and cloned into pHAN-ORA59as to generate pHAN-ORA59sas. For the construction of the RNAi control line *GUS* #5, 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 (XVE), 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 *AtERF1* and *AtERF2* open reading frames were amplified by PCR from Arabidopsis genomic DNA using the following primer sets respectively: 5'-CGG GAT CCA TAT GAC GGC GGA TTC TCA ATC-3' and 5'-CGG GAT CCT TAT AAA ACC AAT AAA CGA TC-3'; 5'-CGG GAT CCA TAT GTA CGG ACA GTG CAA TAT AG-3' and 5'-CGG GAT CCT TAT GAA ACC AAT AAC TCA TC -3'. *AtERF1* and *AtERF2* were cloned as BamHI fragments into pBluescript SK+. The *ORA59*, *AtERF2* and *GUS* ORFs were excised from pRT101-ORA59, pSK-AtERF2 and pSK-GUS respectively, with XhoI/XbaI and cloned into the binary vector pER8 (Zuo et al, 2000) digested with XhoI/SpeI. The *AtERF1* ORF was excised from pSK-AtERF1 with ApaI/SpeI and cloned into pER8 digested with the same enzymes.

For the construction of the XVE-*ERF1-TAP* and XVE-*ORA59-TAP* lines, the *ERF1-TAP* and *ORA59-TAP* cassettes were created by mutating the stop codon at the 3' of the *ERF1* and *ORA59* genes and then fused in frame with a *TAP* tag at the 3'end, generating a protein-tag fusion with the tag at the C-terminus. *ERF1* and *ORA59* were amplified by PCR with the primer set 5'-ACG CGT CGA CAA AAT GGA CCC ATT TTT AAT TCA GTC C- 3' and 5'-CCG CTC GAG CCT TGC CAA GTC CCA CTA TTT TC- 3' and 5'-ACG CGT CGA CAA AAT GGA ATA TCA AAC TAA CTT C- 3' and 5'- CCG CTC GAG CCT TGA GAA CAT GAT CTC ATA AG-3', respectively, and cloned in pGEM-T Easy (Promega, Madison, WI). The *TAP* insert was excised from pBS1479 (Puig et al., 2001) with BglII and cloned into pCAMBIA1300 digested with BamHI. The *ERF1-ΔSTOP* and *ORA59-ΔSTOP* inserts were excised from pGEM-T Easy with SalI/XhoI and cloned into pCAMBIA-*TAP*. The *ERF1-TAP* and *ORA59-TAP* cassettes were excised with SalI/SpeI from pCAMBIA1300-TAP and introduced into the binary vector pER8 digested with XhoI/SpeI.

For the construction of the constitutive *AtERF1*-overexpressing plants, *AtERF1* (*At4g17500*) was amplified by PCR on genomic DNA with the primer set 5'- GGG GTA CCA AAA TGT ACC CAT ACG ATG TTC CAG ATT ACG CTG GTT ACC CAT ACG ATG TTC CAG ATT ACG CTG AGC TCA TGA CGG CGG ATT CTC AAT C–3' and 5'-CGG GAT CCT TAT AAA ACC AAT AAA CGA TC-3' and cloned in pGEM-T Easy. The resulting sequence encodes the AtERF1 protein with a double haemagglutinin (HA) epitope tag at its N-terminal end. The *HA-AtERF1* insert was excised with KpnI/BamHI and cloned into pRT101. The CaMV 35S-cassette containing *HA-AtERF1* was excised with HindIII and introduced into pCAMBIA1300.

The binary vectors pCAMBIA1300-ORA59, pCAMBIA1300-HA-AtERF1 and pCAMBIA1301 were introduced into *Agrobacterium tumefaciens* strain LBA1115 (containing the Vir plasmid pSDM3010). The binary vectors pART27-ORA59, pART27-GUS, pER8-ORA59 and pER8-AtERF2 were introduced into *A. tumefaciens* strain LBA4404 while pER8-GUS, pER8-AtERF1, pER8-ERF1-TAP and pER8-ORA59-TAP 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.

The XVE-*ORA59*; *coi1-1* plants were obtained by fertilizing homozygous *coi1-1* ovules with pollen from transgenic XVE-*ORA59* plants. Heterozygous *coi1*/COI1 F1 siblings containing the transgene were selected on MA medium containing 20 mg/L hygromycin and were allowed to selfpollinate. F2 siblings homozygous for the *coi1* mutation and carrying the XVE-*ORA59* transgene were selected on MA medium containing 50 µM JA and 20 mg/L hygromycin.

Plant infection with pathogens

All genotypes were grown for 2 weeks on solid MA medium containing suitable antibiotics at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux). Seedlings were transferred to sterile soil and cultivated for another 3 weeks in a growth chamber with a 8 h day (1400 lux at 24ºC) and 16 h night (20ºC) cycle at 65% relative humidity. Due to their early stage dwarf phenotype, seeds from the 35S:*ORA59 #17* transgenic plants were sown 2 weeks earlier than the other genotypes. Soil-potted plants from this genetic background were therefore allowed to grow 5 weeks in order to reach a stage with rosette leaf size suitable for pathogen infection.

For inoculation with fungal pathogens, 3-µL droplets of spore suspension were deposited on 4-6 mature leaves of each plant. Inocula used were 5.10^5 and 1.10^6 spores/mL for *Botrytis cinerea* and *Alternaria brassicicola*, respectively. *B. cinerea* spores were incubated in half-strength potato dextrose broth for 2 hours prior to inoculation. Moreover, *Botrytis*-infected leaves were gently wounded with a needle where the spore-containing droplet was deposited. After inoculation, plants were maintained under high relative humidity with the same temperature and photoperiod conditions. In each experiment, 15-20 plants per genotype were inoculated.

Disease ratings were assessed at day 4 and day 7 after inoculation with *B. cinerea* or *A. brassicicola*, respectively. Disease ratings were assigned to the inoculated leaves of each plant, as described by Ton et al. (2002) with minor modifications. Briefly, intensity of disease symptoms and lesion size were classified: I, no visible disease symptoms; II, non-spreading lesion; III, spreading lesion; IV, spreading

lesion surrounded by a chlorotic halo; and V, spreading lesion with extensive tissue maceration and sporulation by the pathogen. For gene expression analysis, infected and non-infected leaves from several inoculated plants of each genotype were collected at day 2 and day 4 for *B. cinerea*-infected plants, and at day 2 and day 7 for *A. brassicicola*-infected plants. Leaf tissue was frozen in liquid nitrogen and stored at –80ºC.

Northern blot analyses

Total RNA was extracted from frozen tissue by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70 % ethanol, and resuspended in water. As described by Memelink et al. (1994), 10 µg RNA samples were subjected to electrophoresis on 1.5% 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 (Chapter 3). Blots were exposed on X-ray films (Fuji, Tokyo, Japan).

DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5'- GGG GTA CCG GAT CCT CTT AAG TGG AGA GTT TTC C-3' and 5'- GCT CTA GAC TCG AGG CCA CGG TGG CTT CTT TTC C-3' for *ORA59* (*At1g06160*); 5'- GGG GTA CCG GAT CCA CGG CGG ATT CTC AAT CTG ATT ATG-3' and 5'- GCT CTA GAC TCG AGC TAA CAT ATC CTC AGA ATC G-3' for *AtERF1* (*At4g17500*); 5'- GGG GTA CCG GAT CCA TGT ACG GAC AGT GCA ATA TAG-3' and 5'- GCT CTA GAC TCG AGC GAT GAT GAC GTG TCA AAA TGG-3' for *AtERF2* (*At5g47220*); 5'- GGG GTA CCG GAT CCT TAA TAA CGG CGT TAA CTC ACG-3' and 5'- GCT CTA GAC TCG AGT CGC CGG AGG CTT ATT TTC CTC-3' for *ORA4* (*At2g44840*); 5'- GGG GTA CCG GAT CCA GAT CCA TGG ATG AAG CAC GAA TC-3' and 5'- GCT CTA GAC TCG AGA ATG GCG GTT TAC GAA TTT GAG-3' for *ORA31* (*At5g47230*); 5'-AAT GAG CTC TCA TGG CTA AGT TTG CTT CC-3' and 5'-AAT CCA TGG AAT ACA CAC GAT TTA GCA CC-3' for *PDF1.2* (*At5g44420*); 5'-GCT TCA GAC TAC TGT GAA CC-3' and 5'-TCC ACC GTT AAT GAT GTT CG-3' for *ChiB* (*At3g12500*); 5'-CGG GAT CCA TAT GAA GAT CAG ACT TAG CAT AAC-3' and 5'-CGG GAT CCT CAA ACG CGA TCA ATG GCC GAA AC-3' for *HEL* (*At3g04720*); 5'-TGT CCC ACT CTC GTT CTT TG-3' and 5'-TCA AGT CCG GCT GGA ACA TTG-3' for *AN5-AT* (*At5g61160*); 5'-ATG ACA AGC TCC GAT CAA TC-3' and 5'-TCA AGC CGA AGC CGG AGA AG-3' for *AIG2* (*At3g28930*); 5'-ATG GCG GCT TCT ACA TTG TAC-3' and 5'-CTA TGT CAG CTT CTC GGA CTC-3' for *ASB1* (*At1g25220*); 5'-CAG GCA CCT CTG CTA CTT TC-3' and 5'-ATA GCT CCA TGG AGG ACA CC-3' for *TSB1* (*At5g54810*); 5'-GCA ATT CTC GAT CCG AGC TC-3' and 5'-CTC TAC TTG GAG AAG CCT TC-3' for *IGPS* (*At2g04400*); 5'-CAG TCC TTT ACT GGC CAA ATG- 3' and 5'- GGG TCT CTA AGC ATC TTT CC- 3' for *DIN11* (*At3g49620*); 5'-TCC ACC AGA TCT ATC TAC GG- 3' and 5'- GCA GCG TAA CCT CCA GTG GC- 3' for *DHS1* (*At4g39980*); 5'-ATG GGA ACA GCG TCG TCT AAG- 3' and 5'- GAG GTT GAC TGG TGA TCC TTC- 3' for *CYP1* (*At4g22710*); 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'-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*); 5'-CGG GAA GGA TCG TGA TGG A-3' and 5'-CCA ACC TTC TCG ATG GCC T-3' for *ROC* (*At4g38740*). The BamHI/NcoI

and EcoRI/HindIII fragments from the *ERF1* (*At3g23240*) and *actA* (Benito et al., 1998) open reading frames, respectively, were used as probes.

Microarray analyses

Wild-type plants (Columbia ecotype), two transgenic Arabidopsis lines expressing *ORA59* in an inducible manner (XVE-*ORA59-6* and *-10*) and two transgenic control lines expressing the *GUS* gene in an inducible manner (XVE-*GUS-7* and *-15*) were used for microarray experiments. Biological samples containing 15-20 seedlings were grown for 2 weeks in polypropylene tubes containing liquid MA medium as described above. Three replicates were used for each treatment or time point. After treatment with estradiol, JA or JA/ethephon (or with appropriate control solutions), total RNA was extracted as described above and subsequent purification was performed using NucleoSpin RNA II columns (Macherey-Nagel, Düren, Germany). RNA integrity of each biological sample was checked using Agilent Lab-on-a-chip 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA). RNAs of the three replicates were then pooled and checked again for RNA integrity. From each pooled sample, 20 µg were labeled using the fluorescent direct label method as described by the supplier (Agilent Technologies). Control-treated samples were Cy5-labeled whereas estradiol-, JA- or JA/ethephon-treated samples were Cy3-labeled. Hybridizations were performed using Agilent Arabidopsis 3 Oligo (44K) Arrays according to manufacturer's recommendations. Scan images were converted to raw data with the Agilent Feature Extraction software (version 7.5) using default parameters. Data were analyzed using the Rosetta Resolver software (Agilent Technologies). For the microarray experiments using wild-type plants, duplicate arrays were performed for each condition using RNA samples from independent plants. To increase confidence in measurements, log ratio values of the two replicates were combined using default parameters of the Rosetta Resolver software.

Data obtained from the two XVE-*GUS* hybridizations were used to remove genes with differential expression presumably due to estradiol or due to the estradiol-activated chimeric XVE transcription factor (Zuo et al., 2000). Genes were considered "induced" when they were upregulated in both XVE-*ORA59* hybridization experiments and not induced in either of the XVE-*GUS* hybridization experiments. Log ratio values of the "induced" genes from the two XVE-*ORA59* lines were combined. Expression differences with fold-change ≥ 2 and with *P*-values ≤ 0.001 were considered significant. The raw data files plus the details of the labelling and hybridization experiments have been deposited in the public microarray database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-460.

Transient expression assays

A 1183 base pair fragment of the *PDF1.2* (*At5g44420*) promoter was amplified by PCR on genomic DNA with the primer set 5'-CGG GAT CCA TGC AGC ATG CAT CGC CGC ATC -3' and 5'-GCG TCG ACG ATG ATT ATT ACT ATT TTG TTT TCA ATG -3' and cloned in pGEM-T Easy (Promega). The *PDF1.2* insert was excised with BamHI/SalI and cloned in the vector GusXX (Pasquali et al., 1994). Protoplasts prepared from *Arabidopsis thaliana* cell suspension ecotype Col-0 were co-transformed with plasmids carrying *PDF1.2*-promoter-*GUS*, effector plasmids carrying *AtERF1*, *AtERF2*, *ERF1* or *ORA59* genes fused to the CaMV 35S promoter and the p2rL7 plasmid (De Sutter et al., 2005) carrying the *Renilla*

luciferase (*LUC*) gene under the control of the CaMV 35S promoter. As a control, co-transformation of *PDF1.2*-promoter-*GUS* with an empty vector (pRT101 or pMOG184 (Mogen International, Leiden, The Netherlands)) and the p2rL7 plasmid was carried out. Protoplasts were transformed using polyethylene glycol as described previously (Schirawski et al., 2000) with the three constructs in a ratio of 1:1:3 (*GUS*:*LUC*:effector plasmid). Protoplasts were harvested 18 hours after transformation and frozen in liquid nitrogen. GUS and LUC activity assays were performed as described by van der Fits and Memelink (1997) and Dyer et al. (2000) respectively, for each individual transformation. *GUS* reporter gene expression was related to *LUC* expression to correct for transformation and protein extraction efficiency. Average GUS/LUC ratios from triplicate experiments were expressed relative to the respective vector controls.

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Chapter 3

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

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Abstract

Jasmonic acid (JA) and related oxylipins are important signaling molecules in plant defense. The regulation of their biosynthesis is not well understood at the molecular level. The expression of several genes encoding JA biosynthetic enzymes is increased by JA, indicating that JA biosynthesis is subject to auto-induction. Here, we report that the AP2/ERF-domain transcription factor ORA47 functions in the regulation of the jasmonate biosynthesis pathway. Overexpression of the *ORA47* gene conferred JA-sensitive phenotypes, such as inhibition of growth, and induced the expression of all biosynthetic genes of the JA pathway tested. Jasmonate measurements in *ORA47*-overexpressing plants showed an increase in the amount of the bioactive JA precursor 12-oxophytodienoic acid (OPDA) while JA levels were similar to those of control plants. Probably, as a consequence of oxylipin biosynthesis, several JA-responsive genes including the gene encoding vegetative storage protein1 (VSP1) were upregulated in *ORA47*-overexpressing plants**.** Our findings demonstrate that ORA47 acts as an important element in the JA-responsive biosynthesis of jasmonate, most likely by controlling the positive feedback regulatory system for JA biosynthesis.

Introduction

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues. Jasmonates are linolenic acid (18:3)-derived cyclopentanone based compounds of wide distribution in the plant kingdom and play crucial roles in responses to mechanical and insect wounding, pathogen infection and UV irradiation (Turner et al., 2002). The defense response involving JAs is a two-step process. First, perception of the external stress induces JA biosynthesis. Then, JA production results in signal transduction leading to the expression of a large number of defense-related genes, including genes encoding vegetative storage proteins (VSPs), the plant defensins (PDFs) or enzymes involved in the biosynthesis of protective secondary metabolites (Turner et al., 2002; Atallah and Memelink, 2004; Pauw and Memelink, 2005). Arabidopsis 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). This indicates that JA is necessary for resistance against certain pathogens. Several molecular players in the jasmonate signaling network have been characterized (Lorenzo and Solano, 2005).

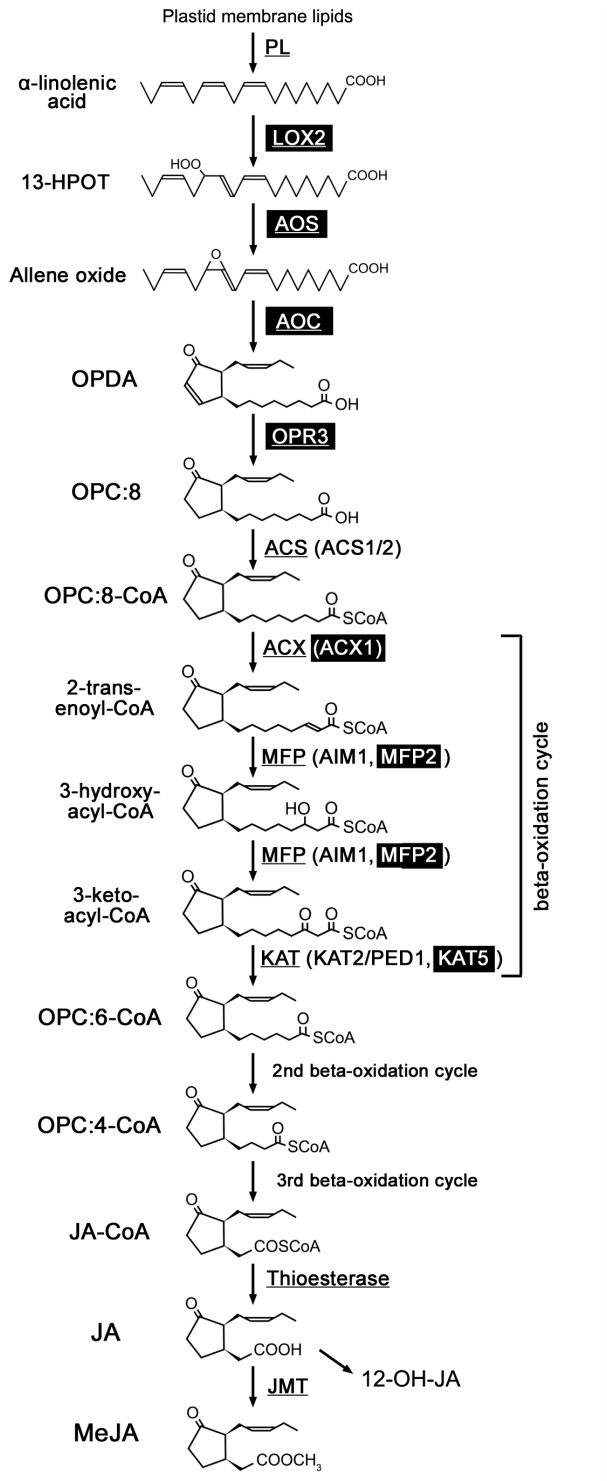

Figure 1. Octadecanoid pathway for JA biosynthesis. Abbreviations for enzyme names are underlined or in brackets. Enzymes whose corresponding genes are upregulated in *ORA47*-overexpressing plants are black-boxed. PL, phospholipase; LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; 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; JMT, *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase. 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; 12-OH-JA, 12-hydroxyjasmonic acid; MeJA, methyl jasmonate.

The transcription factors ORA59 (Chapter 2), ERF1 (Lorenzo et al., 2003) and AtMYC2 (Lorenzo et al., 2004) were shown to regulate the expression of subsets of JA-responsive genes. Whereas it starts to be relatively well understood how JAs regulate defense genes, next to nothing is known about the signal transduction pathway leading to JA biosynthesis.

Most of the enzymes involved in the so-called octadecanoid pathway leading to JA biosynthesis have now been identified by a combination of biochemical and genetic approaches (Creelman and Mulpuri, 2002; Turner et al., 2002). The enzymes are located in two different subcellular compartments (Figure 1; Vick and Zimmerman, 1984; Schaller, 2001; Wasternack and Hause, 2002). The first part of the pathway directs the conversion of α-linolenic acid to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The second part of the pathway takes place in peroxisomes, where OPDA is reduced by OPDA reductase (OPR3) 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 (Figure 1). Subsequently, JA can be metabolized in the cytoplasm by at least seven different reactions. Among them, 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) are of preferential importance.

The expression of all JA biosynthesis genes, including *LOX2*, *AOS*, *AOC, OPR3* and *JMT*, is induced by wounding or treatment with exogenous JA or MeJA (Turner et al., 2002; Sasaki et al., 2001). The observation that wounding induces the expression of JA biosynthesis genes suggests that, at least partly, the wound-induced production of JA is a result of the increased transcription of genes encoding the pathway enzymes. In addition, transcriptional activation of the JA biosynthesis genes by JA indicates that JA signaling is amplified by a positive feedback loop initiated by JA (Turner et al., 2002). However, it is not clear how the JA biosynthesis genes are regulated at the transcriptional level. So far, only WIPK, a mitogen-activated protein kinase, and CEV1, a cellulose synthetase protein, have been implicated as regulatory components of JA production. JA and its methyl ester accumulate in wounded tobacco plants, but do not accumulate in wounded transgenic plants, in which expression of *WIPK* is genetically suppressed (Seo et al., 1995), indicating that WIPK is a positive regulator of wound-induced JA biosynthesis. In Arabidopsis, the *cev1* mutant shows constitutive production of JA and ethylene and constitutive expression of JA-responsive defense-related genes (Ellis and Turner, 2001; Ellis et al., 2002). The CEV1 protein is thought to act as a negative regulator of stress perception or signal transduction, upstream of JA production. A

similar function seems to be affected in the *cet1* mutant which exhibits constitutive elevation of JA and constitutive expression of *THIONIN* (Hilpert et al., 2001), but the corresponding *CET* gene has not yet been cloned.

Several compounds closely related to JA and its precursors are found in plants and it is becoming clear that bioactivity is not limited to JA. Several JA precursors and conjugates have been shown to have biological activity *per se,* and although JA is often regarded as the physiological signal for jasmonate-mediated responses, increasing evidence indicates that JA precursors exert bioactivity in the absence of their conversion to JA. The Arabidopsis *opr3* mutant, in which JA production is blocked downstream of OPDA formation (Figure 1), is male sterile, a phenotype similar to that observed for other mutants impaired in JA biosynthesis or perception. This phenotype was rescued by external application of JA but not OPDA, indicating the absolute requirement for JA in pollen development (Stintzi et al., 2000). In contrast to the *fad* triple mutant which is unable to make any jasmonate, the *opr3* mutant shows wild-type resistance to insect and fungal pests, suggesting that OPDA can act as a signal in the activation of defense responses (Stintzi et al., 2001). Exogenously applied OPDA was able to induce many JA-dependent genes in the *opr3* mutant while a subset of defense-related genes was activated by OPDA but not by JA, indicating overlapping as well as distinct signaling functions.

The activation of distinct or common subsets of target genes might be due to the recruitment of specific transcription factors in response to signaling molecules such as JA or OPDA. Several JA responses in plants are regulated by members of the AP2/ERF-domain transcription factor family. In *Catharanthus roseus,* the jasmonate-dependent activation of genes encoding terpenoid indole alkaloid biosynthetic enzymes is mediated by two ORCA proteins, which are members of the AP2/ERF-domain transcription factor family (Menke et al., 1999; van der Fits and Memelink, 2000). In Arabidopsis, the AP2/ERF-domain transcription factor ORA59 was shown to be involved in JA signal transduction as well as in ethylene signaling (Chapter 2). The related transcription factor ERF1 was also reported to have a similar function as ORA59 (Lorenzo et al., 2003), although its importance is questionable in view of the results presented in Chapter 2. Atallah (2005) previously characterized 14 genes encoding AP2/ERF proteins, including ORA59, which were rapidly induced by JA treatment in 10-days-old Arabidopsis seedlings. The JA-induced expression of these genes, named *Octadecanoid-Responsive Arabidopsis AP2/ERF* (*ORA*) genes, was severely reduced in the JA-insensitive *coi1-1* mutant, further supporting a role for these ORA proteins in the JA signal transduction pathway.

In this study we investigated the possible function of the ORA47 transcription factor in JA signaling. We found that plants constitutively overexpressing the *ORA47* gene showed a

strong dwarf phenotype and produced anthocyanins. *ORA47* overexpression led to the activation of a large number of genes involved in JA biosynthesis. Oxylipin measurements in plants overexpressing *ORA47* revealed an increased OPDA level, but did not record altered levels of JA. In addition, many JA-responsive genes, such as *VSP1*, were highly expressed in *ORA47*-overexpressing plants. The AP2/ERF-domain protein ORA47, encoded by a jasmonate-responsive gene, is the first transcription factor shown to regulate genes involved in jasmonate biosynthesis. Our results suggest that ORA47 is likely to be responsible for the regulation of the auto-stimulatory loop in JA biosynthesis.

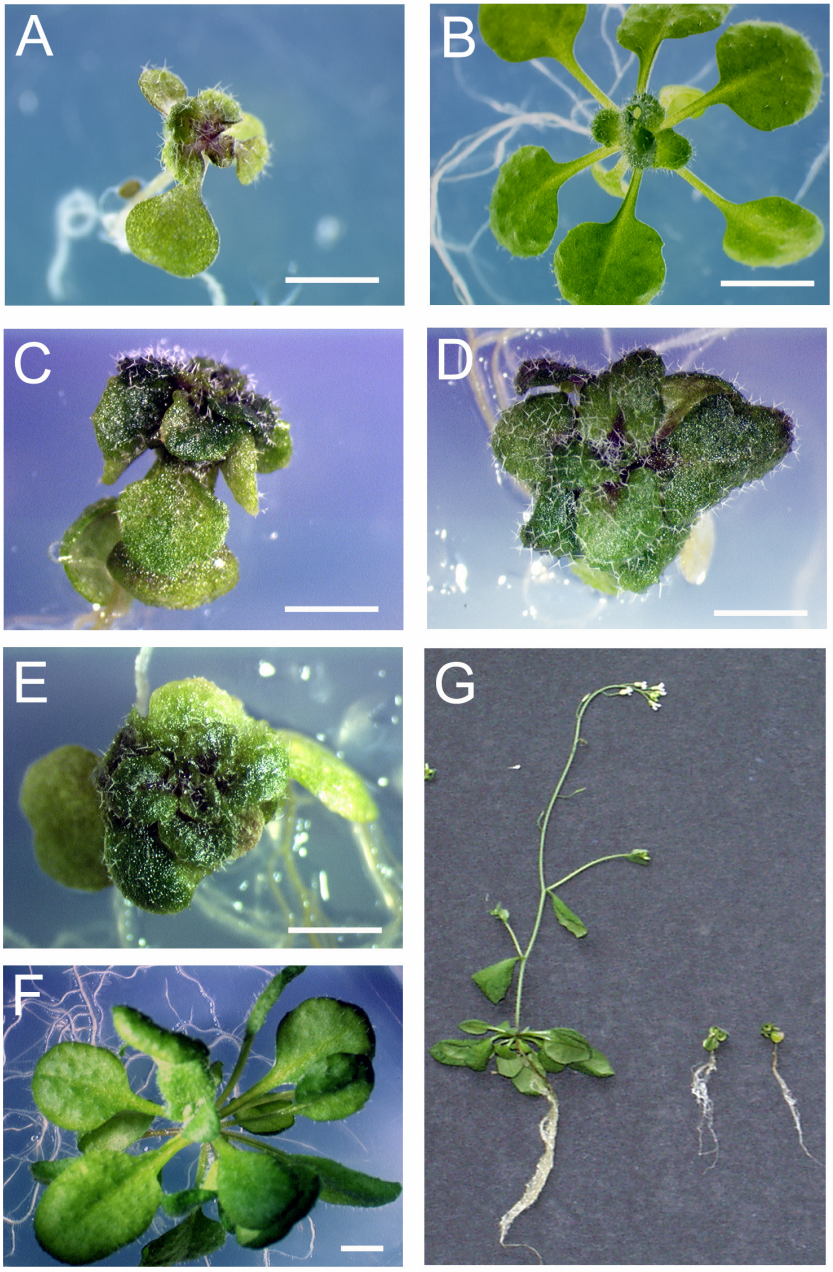

Figure 2. Arabidopsis plants constitutively overexpressing *ORA47* show dwarfism and anthocyanin production. Primary transformants carrying the 35S:*ORA47* construct **(A, C, D, E, G** (right)**)** or empty vector **(B, F, G** (left)**)** were germinated on selective medium. Bars represent 5 millimeters. (**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).

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 2, three-weeks-old T1 transformants carrying a 35S:*ORA47* construct (Figure 2A) were smaller than control plants (Figure 2B) and accumulated anthocyanins in the shoot apex. After 5 weeks of growth, 35S:*ORA47* plants (Figure 2C-E) were showing a severe dwarf phenotype with no stem elongation compared to control plants (Figure 2F). Small and disorganized dark rosette leaves were initiated (Figure 2C-E). At a later stage, two-months-old plants remained extremely small compared to control plants (Figure 2G). 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 (Figure 3A).

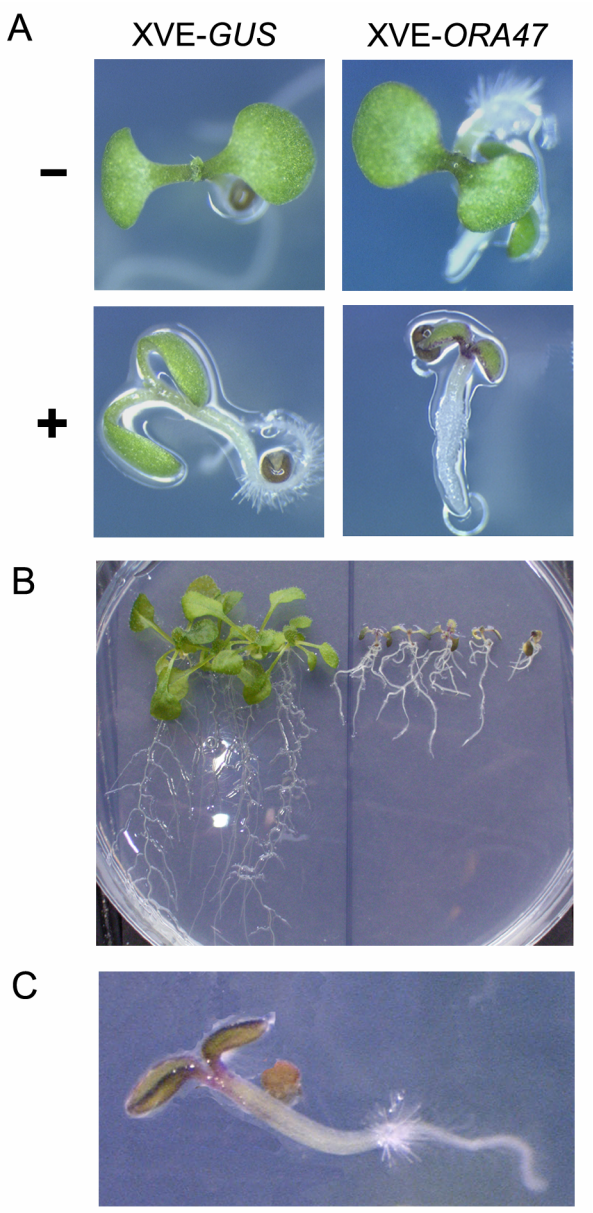

Figure 3. Growth inhibition and anthocyanin production in estradiol-induced *ORA47* overexpressing plants. **(A).** Transgenic seeds carrying an overexpression module with the *ORA47* or *GUS* gene under the control of an inducible promoter (XVE-*ORA47* and XVE-*GUS*, respectively) were germinated for 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.

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 (Figure 3A). 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 (Figure 3B), 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 (Figure 3C; Feys et al., 1994). Similarly, the *cev1* mutant plants carrying 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 jasmonate production.

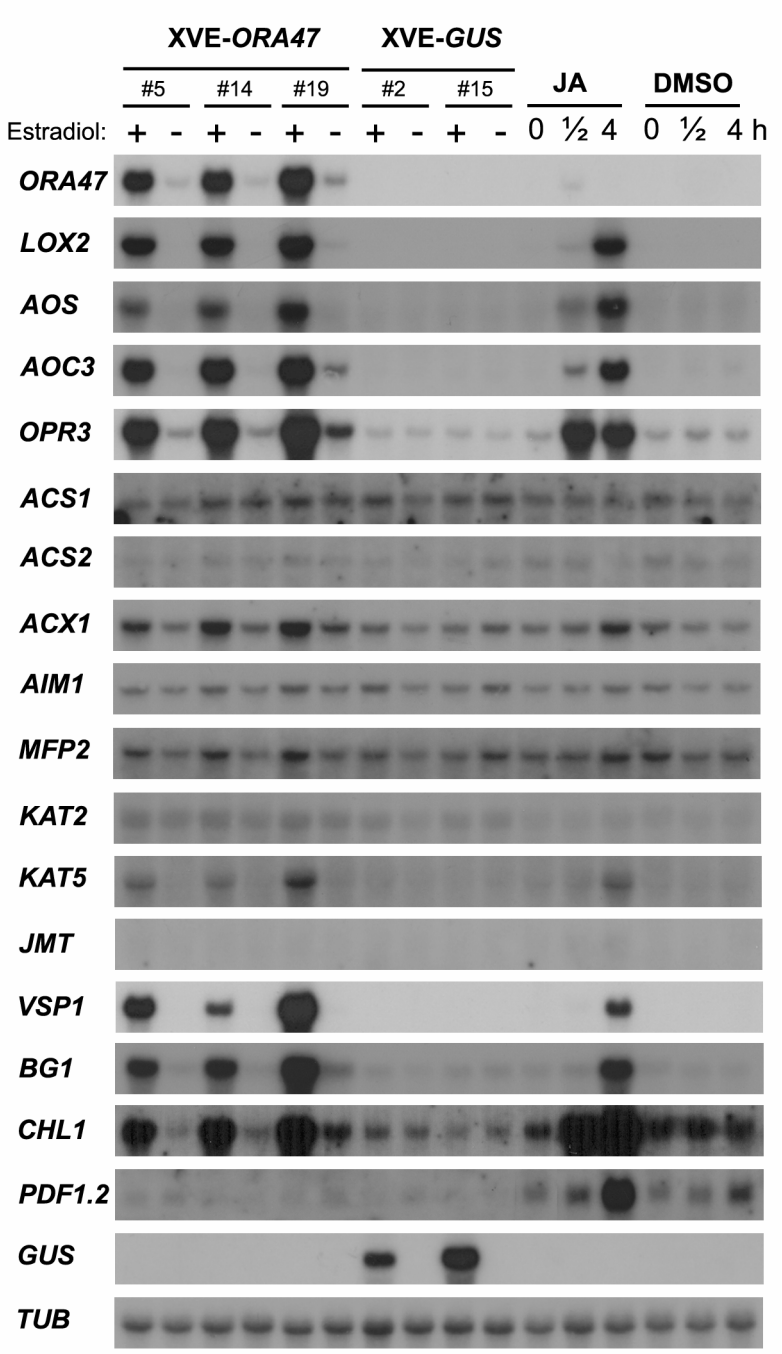

Figure 4. *ORA47* overexpression increases the expression of JA biosynthesis genes. RNA gel blot analyses with two-weeks-old independent XVE-*ORA47* and XVE-*GUS* lines treated for 24 hours with 2 µM estradiol (+) or with the solvent DMSO (-). To study JA-responsive gene expression, two-weeks-old wild-type 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.

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

The biosynthesis of JA occurs through the octadecanoid pathway (Turner et al., 2002) and involves a series of well-characterized enzymatic steps (Figure 1). To test whether ORA47 might transcriptionally control jasmonate biosynthesis, we examined the expression of the JA biosynthesis genes in XVE-*ORA47* plants in response to estradiol treatment. RNA gel blots revealed that expression of the *LOX2, AOS, AOC3* and *OPR3* genes, encoding enzymes of the first part of JA biosynthesis, were strongly induced in XVE-*ORA47* plants treated with estradiol (Figure 4). Similar results were obtained in the three independent XVE-*ORA47* transgenic lines. The expression level of the JA biosynthetic genes was tightly correlated with *ORA47* gene induction in each XVE-*ORA47* transgenic line. No expression of the biosynthetic genes was observed in the XVE-*ORA47* lines without inducer, except for line 19 where *AOC3* 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 JA biosynthesis gene expression was detected in the XVE-*GUS* control plants after estradiol treatment. *GUS* mRNA accumulation on the other hand was strongly induced, demonstrating the effectiveness of estradiol treatment. Hybridization with a *TUBULIN* (*TUB*) probe showed equal RNA loading. This demonstrates that the induced expression of *LOX2, AOS, AOC3* and *OPR3* genes is due to the specific activation of *ORA47* transgene expression by estradiol. All these genes were induced by JA treatment in 10-days-old wild-type Arabidopsis seedlings (Figure 4), as previously reported (Turner et al., 2002).

Although the first enzymatic steps leading to OPC:8 synthesis are well characterized (Figure 1), less is known about the identity and regulation of the β-oxidation genes involved in the biosynthesis of jasmonates. 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 4 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.

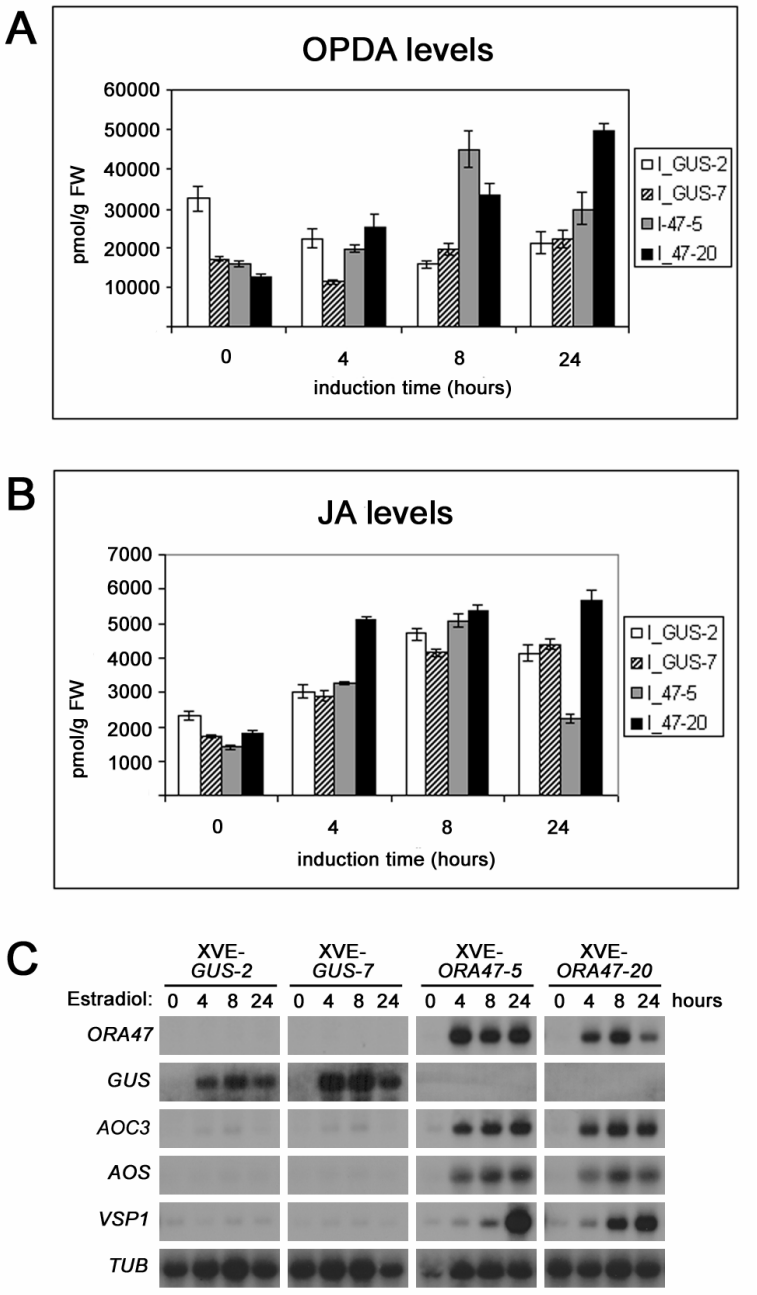

Figure 5. *ORA47* overexpression increases endogenous OPDA levels. Sixteen-days-old seedlings from two XVE-*ORA47* and two XVE-*GUS* lines were treated with 5 µM estradiol and tissue samples were collected after 4, 8 and 24 hours. OPDA **(A)** and JA **(B)** contents in XVE-*ORA47*-5 (grey bars), XVE-*ORA47*-20 (black bars), XVE-*GUS*-2 (open bars) and XVE-*GUS*-7 (hatched bars) are indicated in pmol/g of fresh weight (FW). Values represent means ± SE of three measurements per sample. **(C)**. RNA was collected from the different tissue samples used for oxylipin measurements. The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

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; Figure 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 JA biosynthesis in wounded leaves (Afitlhile et al., 2005). As shown in Figure 4, 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. The *JMT* gene, encoding an enzyme responsible for the methylation of JA to form MeJA (Figure 1), is induced by JA in leaves of mature plants. In contrast, *JMT* expression is undetectable in young seedlings even after JA treatment (Seo et al., 2001). Our results shown in Figure 4 confirmed the absence of *JMT* expression in JA-treated wild-type seedlings, and show that ORA47 induction failed to induce *JMT* gene expression at this developmental stage.

In addition to JA biosynthesis genes, overexpression of *ORA47* gene induced the expression of a large number of JA-responsive genes including *VSP1*, *β-glucosidase1* (*BG1*) and *chlorophyllase1* (*CHL1*; Figure 4 and Chapter 5). In contrast and surprisingly, the JA- and ethylene-responsive gene *PDF1.2* was not expressed in *ORA47*-overexpressing plants. In conclusion, gene expression analysis in *ORA47*-overexpressing plants revealed that all the established JA biosynthesis genes showed induced expression, suggesting that ORA47 overexpression might result in elevated amounts of endogenous JAs.

***ORA47* overexpression increases endogenous levels of OPDA**

The increase in transcript abundance of JA biosynthetic genes in *ORA47*-overexpressing plants prompted us to determine the amounts of JA and its biologically active precursor OPDA in these plants. Sixteen-days-old seedlings from two XVE-*ORA47* and two XVE-*GUS* lines were treated with 5 µM estradiol and tissue samples were collected after 0, 4, 8 and 24 hours. Endogenous JA and OPDA contents were determined by GC-MS analysis. Estradiol-induced expression of *ORA47* led to a 2- to 4-fold increase in the endogenous OPDA level compared to the OPDA content at time point 0 in the different XVE-*ORA47* lines (Figure 5A). Transgenic line XVE-*ORA47*-5 reached a maximum OPDA content of about 45 nmol/g of fresh weight (FW) after 8 hours of induction and the level was decreased at 24 hours, whereas line XVE-*ORA47*-20 reached a similar level after 24 hours. In contrast, OPDA

contents remained at a basal level for all time points in XVE-*GUS* control lines. Treatment with estradiol resulted in elevated amounts of JA at all time points and in all lines, with no differences between XVE-*ORA47* and XVE-*GUS* lines (Figure 5B). Therefore, the estradiol-induced *ORA47* expression did not lead to an increase in the JA level compared with the appropriate controls. RNA was collected from the different samples used to measure JA and OPDA levels. As shown by RNA gel blot analyses, estradiol treatment induced the expression of the *ORA47* and *GUS* genes in the XVE-*ORA47* and XVE-*GUS* lines, respectively (Figure 5C). Consistent with the results from Figure 4, the *AOC3, AOS* and *VSP1* genes were expressed exclusively in the XVE-*ORA47* lines.

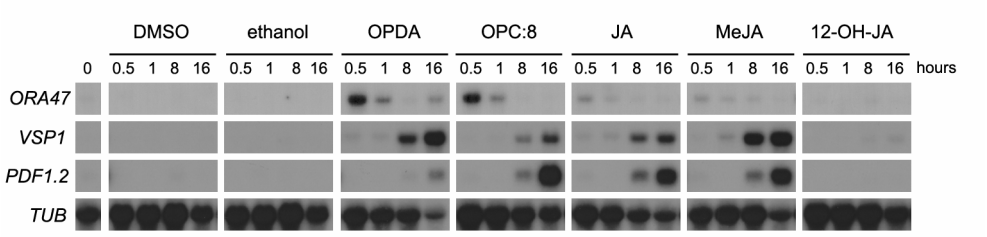

Figure 6. OPDA induces *VSP1* gene expression but has limited effect on the *PDF1.2* gene expression. Two-weeks-old Arabidopsis seedlings were treated for the number of hours indicated with 50 µM OPDA, OPC:8, JA, MeJA or 12-OH-JA, or with the solvents DMSO and ethanol. The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

Differential expression of the *VSP1* and *PDF1.2* genes to several JA precursors and derivatives

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). Overexpression of *ORA47* led to increased expression of the *VSP1* gene, but not the *PDF1.2* gene. Furthermore, plants overexpressing the *ORA47* gene accumulated a high level of OPDA, but not of JA. These results led us to hypothesize that a JA-related signaling molecule synthesized in *ORA47*-overexpressing plants (e.g. OPDA) would activate *VSP1* gene expression, whereas *PDF1.2* gene expression would require another JA-related signaling molecule (e.g. JA) that is not produced in *ORA47*-overexpressing plants. To test whether exogenous application of different JA-related compounds in wild-type plants results in differential expression of the *VSP1* and *PDF1.2* genes, two-weeks-old wild-type seedlings were treated for various time periods with JA or the JA-precursors OPDA and OPC:8 and the JA-derivatives MeJA and 12-hydroxyjasmonic acid (12-OH-JA; Figure 1). Several lines of evidence suggest that a number of JA-related oxylipins, such as OPDA or 12-OH-JA, might have a biological activity distinct from JA or MeJA (Schaller et al., 2005). All treatments were performed using an identical concentration of all compounds for direct comparison of gene induction. As shown in Figure

6, *VSP1* expression was responsive to all oxylipin treatments except for 12-OH-JA. *VSP1* transcript levels in response to OPDA and MeJA treatments after 8 and 16 hours were higher than with OPC:8 and JA treatments. In contrast, the *PDF1.2* gene was highly expressed in response to OPC:8, JA and MeJA treatments. Only low induction of *PDF1.2* expression was detected after 16 hours of treatment with OPDA. Control treatments with the solvents DMSO and ethanol did not induce gene expression. These results suggest that the *VSP1* gene is highly responsive to OPDA, whereas the *PDF1.2* gene is weakly induced by OPDA. These findings are in agreement with the observations that *ORA47*-overexpressing plants accumulate OPDA but not JA, and induce *VSP1* but not *PDF1.2* transcript levels.

Plant infection with necrotrophic fungi activates *ORA47* expression in a COI1-dependent manner

The jasmonate signal pathway involves several signal transduction events: (i) the perception of the primary wound or stress stimulus, including pathogen attack; (ii) the perception of this signal and induction of jasmonate biosynthesis; and (iii) the perception of jasmonate and induction of responses. The later is controlled by the JA signaling component COI1, as mutation of the *COI1* gene blocks the JA-induced expression of JA-responsive genes. In *coi1-1* mutant seedlings, exogenous application of JA failed to induce the expression of the *ORA47* gene (Atallah, 2005), indicating that *ORA47* expression in response to JA is dependent on COI1. This also suggests that ORA47 functions downstream of COI1 in the JA signaling pathway. However, the observation that ORA47 regulates jasmonate biosynthesis suggests that ORA47 plays a role upstream of jasmonate biosynthesis in early events of JA signaling. To test the latter hypothesis, expression of the *ORA47* gene was analyzed in response to infection with the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola.* In Arabidopsis, resistance against both pathogens is dependent on intact JA signaling responses (Thomma et al., 1998; Penninckx et al., 1996). RNA gel blot analyses in wild-type plants showed that *ORA47* gene expression was induced after infection with *B. cinerea* (Figure 7A) and *A. brassicicola* (Figure 7B). In response to infection with *B. cinerea*, *ORA47* was predominantly expressed in locally infected leaves 2 days after inoculation. In response to infection with *A. brassicicola*, *ORA47* gene expression occurred in infected and non infected leaves with a peak of expression after 7 days in non-inoculated leaves. In *coi1-1* mutant plants infected with *B. cinerea*, induction of *ORA47* gene expression was totally blocked both locally and systemically. After infection with *A. brassicicola*, *ORA47* gene expression in *coi1-1* mutant plants was dramatically reduced. This indicates that the activation of *ORA47* by these pathogens requires COI1, suggesting that ORA47 acts downstream of COI1 in the regulation of JA biosynthesis genes.

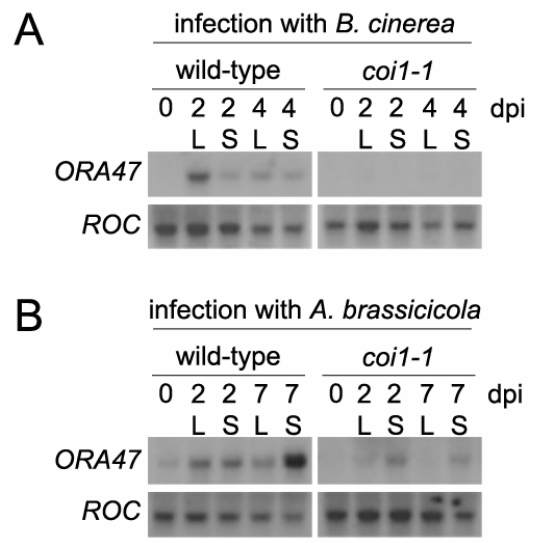

Figure 7. Infection with necrotrophic fungi activates *ORA47* expression in a COI1-dependent manner. Four-weeks-old wild-type and *coi1-1* mutant plants were infected with *Botrytis cinerea* (**A**) or *Alternaria brassicicola* (**B**) and RNA was extracted from infected local (L) and non-infected systemic (S) leaves from several inoculated plants of each genotype after the number of days indicated (dpi, days post inoculation). The *ROC* probe was used to verify RNA loading.

Activation of JA biosynthetic gene expression by ORA47 requires COI1

Induction of *ORA47* expression led to increased expression of JA biosynthesis genes as well as JA-responsive defense genes including *VSP1* (Figure 4). We speculate that the JA biosynthesis genes are direct target genes of ORA47, although this remains to be demonstrated. The defense genes might also be direct target genes, or alternatively, they might respond to the ORA47-mediated biosynthesis of oxylipin signaling molecules including OPDA. To distinguish between these possibilities, we introduced the XVE-*ORA47* expression module in the JA-insensitive *coi1-1* mutant background. ORA47 is expected to regulate direct target genes without a requirement for COI1, whereas genes responding to elevated levels of JAs depend on an intact COI1 protein.

JA-insensitive *coi1-1* mutant plants carrying the XVE-*ORA47* expression module were generated and expression of the ORA47-regulated genes in response to estradiol or JA was analyzed using RNA gel blots (Figure 8). In XVE-*ORA47* plants with a functional *COI1* gene (XVE-*ORA47;* wild-type), *VSP1* and JA biosynthetic genes were expressed in response to JA, whereas exogenous JA treatment did not induce the expression of these genes in XVE-*ORA47*; *coi1-1* plants*,* consistent with the *coi1* phenotype. *O*verexpression of the *ORA47* gene in response to estradiol did not lead to induction of the JA biosynthetic genes *AOS* and *LOX2*, or the defense-related gene *VSP1* in XVE-*ORA47*; *coi1-1* plants (Figure 8). Similar

results were obtained with the JA biosynthetic genes *OPR3, ACX1, MFP2* and *KAT5* (data not shown). This indicates that upregulation of these genes in transgenic *ORA47*-overexpressing plants requires the JA signaling component COI1. Although dramatically reduced compared to the estradiol-induced expression in the COI1 background, a slight increase in the *AOC3* transcript level was observed in response to estradiol in the *coi1-1* mutant background compared to the control treatment. This indicates that ORA47-mediated expression of the *AOC3* gene is, to a large extent, dependent on COI1. Nevertheless, ORA47 is also able, to a certain degree, to activate *AOC3* expression in a COI1-independent manner.

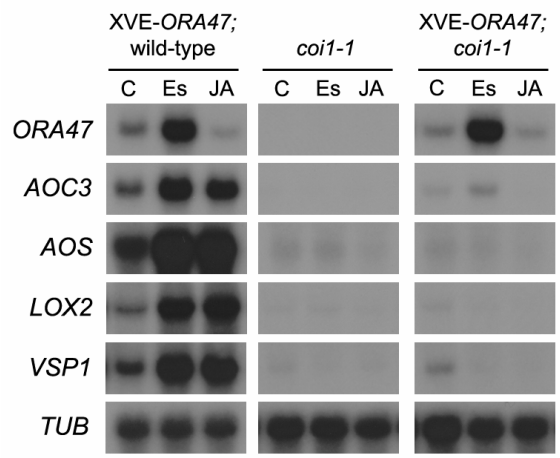

Figure 8. Activation of JA biosynthetic gene expression by ORA47 requires COI1.
RNA gel blot analyses with two-weeks-old *coi1-1* mutant plants and transgenic plants carrying the XVE-*ORA47* expression module in the *coi1-1* (XVE-*ORA47*; *coi1-1*) or wild-type (XVE-*ORA47*; wild-type) backgrounds treated for 8 hours with 50 µM jasmonic acid (JA), 2 µM estradiol (Es) or the solvent DMSO (C). The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

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

To further investigate the role of ORA47 in JA signaling leading to gene expression, we analyzed the expression of the JA biosynthetic genes in 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 9A, 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 JA biosynthesis genes, such as *AOC3* and *LOX2*, was similar in the *ora47-1* mutant compared to wild-type plants in response to JA (Figure 9A).

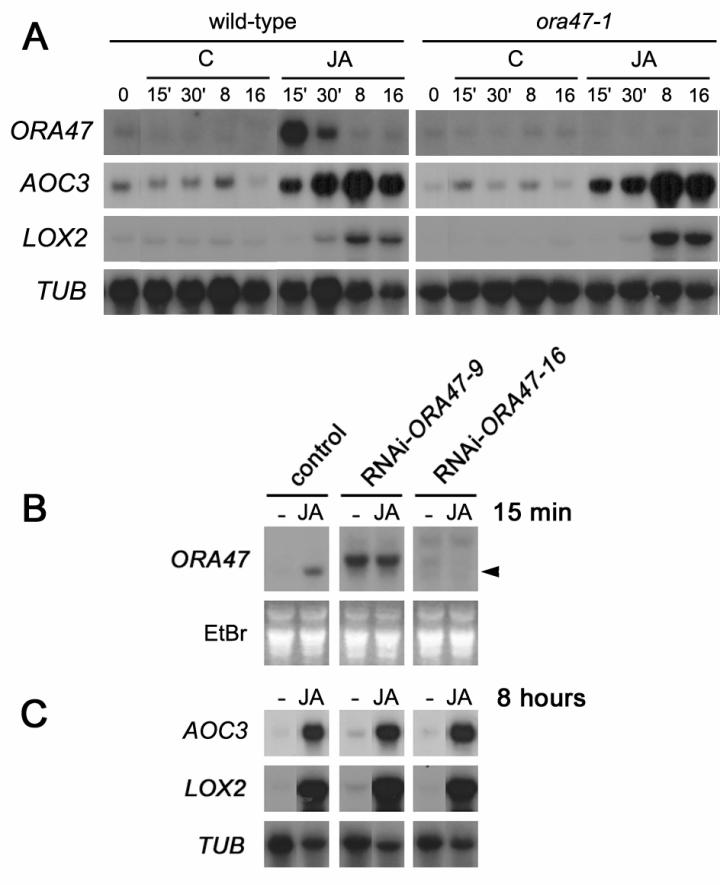

Figure 9. Expression of JA biosynthetic genes in response to JA in the *ora47* mutant and transgenic *ORA47*-silenced plants. **(A)**. RNA gel blot analyses with two-weeks-old *ora47-1* mutant and wild-type plants treated for 15 and 30 minutes or for the number of hours indicated with 50 µM jasmonic 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 jasmonic acid (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**).

This indicates that a JA-induced increase in the *ORA47* transcript level is not required for the JA-responsive expression of the JA biosynthesis genes *AOC3* and *LOX2*. 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 9B). 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 9B). 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 *AOC3* and *LOX2* genes in response to 8 hours treatment with JA was similar in the *ORA47*-silenced lines compared to the control line (Figure 9C), indicating that JA can induce *AOC3* and *LOX2* gene expression in the absence of *ORA47* gene expression. This suggests that the ORA47 transcription factor is not strictly required for the expression of the JA biosynthesis *AOC3* and *LOX2* genes in response to JA.

Discussion

Jasmonic acid is a signaling molecule that regulates certain aspects of development as well as diverse responses to stress. Little is known about the regulatory mechanisms controlling JA 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 jasmonate biosynthesis. We show that overexpression of the *ORA47* gene resulted in the activation of JA biosynthesis genes, and led to elevated amounts of endogenous OPDA, a bioactive signaling molecule as well as a precursor of JA. This is the first identification of a plant transcription factor involved in the regulation of jasmonate biosynthesis.

ORA47 positively regulates the JA biosynthesis genes

Overexpression of *ORA47* gene in Arabidopsis activated the expression of all established genes encoding enzymes of the JA biosynthetic pathway, such as *LOX2, AOS, AOC3* and *OPR3*. Additionally, several genes encoding enzymes of the β-oxidation steps, including *ACX1, MFP2* and *KAT5,* were also expressed at higher 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) showed 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 JA production. Moreover, He et al. (2002) suggested a role for KAT2/PED1 in senescence-induced JA synthesis. Therefore it is likely that the *KAT2/PED1* gene is expressed in response to wounding or senescence without the requirement for ORA47, whereas a different signal initiating the auto-stimulatory loop would recruit ORA47 to activate the *KAT5* gene.

ORA47 controls OPDA production

Activation of all established JA biosynthetic genes tested in *ORA47*-overexpressing plants suggested that these plants might contain enhanced levels of endogenous JA. However, despite the induced expression of *OPR3* and the β-oxidation genes, we did not observe an increase in JA levels in *ORA47*-overexpressing plants compared to control plants. In contrast, overexpression of the *ORA47* gene in plants led to increased levels of the JA precursor OPDA. In recent years, studies on JA signaling have provided compelling evidence that bioactivity is not limited to jasmonic acid. Several JA intermediates, such as OPDA, and JA derivatives, collectively called jasmonates, were shown to have bioactivity *per se*. Genetic studies with the Arabidopsis *opr3* mutant, in which JA synthesis is blocked downstream of OPDA formation (Figure 1), indicate that OPDA is active as a defense signal against insect and fungal attack without conversion to JA (Stintzi et al., 2001). In contrast, Stintzi et al. (2001) have provided evidence that exogenous JA, but not OPDA, is able to induce *VSP1* gene expression in the *opr3* mutant, suggesting that synthesis of JA is strictly required for *VSP1* gene expression. This finding is in contradiction with our results showing that the *VSP1*

gene was induced in the *ORA47*-overexpressing plants which contained high levels of OPDA, but not JA. In wild-type plants, exogenous application of OPDA induced the expression of the *VSP1* gene to a higher level than application of the same concentration of JA. Although we cannot exclude that OPDA was converted to JA in the OPDA-treated wild-type plants, this difference in *VSP1* transcript levels suggests that the *VSP1* gene is more responsive to OPDA than to JA. In contrast, the JA-responsive gene *PDF1.2* was not induced in *ORA47*-overexpressing plants, indicating that the OPDA produced in *ORA47*-overexpressing plants was not able to activate the expression of the *PDF1.2* gene. This is supported by the observation that the *PDF1.2* gene was weakly expressed in OPDA-treated wild-type plants compared to OPC:8-, JA- or MeJA-treated plants. Again, *PDF1.2* gene induction by OPDA in wild-type may be due to conversion of OPDA to JA. However, the low responsiveness of the *PDF1.2* gene to OPDA compared to JA indicates that there is little or no conversion of OPDA to JA. The consistency of differential *VSP1* and *PDF1.2* gene expression patterns in *ORA47*-overexpressing plants and in response to different JA-related signal molecules indicates that these genes are regulated by different jasmonate species.

It is not clear why elevated amounts of OPDA, together with the activation of genes coding for downstream JA biosynthesis enzymes, did not lead to higher levels of JA in the transgenic plants. The synthesis of OPDA occurs in the chloroplasts whereas the OPR3 and β-oxidation enzymes are located in the peroxisomes (Schaller et al., 2005). Therefore, OPDA or the already activated form, OPDA-CoA, must be transported from the chloroplasts to the peroxisomes, and this transport is likely to be regulated (Stenzel et al., 2003). The peroxisomal ATP-binding cassette (ABC) transporter COMATOSE (CTS) is thought to be responsible for the transport of OPDA into the peroxisomes, as *cts* mutants showed a lower JA levels than wild-type plants after wounding (Theodoulou et al., 2005). It is possible that although *OPR3* and the β-oxidation genes are induced in *ORA47*-overexpressing plants, conversion of OPDA to JA does not occur due to lack of transport of OPDA from the plastids to the peroxisomes.

Though reduced compared to wild-type plants, the presence of JA in wounded *cts* mutant plants prompted the authors to propose a model with two parallel pathways for peroxisomal import of OPDA from the chloroplasts: a pathway that requires the active transport of OPDA in a CTS-dependent manner and another pathway involving ion trapping where the free acid OPDA would enter the peroxisomes via passive diffusion through the membranes without the need for a transport protein (Theodoulou et al., 2005). With respect to these findings, it is possible that a small proportion of the OPDA produced in *ORA47*-overexpressing plants is transported to the peroxisomes via the less predominant passive pathway, resulting in the production of a sufficient amount of JA to activate the expression of *VSP1*, but not of *PDF1.2*.

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 jasmonic acid, 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 jasmonates 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 JA-responsive genes and increased resistance against *B. cinerea* (Seo et al., 2001). The *cas1* and *cet1* mutant plants contain constitutively high levels of jasmonate (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 jasmonate 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 OPDA levels and downstream gene activation. The biosynthesis of OPDA is likely to induce the constitutive expression of a large number of jasmonate-responsive genes, thereby generating a stress condition that compromises plant development and overall fitness. Indeed, overexpression of *ORA47* induced the expression of several JA-responsive genes, including *VSP1, BG1* and *CHL1*.

ORA47 is involved in the JA auto-stimulatory loop

Here we showed that overexpression of *ORA47*, encoding an AP2/ERF-domain transcription factor, induces the expression of all established JA biosynthesis genes tested and results in elevated OPDA levels. These results tend to place ORA47 upstream of jasmonate 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 jasmonate production. However, several lines of evidence indicate that ORA47 is a downstream component in JA signaling where ORA47 regulates the positive feedback amplification loop. First, induction of the *ORA47* gene by JA (Atallah, 2005) and fungal infection (Figure 8) require the JA signal transduction component COI1, indicating that perception of the JA signal is required for *ORA47* expression in response to stress. Therefore, ORA47 is likely to act as a terminal component in JA signal transduction, rather than as an integrator of stress signals leading to JA production. We speculate that the function of ORA47 is to regulate the amplification loop that leads to the expression of the JA biosynthesis genes in response to JA. Up to now, it is

still unclear how JA production initially occurs in response to stress and what is the integrator of such stress that leads to JA 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. Activation of ORA47 and resulting expression of the JA biosynthesis genes would lead to production of a small amount of jasmonate that would activate transcription of the *ORA47* gene and subsequent amplification of the signal by the feedback loop. Overexpression of the *ORA47* gene in a *coi1* mutant background did not lead to activation of the JA biosynthesis genes, suggesting that these genes are not primary targets of ORA47. However, it is possible that the stress-induced activation of ORA47 requires COI1-dependent modifications (or COI1-dependent co-factors) to bind to the promoters of the JA biosynthesis genes. Within that scenario, some JA should be produced to initiate these modifications. The low but significant induction of *AOC3* observed in the *coi1* mutant background in response to *ORA47* overexpression might lead to the production of that small quantity of JA.

Analyses of the *ora47* knock-down mutant and the *ORA47*-silenced plants showed that expression of the JA biosynthesis genes in response to JA was not altered in these plants compared to control plants. This indicates that, in addition to ORA47, (an)other transcription factor(s) are(is) very likely to regulate the JA biosynthesis genes. The results presented in chapter 5 reveal that, within the set of analysed *ORA* genes, only overexpression of *ORA47* leads to induction of the JA biosynthesis genes, excluding the possibility of functional redundancy among ORAs. Therefore, ORA47, together with (an) unidentified transcription factor(s), regulate the JA-induced auto-stimulatory loop resulting in activation of the JA biosynthesis genes.

Materials and Methods

Biological Materials, Growth Conditions and Treatments

Arabidopsis thaliana ecotype Columbia (Col-0) is the genetic background for all wild-type, transgenic and *coi1-1* 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 3A).

Seeds from the *coi1-1* mutant were screened on solid MA medium containing 50 μM JA dissolved in DMSO (0.1% final concentration) for JA insensitivity.

Treatments with the different oxylipins were performed at a final concentration of 50 μM. The compounds JA, MeJA, OPC:8 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.

Plant infection with *Botrytis cinerea* and *Alternaria brassicicola* was performed as described in Chapter 2.

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.

The binary vector pCAMBIA1300-ORA47 was introduced into *Agrobacterium tumefaciens* strain LBA1115 (containing the Vir plasmid pSDM3010). The binary vectors pART27-ORA47 and pART27-GUS were introduced into *A. tumefaciens* strain LBA4404 while pER8-ORA47 and pER8-GUS 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.

The XVE-*ORA47*; *coi1-1* plants were obtained by fertilizing homozygous *coi1-1* ovules with pollen from transgenic XVE-*ORA47* plants. Heterozygous *coi1*/COI1 F1 siblings containing the transgene were selected on MA medium containing 20 mg/L hygromycin and were allowed to self-pollinate. F2 siblings homozygous for the *coi1-1* mutation and carrying the XVE-*ORA47* transgene were selected on MA medium containing 50 µM JA for JA-insensitivity and subsequently transferred to medium containing 20 mg/L hygromycin for selection of the transgene.

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 65ºC before addition of denatured ^{32}P-labeled DNA probes. After overnight hybridization, blots were washed twice at 42ºC 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 cyclase3* (*AOC3, 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'- 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 few 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 JA and OPDA levels

For JA and OPDA measurements, two independent transgenic lines containing the XVE-*ORA47* expression module and two independent transgenic lines containing the XVE-*GUS* expression module were used. Per treatment and per line, 10 mg of surface-sterilized seeds were germinated in 250 ml Erlenmeyer flasks containing 50 ml MA medium and grown for 16 days. 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. One aliquot was kept for RNA extraction while the rest of the powdered tissue was used to measure OPDA and JA levels. Plant material from 20 seedlings was pooled to minimize biological differences and this was done in triplicate. The powdered tissue (500 mg) was homogenized with 10 ml 80% (v/v) methanol after adding 100 ng ($^2H_6$)JA and 100 ng ($^2H_5$)OPDA, respectively, as internal standards. The homogenate was filtered, and the eluate passed through a column filled with 3 ml DEAE-Sephadex A25 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) (Ac^--form, methanol). The column was washed with 3 ml methanol and subsequently with 3 ml 0.1 M acetic acid in methanol. Eluents with 3 ml of 1 M acetic acid in methanol and 3 ml of 1.5 M acetic acid in methanol were collected, evaporated and separated on preparative HPLC using an Eurospher 100-C18 column (5 µm, 250 x 4 mm) (Knauer, Berlin, Germany). A gradient of methanol and 0.2 % acetic acid in H_2O (40% to 100%) was used within 25 min. Fractions at R_t 13 to 14.50 min (corresponding to JA) and at R_t 21.75 to 22.50 min (corresponding to OPDA) were combined separately and evaporated. The evaporated samples were

dissolved in 200 µl $CHCl_3$ / *N*,*N*-diisopropylethylamine (1 : 1; v/v) and derivatized with 10 µl pentafluorobenzylbromide at 20 °C overnight. The evaporated samples corresponding to the JA and OPDA fractions from HPLC, respectively, were dissolved in 5 ml n-hexane and passed through a Chromabond-SiOH-column (500 mg; Macherey-Nagel, Düren, Germany). The pentafluorobenzyl esters were eluted with 7 ml n-hexane / diethylether (1 : 1; v/v). Eluates were evaporated, dissolved in 100 µl MeCN and analyzed by GC-MS. GC-MS analysis was performed with a Polaris Q Thermo-Finnigan instrument at 100 eV with negative chemical ionisation mode using NH_3 as the ionization gas, at an ion source temperature of 140 °C, with a column Rtx-5w/Integra Guard (Restek Corp., Bad Homburg, Germany) (5m inert precolumn connected with a column of 15 m x 0.25 mm, 0.25 µm film thickness, crossbond 5% diphenyl – 95% dimethyl polysiloxane). Injection temperature was 220°C, interface temperature was 250°C. A helium flow of 1 ml min^{-1} was used. Injection was used splitless with 1 µl sample each. The following column temperature program was used: 1 min 60°C, 25°C min^{-1} to 180°C, 5 °C min^{-1} to 270°C, 10°C min^{-1} to 300°C, 10 min 300°C; R_t of pentafluorobenzyl esters: ($^2H_6$)JA 11.80 min, ($^2H_6$)-7-*iso*-JA 12.24 min, JA 11.86 min, 7-*iso*-JA 12.32 min, *trans*-($^2H_5$)OPDA 21.29 min, *cis*-($^2H_5$)OPDA 21.93 min, *trans*-OPDA 21.35 min, *cis*-OPDA 21.98 min. Fragments *m/z* 209, 215 (standard) and *m/z* 291, 296 (standard) were used for the quantification of JA and OPDA, respectively.

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Chapter 4

The Arabidopsis AP2/ERF-domain transcription factor ORA37 represses jasmonic acid- and ethylene-responsive genes, but also stimulates another set of jasmonic acid-responsive genes

Martial Pre and Johan Memelink

Abstract

Jasmonic acid (JA) is an important plant hormone involved in defense responses against external threats. JA perception leads to the activation of a specific set of defense genes adapted to the nature of the threat. Here we describe the role of the *Arabidopsis thaliana* AP2/ERF-domain transcription factor ORA37 in JA and ethylene signaling. JA treatment induces the expression of the *ORA37* gene in wild-type plants. In response to JA or ethylene treatment, expression of a subset of defense-related genes was repressed and enhanced in *ORA37*-overexpressing and *ORA37*-silenced plants, respectively. This indicates that ORA37 acts as a negative regulator of this subset of JA-responsive genes. In contrast, overexpression of the *ORA37* gene resulted in enhanced JA-induced expression of a distinct subset of JA-responsive genes, indicating that ORA37 can also function as a positive regulator of gene expression. Our results suggest that ORA37 is able to differentially regulate distinct branches of the JA signaling pathway to allow plants to establish a specific defense gene expression response.

Introduction

In their natural environment, plants are continuously threatened by various biotic and abiotic stresses. Their survival under such conditions is dependent on the ability to perceive external signals and to quickly induce appropriate responses. The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene are major endogenous low molecular weight signal molecules involved in regulating defense responses in plants. Depending on the type of external stimuli, specific synthesis of these hormones activates different signaling pathways with a cascade of events leading to the production of proteins with direct roles in defense against the particular environmental stress.

JA synthesis is induced by a range of biotic and abiotic stresses, including osmotic stress, wounding, drought, exposure to elicitors, insect attack and pathogen infection (Creelman and Mullet, 1997; Turner et al., 2002). Several studies have revealed complex cross-talk relationships between JA, ethylene and SA, which can act synergistically or antagonistically, in order to fine-tune the defense response (Kunkel and Brooks, 2002). The resistance of many pathogens, such as the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola*, requires both JA and ethylene, as demonstrated by the enhanced susceptibility of loss-of-function mutations in components of the respective signaling pathways such as *ETHYLENE-INSENSITIVE2* (*EIN2*) and *CORONATINE-INSENSITIVE1* (*COI1;* Thomma et al., 1998 and

1999; Penninckx et al., 1996). Moreover, JA and ethylene synergistically cooperate to activate the expression of some genes encoding defense-related proteins such as the antimicrobial defensin PDF1.2, the hevein-like protein HEL or the basic chitinase CHI-B (Penninckx et al., 1998; Potter et al., 1993; Thomma et al., 1998). Conversely, ethylene has been shown to down-regulate the JA signaling pathway in wounded tissues. In wounded or JA-treated Arabidopsis plants, mutation in the ethylene pathway results in increased induction of a subset of JA-responsive genes (Rojo et al., 1999) and exogenous application of ethylene compromises the JA-induced expression of those genes, including genes encoding vegetative storage proteins (VSPs) and glucosinolate biosynthetic enzymes (Rojo et al., 1999; Matsushima et al., 2002; Mikkelsen et al., 2003). Therefore, understanding the molecular mechanisms whereby these genes are differentially regulated is of major importance to unravel the complexity of positive or negative cross-talk between these signaling molecules in response to specific environmental stimuli.

In plants, a number of AP2/ERF-domain transcription factors have been implicated in the regulation of stress responses, including several JA responses. The ORCA2 and ORCA3 transcription factors from *Catharanthus roseus* were shown to act as regulators of JA-responsive gene expression and *ORCA* gene expression was rapidly induced after treatment with methyl-jasmonate, a natural JA derivative (Menke et al., 1999; van der Fits and Memelink, 2000 and 2001). In Arabidopsis, Atallah (2005) previously characterized 14 genes encoding AP2/ERF-domain proteins, which were rapidly induced by JA treatment in 10-days-old seedlings. The JA-induced expression of these genes, named *Octadecanoid-Responsive Arabidopsis AP2/ERF* (*ORA*) genes, was severely reduced in the JA-insensitive *coi1* mutant, further supporting their role in JA signaling. The AP2/ERF genes *ORA59*, *ERF1* and *AtERF2* are induced by JA and ethylene (Atallah, 2005; Lorenzo et al., 2003; Fujimoto et al., 2000) and ectopic expression of these genes led to elevated transcript levels of several defense genes including *PDF1.2* and *ChiB* (Chapter 2; Solano et al., 1998; Lorenzo et al., 2003; Brown et al., 2003). Moreover, *ORA59*- and *ERF1*-overexpressing plants were more resistant to fungal infection (Chapter 2; Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). Analysis of *ORA59*-silenced plants, which failed to induce *PDF1.2* and *ChiB* gene expression after JA and ethylene treatment and which were less resistant against fungal infection (Chapter 2), indicated that ORA59 has a more essential role than ERF1 and AtERF2 in fungal resistance and in regulation of JA- and ethylene-responsive gene expression.

The transcription factor ORA37 differs from the other ORA proteins identified by Atallah (2005) by the presence of an ERF-associated amphiphilic repression (EAR) motif in the C-terminal part of the protein. This LxLxL EAR motif has been shown to function as an active repressor of transcription (Otha et al., 2001; Fujimoto et al., 2000; Hiratsu et al., 2003). Seven

other AP2/ERF-domain proteins encoded by the Arabidopsis genome contain such an EAR repression domain (Atallah, 2005). However, only the *ORA37* gene was induced by JA treatment in the screening performed by Atallah (2005). The *ORA37* gene, also referred to as *AtERF4*, is induced by ethylene treatment or wounding (Fujimoto et al., 2000; Cheong et al., 2002) and the ORA37/AtERF4 transcription factor was previously shown to repress the expression of a GCC-box-containing reporter gene (Ohta et al., 2001; Fujimoto et al., 2000). Rapid induction of the *ORA37* gene in response to JA treatment indicates that the ORA37 protein plays a role in JA signaling pathway.

Here we report that ORA37 is a negative regulator, which modulates JA and ethylene responses. Overexpression of *ORA37* had no effect on the basal transcript level of several JA-responsive genes in untreated plants. However, upon JA and/or ethylene treatment, *ORA37*-overexpressing plants showed significantly lower induction of *PDF1.2, HEL* and *ChiB* genes than control plants. Conversely, transgenic plants silencing the *ORA37* gene showed increased *PDF1.2, HEL* and *ChiB* transcript levels after JA- and/or ethylene-treatment compared to control plants. These results indicate that ORA37 acts as a negative regulator of JA and ethylene responses. Surprisingly, JA-treated plants overexpressing *ORA37* also showed enhanced expression of several other JA-responsive genes, including *VSP1,* compared to JA-treated control plants. These results indicate that ORA37 not only represses JA and ethylene responses, but also stimulates other JA responses.

Results

Overexpression of *ORA37* decreases certain JA and ethylene responses, but enhances other JA responses

To investigate the role of the transcription factor ORA37 in JA signaling, transgenic Arabidopsis plants constitutively expressing the *ORA37* mRNA under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter were constructed. Several independent transformants were selected and allowed to self-pollinate. RNA gel blot analyses performed with two-weeks-old transgenic lines from the T2 generation showed that the *ORA37* gene was highly expressed in a large number of independent lines compared to control lines (Figure 1A). In general, plants overexpressing the *ORA37* gene were slightly smaller than control plants (Figure 1B and data not shown). Transgenic plants from line #40 displayed both mild and strong dwarfism compared with control plants. The expression level of the transgene was positively correlated with the reduction in plant size (Figure 1A and B), indicating that expression of *ORA37* has a negative effect on plant growth. Three

independent transgenic lines showing high expression of the *ORA37* gene (lines #6, #21 and #40) were selected for further analyses.

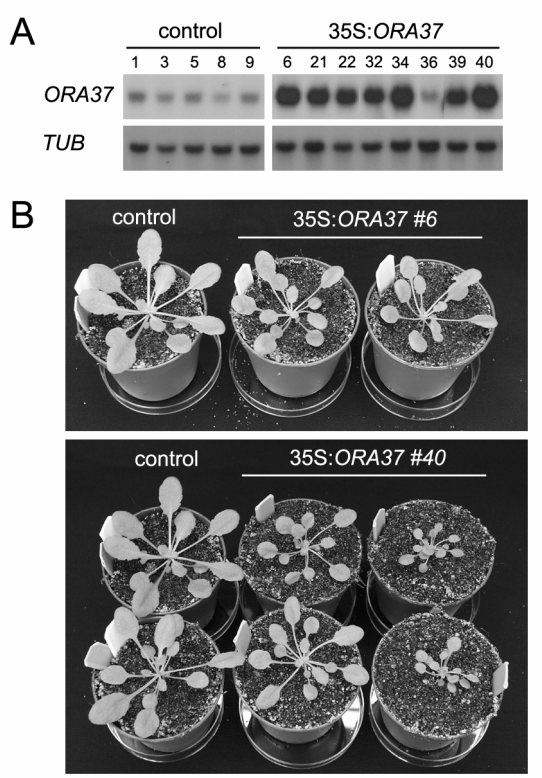

Figure 1. Overexpression of the *ORA37* gene in plants results in a mild dwarf phenotype. **(A).** RNA gel blot analyses with two-weeks-old independent transgenic lines constitutively overexpressing the *ORA37* gene (35S:*ORA37*) and control lines. The control lines (1301 lines) carry the *GUS* gene under the control of the CaMV 35S promoter. Independent lines are indicated by numbers. The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading. **(B).** Four-weeks-old 35S:*ORA37* #6 and #40 lines and the control line (1301-5) were grown under the conditions described in Materials and Methods.

ORA37 has been suggested to act as a transcriptional repressor of ethylene responses (Yang et al., 2005). Treatment with ethylene induced the expression of the *ORA37* gene after 12 hours (Fujimoto et al., 2000), indicating a role of ORA37 in ethylene signaling. However, induction of the *ORA37* gene is observed within 30 minutes to 1 hour after JA treatment (Atallah, 2005), suggesting that ORA37 has a more rapid regulatory effect on JA responses than on ethylene responses. We speculated that ORA37 is negatively regulating the JA-responsiveness of certain genes. Therefore, we analyzed the JA response of several genes

in *ORA37*-overexpressing plants. The 26 selected genes are known to be induced by JA treatment. Figure 2 shows the RNA gel blot analyses for these genes.

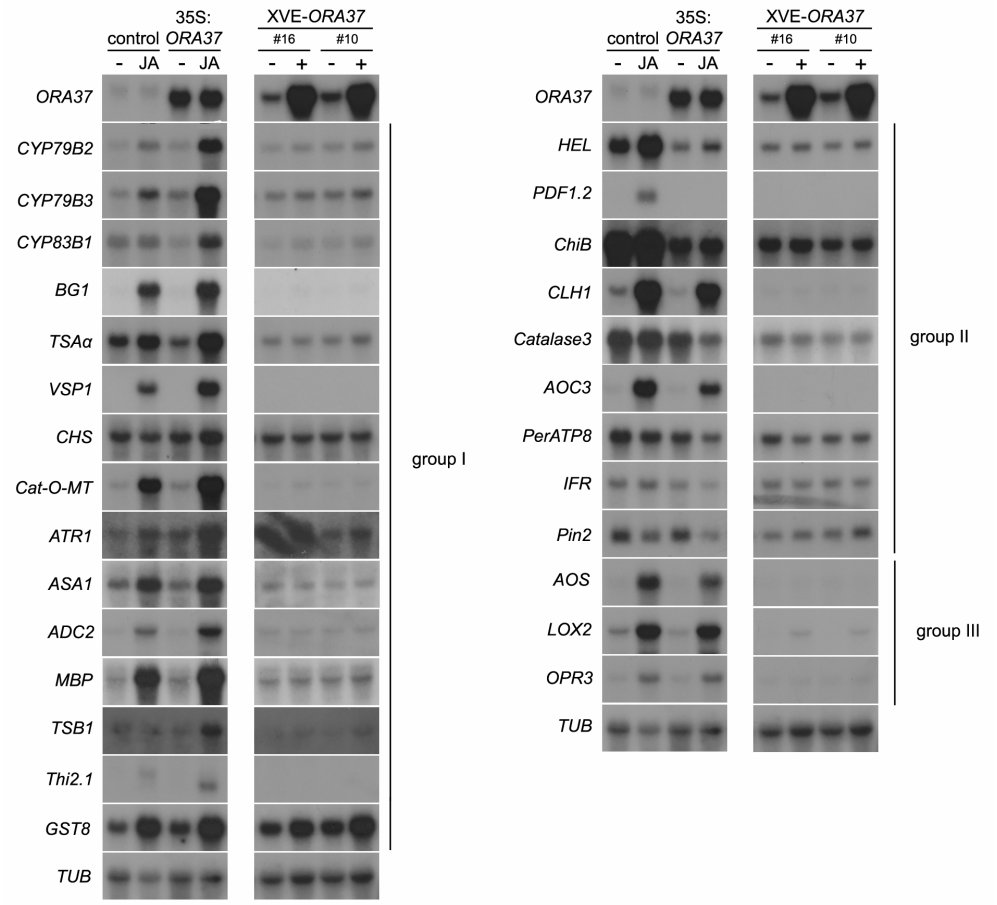

Figure 2. Overexpression of *ORA37* positively or negatively affects the JA-induced expression of distinct sets of JA-responsive genes. Two-weeks-old seedlings from transgenic line 35S:*ORA37* (line #40) and the control line (1301-5) were treated for 8 hours with 50 µM JA or with 0.1% of the solvent DMSO (-). Two-weeks-old seedlings from independent transgenic lines carrying an inducible *ORA37*-overexpression module (XVE-*ORA37* lines #10 and #16) were treated for 24 hours with the inducer estradiol (+) or with the solvent DMSO (-).The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

A control line treated with JA for 8 hours was used to monitor the JA-responsiveness of the selected target genes (Figure 2). Due to its rapid and transient expression in response to JA (Atallah, 2005; see also Figures 4 and 5), induction of the *ORA37* gene could not be detected in the JA-treated control line for that time point (Figure 2).

In most cases, gene expression in untreated *ORA37*-overexpressing plants was similar to the level observed in untreated control plants. Additionally, in transgenic XVE-*ORA37* plants

inducibly overexpressing the *ORA37* gene, genes showing a detectable basal expression level, including *CYP79B2*, *CYP79B3, TSAα* and *HEL*, were expressed at the same level in induced and noninduced conditions. Only the *GST8* gene showed estradiol-induced expression in the XVE-*ORA37* lines (Figure 2). These results show that in normal conditions, ORA37 has no direct effect on the expression of the majority of the tested genes.

In contrast, ORA37 overexpression had an effect on the JA-induced expression of a large number of the tested genes. This effect allowed us to cluster these genes in three groups (Figure 2). Group I, with 15 genes, comprises genes with a higher expression level in response to JA in plants overexpressing the *ORA37* gene compared with control plants. This indicates that the presence of ORA37 enhances the JA-induced expression of the genes belonging to group I. In group II are 9 genes with a severely reduced expression level in response to JA in *ORA37*-overexpressing plants compared with control plants. Within this group II, expression of the *PDF1.2* gene in response to JA was totally blocked in the *ORA37*-overexpressor. This indicates that the presence of ORA37 severely decreases the JA-induced expression of the genes belonging to group II. Group III, with 3 genes, represents genes with similar transcript levels after JA treatment in *ORA37*-overexpressing plants and control plants. These results suggest that ORA37 can act either as a positive (group I) or a negative regulator (group II) of JA-responsive gene expression. Furthermore, the JA-induced expression of a number of genes (group III) is not affected by the overexpression of the *ORA37* gene, suggesting that regulation of the expression of these genes by JA is independent of ORA37 and that ORA37 plays a role in a specific branch of the JA signal transduction pathway.

The group I genes *VSP1* and *CYP79B2* were shown to be induced after wounding (Rojo et al., 1999; Mikkelsen et al., 2000). The ethylene signaling pathway was shown to antagonize the expression of some wound-induced genes (Rojo et al., 1999). In contrast, several genes from group II, including *PDF1.2* and *HEL*, were shown to be synergistically induced by a combined treatment with JA and ethylene. This prompted us to study the expression of the *ORA37*-regulated genes in response to JA and/or ethylene treatments in *ORA37*-overexpressing plants. In accordance with previous reports, the expression of the JA-responsive gene *VSP1* was strongly induced by JA alone and its JA-responsive expression was dramatically decreased in the presence of the ethylene-releaser ethephon in the control line (Figure 3). In contrast, *VSP1* gene expression in response to JA and JA/ethylene was clearly higher in the *ORA37*-overexpressing line compared to the control line. Moreover, induction of the *VSP1* gene expression in response to JA alone was occurring earlier in the overexpressing line compared to the control. A similar expression profile was observed with the *CYP79B2* and *CYP79B3* genes. These results indicate that overexpression of the *ORA37*

gene in plants enhances the positive effect of JA on the expression of the *VSP1*, *CYP79B2* and *CYP79B3* genes. In addition, these results suggest that ORA37 might act negatively on the negative effect of ethylene on the expression of these genes.

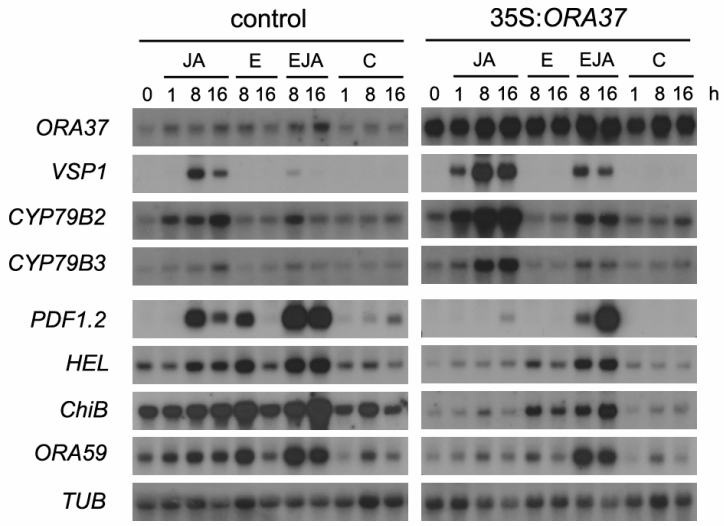

Figure 3. Overexpression of *ORA37* positively or negatively affects the JA- and/or ethylene-induced expression of distinct sets of JA-responsive genes. Two-weeks-old seedlings from transgenic line 35S:*ORA37* (line #40) and the control line (1301-5) were treated for the number of hours (h) indicated with 50 µM jasmonic acid (JA), 1 mM ethephon (E), a combination of both (EJA), or the solvents DMSO and Na-phosphate (C). The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

Conversely, expression of the *PDF1.2* gene in response to hormone treatments was severely compromised in the *ORA37*-overexpressing plants. Induction of *PDF1.2* gene was impaired in response to JA or ethephon treatment (Figure 3). A combination of JA and ethephon resulted in reduced *PDF1.2* gene expression after 8 hours treatment, whereas *PDF1.2* mRNA accumulation in the *ORA37*-overexpressing plants after 16 hours treatment with JA and ethephon was comparable with that observed in the control plants. This indicates that the response to these signaling molecules is not totally blocked in plants overexpressing the *ORA37* gene. Similarly, expression of the *HEL* and *ChiB* genes in response to JA and/or ethephon was clearly reduced in the overexpressor. The basal expression of the *HEL* and *ChiB* genes was also lower in plants overexpressing the *ORA37* gene compared to the control line. These results indicate that ORA37 can act as a negative regulator of a branch of the JA and ethylene signaling pathways. Similar results were observed in two other independent transgenic lines overexpressing the *ORA37* gene (lines #6 and #21; data not shown).

Silencing the *ORA37* gene enhances the JA and ethylene responsiveness of group II genes

To confirm the results obtained from the ectopic overexpression approach, we constructed transgenic Arabidopsis plants in which the *ORA37* gene was silenced using the RNA interference (RNAi) technique. Transgenic *ORA37*-silenced plants growing under normal conditions displayed no visible aberrant phenotype compared to wild-type plants. RNA blot analyses showed that the *ORA37* mRNA was undetectable after treatment with JA in independent lines #1, #4, #7 and #9 (Figure 4). Instead, a prominent band corresponding to a large RNA species together with a smeary signal caused by smaller RNAs was observed with a probe specific for *ORA37*. The presence of such a smeary signal was previously correlated with efficient silencing (Chapter 2), and the large RNA species is most likely the complete unspliced hairpin RNA encoded by the silencing transgene. In transgenic lines #5 and #8, the *ORA37* mRNA was detectable in response to JA, indicating that these lines are not completely silencing the *ORA37* gene. Transgenic lines #2, #3 and #6 showed an intermediate phenotype. A total of 17 independent transgenic silenced lines were screened (Figure 4 and data not shown).

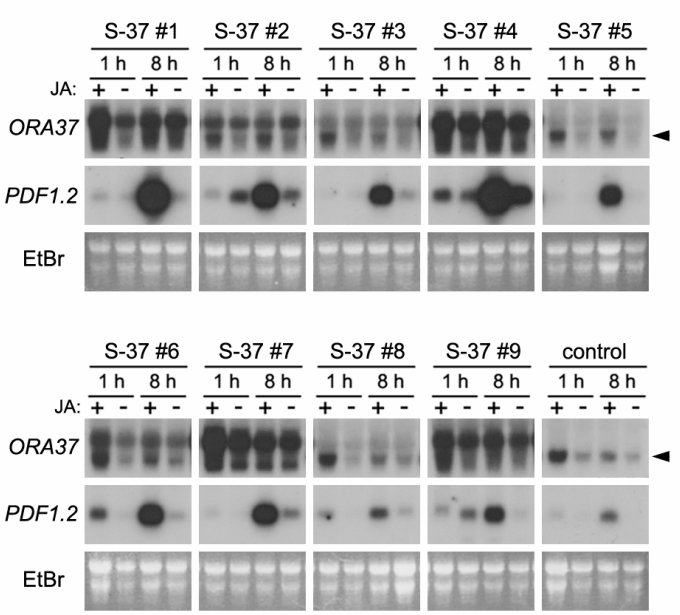

Figure 4. Post-transcriptional silencing of *ORA37* causes increased JA-responsive expression of *PDF1.2.* Two-weeks-old seedlings from nine independent transgenic lines carrying the *ORA37*-silencing construct (S-37) and a transgenic control line were treated for 1 or 8 hours with 50 µM JA (+) or with the solvent DMSO (-). The RNA gel blot was hybridized with the indicated probes. Black arrowheads indicate the position of the *ORA37* mRNA on the RNA gel. Equal loading was verified by ethidium bromide (EtBr) staining of the RNA gel prior to blotting.

As shown in Figure 4, expression of the *PDF1.2* gene in response to JA was dramatically increased in all independent *ORA37*-silenced lines compared with the control line. Enhancement of the JA-induced *PDF1.2* transcript level in *ORA37*-silenced lines was positively correlated with the efficiency of silencing the *ORA37* gene in these individual lines (Figure 4), indicating that the effect observed on *PDF1.2* gene induction was directly linked to ORA37. These results further confirm the role of ORA37 as a negative regulator of a subset of JA-responsive genes including *PDF1.2.* Transgenic lines #4, #10 and #12 were selected for further experiments.

In addition to JA, treatment with a combination of JA and ethephon in the *ORA37*-silenced line resulted in higher expression of the *PDF1.2, ChiB* and *HEL* genes compared to the control line (Figure 5). Moreover, expression of the *ChiB* and *HEL* genes in response to ethephon alone, as well as in non-treated conditions, was significantly enhanced in the *ORA37*-silenced line compared to the control line.

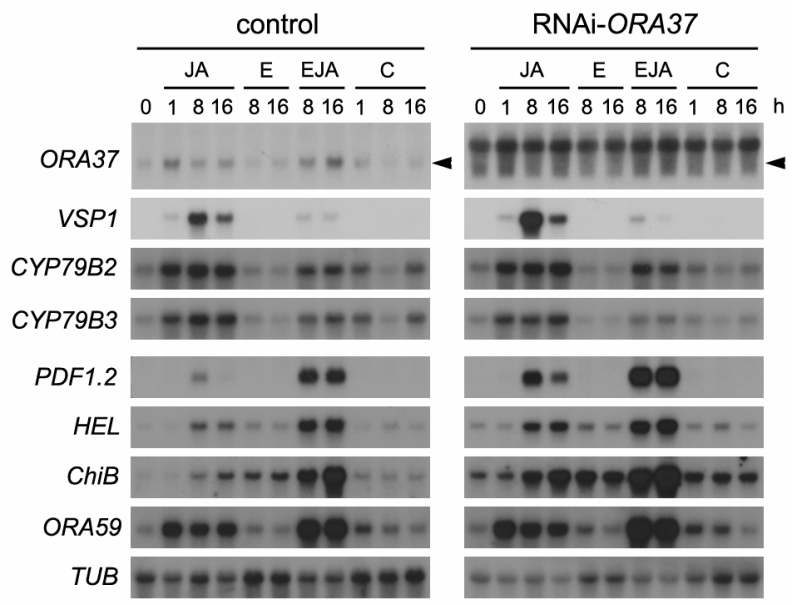

Figure 5. Post-transcriptional silencing of the *ORA37* gene enhances the JA- and/or ethylene-responsiveness of several defense genes. Two-weeks-old seedlings from transgenic *ORA37*-silencing line # 4 (RNAi-*ORA37*) and a control line were treated for the number of hours (h) indicated with 50 µM jasmonic acid (JA), 1 mM ethephon (E), a combination of both (EJA), or the solvents DMSO and Na-phosphate (C). The RNA gel blot was hybridized with the indicated probes. Black arrowheads indicate the position of the *ORA37* mRNA on the RNA gel. The *TUB* probe was used to verify RNA loading.

This indicates that the absence of ORA37 has a positive effect on the JA- and/or ethylene-induced expression of the *PDF1.2, ChiB* and *HEL* genes, as well as on their basal transcript level. These results further demonstrate that ORA37 acts as a negative regulator of the branch of the JA and ethylene signaling pathways involved in *PDF1.2, HEL* and *ChiB* gene

expression. These observations are in accordance with the results obtained with *ORA37*-overexpressing plants (Figure 3).

Treatment of the *ORA37*-silenced line with JA and/or ethephon resulted in *VSP1*, *CYP79B2* and *CYP79B3* induction ratios that were similar to those observed in JA- and/or ethephon-treated control plants (Figure 5). This indicates that silencing of *ORA37* gene expression had no significant effect on the induction of these genes in response to hormone treatments.

ORA37 acts antagonistically to the transcription factor ORA59

In Chapter 2, the JA- and ethylene-induced expression of the *PDF1.2*, *HEL* and *ChiB* genes was shown to be controlled by ORA59, another member of the AP2/ERF-domain family of transcription factors. Our results show that ORA37 seems to have an opposite role in regulating the expression of this group of genes. To test whether the negative effect of *ORA37* overexpression on the JA- and/or ethylene-induced expression of these genes could be due to ORA37-mediated repression of the *ORA59* gene, we analyzed the expression of *ORA59* in response to these signals in plants overexpressing (Figure 3) and silencing (Figure 5) the *ORA37* gene. Expression of the *ORA59* gene in response to JA or ethephon was slightly reduced in *ORA37*-overexpressing plants compared with control plants (Figure 3). However, no significant repression was observed with a combination of both hormones. Furthermore, *ORA59* expression in response to hormone treatments was identical in the RNAi-*ORA37* line compared to the control line (Figure 5). These results indicate that ORA37 does not regulate the JA- and/or ethylene-induced expression of the *ORA59* gene.

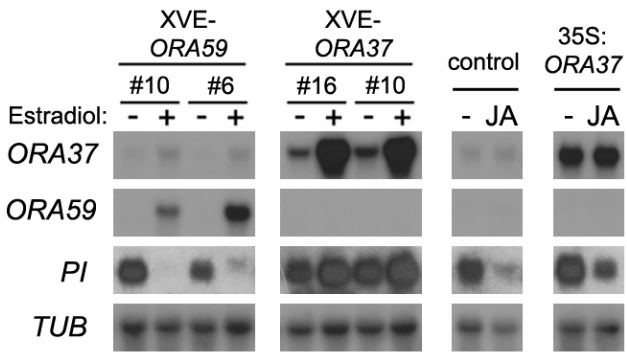

Figure 6. ORA37 and ORA59 have opposite effects on the expression of the *PI* gene. Two-weeks-old seedlings from transgenic line 35S:ORA37 (line #40) and the control line (1301-5) were treated for 8 hours with 50 µM JA or with 0.1% of the solvent DMSO (-). Two-weeks-old seedlings from independent transgenic lines carrying an inducible *ORA37*-overexpression module (XVE-*ORA37* lines #10 and #16) or an inducible *ORA59*-overexpression module (XVE-*ORA59* lines #6 and #10) were treated for 24 hours with the inducer estradiol (+) or with the solvent DMSO (-).The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

Microarray experiments performed with plants overexpressing the *ORA59* gene allowed us to identify a number of genes that were down-regulated in *ORA59*-overexpressing plants (data not shown). The expression of a gene encoding a protease inhibitor (PI) was 15-fold lower in *ORA59*-overexpressing plants compared with the control. Expression of the *PI* gene was also down-regulated by treatment with JA and/or ethephon in wild-type plants. In accordance with the microarray data (not shown), RNA gel blot analyses showed that *PI* gene expression was highly repressed in estradiol-induced *ORA59*-overexpressing transgenic plants (Figure 6). Moreover, the *PI* transcript level was lower in JA-treated control plants compared with untreated plants. In contrast, *PI* mRNA accumulation was increased in plants overexpressing the *ORA37* gene, and JA treatment failed to repress *PI* expression to the same level as in the control plants. These results suggest that ORA37 acts as a positive regulator of *PI* gene expression. Due to the presence of an EAR repression domain in the C-terminal region of the protein, this effect of ORA37 is most likely the result of its activity as a negative regulator of the inhibitory effect of JA or *ORA59* on the expression of the *PI* gene.

Discussion

In this study, we characterized the function of ORA37 as a transcriptional repressor of a subset of JA and ethylene responses and we provided evidence that suggests a role of ORA37 in positively regulating a branch of the JA signaling pathway. Transgenic lines overexpressing *ORA37* showed a reduced effect of JA and ethylene on the expression of genes that are responsive to a combination of these signals. Consistent with this finding, Arabidopsis RNAi lines with reduced *ORA37* expression displayed an increased effect of JA and ethylene on the expression of these same genes. Our results indicate that *ORA37* overexpression may down-regulate the expression of some JA- and ethylene-induced genes. The role of *ORA37* may be to attenuate JA and ethylene responses in Arabidopsis.

ORA37 negatively regulates GCC-box-containing gene expression by JA and/or ethylene

Our results demonstrated that ORA37 is able to repress the JA- and/or ethylene-induced expression of *PDF1.2*, *ChiB* and *HEL* genes when overexpressed in plants. This is in accordance with recent findings from Yang et al. (2005) showing that *AtERF4/ORA37* overexpression led to reduced expression of the *ChiB* gene in untreated and JA- or ethylene-treated plants compared to control plants.

The AtERF4/ORA37 transcription factor contains an ERF-associated amphiphilic repression (EAR) motif in its C-terminal part, which has been demonstrated to be essential for active

gene repression (Otha et al., 2001; Fujimoto et al., 2000; Hiratsu et al., 2003). It has also been demonstrated that AtERF4/ORA37 specifically binds to a GCC-box *cis*-acting sequence *in vitro* and represses the expression of a reporter gene fused to a promoter containing tetramers of a synthetic GCC-box in transient experiments (Fujimoto et al., 2000). The GCC-box motif has been first identified as an ethylene-responsive element that is necessary, and in some cases sufficient, for the regulation of transcription by ethylene (Ohme-Takagi and Shinshi, 1995). A perfect GCC-box (GCCGCC motif) is present in the promoter region of the JA- and ethylene-responsive *PDF1.2*, *ChiB* and *HEL* genes. Therefore, it is likely that ORA37 represses the expression of these genes *via* direct binding to the promoter. Evidence showing the direct binding of ORA37 to the promoter of these genes *in planta* is needed to confirm this hypothesis.

ORA37 is likely to act as a negative regulator of the ethylene-mediated negative effect on JA responses

Surprisingly, our results indicate that *ORA37* overexpression may also upregulate the JA-induced expression of a subset of JA-responsive genes, including *VSP1*. It is not clear how the positive effect of *ORA37* overexpression on JA signaling is operating at the molecular level.

In silico promoter analysis of the *VSP1* gene did not reveal the presence of a GCC-box(-like) motif, making it unlikely that *VSP1* is directly regulated by an AP2/ERF-domain transcription factor. Furthermore, the EAR repression domain present in ORA37 was demonstrated to be responsible for the active repressor activity of ORA37. Overexpression of a transcription factor fused to an EAR domain in transgenic Arabidopsis plants resulted in a loss-of-function phenotype, because the hybrid version of the transcription factor acted as a dominant repressor of the genes regulated by this transcription factor (Hiratsu et al., 2003). It is therefore unlikely that ORA37 can function as a transcriptional activator and that the enhanced gene expression observed in JA-treated *ORA37*-overexpressing plants is due to direct binding of ORA37 to the promoter of genes, including *VSP1*, and subsequent activation of gene expression. The *VSP2* gene, a close homologue of the *VSP1* gene, was not expressed in response to JA in *jai1/jin1* mutant plants. The *JAI1/JIN1* locus encodes the helix-loop-helix-leucine zipper-type transcription factor AtMYC2 (Lorenzo et al., 2004). AtMYC2 was shown to activate gene expression *via* recognition of a G-box-like *cis*-acting element (Abe et al., 1997). A G-box-like element has been reported to drive JA-mediated expression of the *VSP1* gene (Guerineau et al., 2003). Thus, it is likely that AtMYC2, instead of ORA37, is the positive regulator of the JA-induced expression of the *VSP2* gene (Lorenzo et al., 2004), as well as the *VSP1* gene.

The ethylene 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). Similarly, the JA-induced expression of the *VSP1* and *CYP79B2* genes was reduced in control plants treated with a combination of ethephon and JA compared to control plants treated with JA alone (Figure 3). This indicates that ethylene has a negative effect on a branch of the JA signaling pathway. Therefore, the role of ORA37 might be to repress the inhibitory effect of ethylene on the expression of a subset of JA- and/or wound-inducible genes. Overexpression of the ethylene-responsive AP2/ERF gene *ERF1* has been shown to inhibit the expression of the *VSP2* gene in response to JA (Lorenzo et al., 2004). It was suggested that the negative regulation of the *VSP2* gene by ethylene is executed through ERF1. It is possible that ORA37 antagonizes the ERF1-mediated negative effect of ethylene, presumably by competing for the same AP2/ERF-domain binding site. Identification of the direct target genes of ORA37 is an important prerequisite to unravel the precise function of ORA37 in the JA and/or ethylene signaling pathways. In the absence of ethylene treatment, JA treatment in *ORA37*-overexpressing plants resulted in higher expression of the *VSP1* and *CYP79B2* genes compared to JA-treated control plants. The increased expression of these genes in *ORA37*-overexpressing plants in response to JA alone may be due to ORA37-mediated repression of the negative effect of the basal level of ethylene.

ORA37 versus ORA59

Our results show that ORA37 negatively regulates the expression of the *PDF1.2*, *ChiB* and *HEL* genes in response to JA and/or ethylene. In contrast, positive regulation of these genes by these signals was demonstrated to be controlled by ORA59, another member of the AP2/ERF-domain transcription factor family (Chapter 2). The results obtained with *ORA37*-overexpressing and *ORA37*-silencing plants demonstrated that *ORA59* gene expression in response to JA and/or ethylene was not controlled by ORA37, ruling out the possibility that the *PDF1.2*, *ChiB* and *HEL* gene repression by ORA37 was due to *ORA59* gene repression. ORA59 and ORA37 can both bind to GCC-box-containing promoters (Fujimoto et al., 2000; Adel Zarei, personal communication), suggesting that both proteins are targeted to the same gene promoters, including those of *PDF1.2*, *ChiB* and *HEL*, with antagonistic effects on gene expression. Binding of ORA37 to the GCC-box could prevent the association of the positive regulator ORA59 with these promoters, thereby preventing gene expression. However, due to the presence of the EAR domain, ORA37 actively represses gene expression. Therefore, such antagonism is not only passive (due to the competition for the same binding site) but also active due to EAR-mediated active gene repression.

ORA37 also seems to antagonize ORA59 for the regulation of the *PI* gene. In contrast to the previous scenario, ORA59 appeared to have a negative effect on *PI* gene expression, whereas ORA37 appeared to act positively. *PI* gene expression was impaired in JA-treated plants or in plants overexpressing the *ORA59* gene and *ORA37* overexpression resulted in reduced inhibition of gene expression in response to JA, further supporting the reverse and opposite functions of ORA37 and ORA59. It is possible that ORA37 represses the expression of an ORA59-regulated gene encoding an inhibitor of *PI* expression.

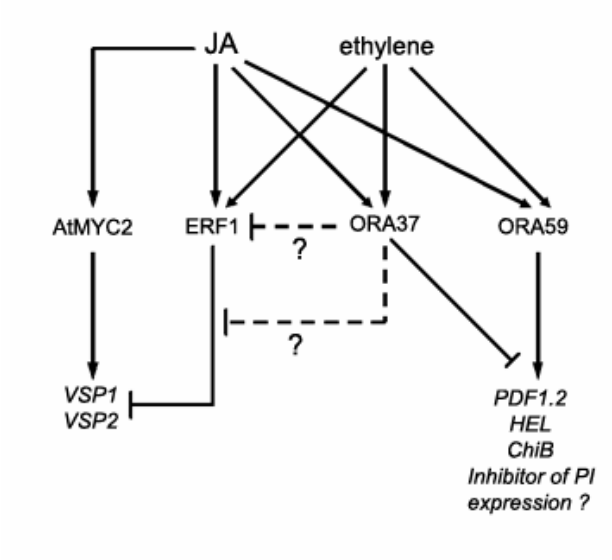

Figure 7. Model showing the role of several transcription factors in JA and ethylene signaling. Perception of JA and/or ethylene leads to the transcriptional activation of the transcription factors *ORA37*, *ORA59*, *ERF1* and *AtMYC2*. ORA59 integrates both JA and ethylene signals to control the expression of a large number of defense genes including the *PDF1.2, HEL* and *ChiB* genes, as well as presumably a gene encoding an inhibitor of *PI* expression. In contrast, ORA37 represses the expression of these genes. In response to JA, AtMYC2 activates the expression of the JA- and wound-inducible genes *VSP1* and *VSP2*. The ethylene-responsive ERF1 transcription factor represses the expression of the *VSP1* and *VSP2* genes. ORA37 enhances *VSP1* and *VSP2* gene expression, presumably by acting negatively on the negative effect of ERF1 on *VSP1* and *VSP2* gene expression.

Induction of the *ORA37* gene occurs within 0.5-1 hour after JA treatment (Atallah, 2005). The Arabidopsis genome encodes 8 AP2/ERF-domain transcription factors with an EAR repression domain. Among these 8 genes, only the *ORA37* gene was induced by JA treatment in the screening performed by Atallah (2005). This suggests that ORA37 is a key regulator of the JA signaling pathway. Moreover, treatment with ethylene induced the expression of the *ORA37* gene after 12 hours (Fujimoto et al., 2000). Expression analyses performed in Arabidopsis mutants insensitive to JA (*coi1*) or ethylene (*ein2* and *etr1*) revealed that JA-induced *ORA37* expression requires both intact JA and ethylene signaling pathways

(Atallah, 2005). Moreover, a combination of JA and ethylene prolonged the expression of the *ORA37* gene (Atallah, 2005). This strongly indicates that ORA37 is involved in the JA and ethylene signaling pathway.

Therefore, JA and ethylene induce both activators (e.g. ORA59 and ERF1) and repressors (e.g. ORA37) of gene expression responses. The model presented in Figure 7 summarizes the findings from this study. The functional importance of the induction of both positive and negative regulators by JA and ethylene remains unclear. The balance between AP2/ERF-domain activators and repressors on common target promoters may provide an important mechanism in transcriptional control. In the natural situation, it is probable that ORA37 and ORA59 have different expression levels at any given time point, as is also suggested by the differential kinetics of *ORA37* and *ORA59* mRNA accumulation in response to JA (Atallah, 2005). A similar dual function was previously described in Arabidopsis for TGA5 and TGA4, two bZIP transcription factors (Foley and Singh, 2004). TGA5 positively regulates a reporter gene under the control of a minimal promoter driven by a stress-related regulatory element, whereas TGA4 negatively regulates the same reporter construct. In *Catharanthus roseus,* the EAR-motif-containing zinc finger transcription factors ZCT1, ZCT2 and ZCT3 were shown to repress the activating activity of the ORCA2 and ORCA3 AP2/ERF-domain transcription factors on the *STR* promoter (Pauw et al., 2004). The expression of both the ZCT repressors and the ORCA activators is induced by JA and fungal elicitors. The simultaneous induction of repressors and activators may serve to fine-tune the amplitude and timing of gene expression. Such a fine-tuning may in part be achieved by the differential effect of JA and ethylene on the amplitude and kinetics of *ORA37* and *ORA59* mRNA accumulation. Alternatively, in analogy to models used to explain switch-like transcriptional control by developmental signals (Barolo and Posakony, 2002), induction of a combination of activators and repressors may be necessary to achieve a switch-like on/off state of gene expression in response to stress signals. Such a mechanism could be adapted for a quick and transient response against stress.

Materials and Methods

Biological materials, growth conditions and treatments

Arabidopsis thaliana ecotype Col-0 is the genetic background for wild-type plants and all transgenic 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 the T2 generation were selected on solid MA

medium containing 100 mg/L timentin and either 25 mg/L kanamycin for *ORA37*-silenced plants and related control plants or 20 mg/L hygromycin for *ORA37*-overexpressing plants and related control plants. Following stratification for 3 days at 4ºC, seeds were incubated at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days, after which 15 to 20 seedlings 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 for different time periods with 50 μM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.1% final concentration), 1 mM of the ethylene-releasing compound ethephon (Sigma) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) or a combination of JA and ethephon. As controls, seedlings were treated with 0.1% DMSO, 0.5 mM sodium phosphate or a combination of both. Transgenic plants carrying an XVE expression module containing the *ORA37* or *GUS* gene were treated with 2 μM estradiol (Sigma) dissolved in DMSO (0.2% final concentration). As a control, seedlings were treated with 0.2% DMSO.

Binary constructs and plant transformation

The *ORA37* (*At3g15210*) open reading frame (ORF) was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-CGG GAT CCA TAT GGC CAA GAT GGG CTT GAA AC-3' and 5'-CGG GAT CCT CAG GCC TGT TCC GAT GGA G-3'. The *ORA37* ORF digested with BamHI was inserted in pBluescript SK+ (Stratagene, La Jolla, CA) such that the 5'-end of the *ORA37* ORF flanked the EcoRI site.

For the construction of transgenic lines constitutively overexpressing *ORA37*, the *ORA37* ORF digested with BamHI was cloned into pRT101 (Töpfer et al., 1987). The Cauliflower Mosaic Virus (CaMV) 35S cassette containing the *ORA37* ORF in sense orientation was excised from pRT101-ORA37 with PstI and cloned into the binary vector pCAMBIA1300 (accession number AF234296). The binary vector pCAMBIA1301 (accession number AF234297) carrying the *GUS* gene under the control of the CaMV 35S promoter was used to generate the control line (line 1301-5).

For the construction of transgenic lines showing post-transcriptional gene silencing of the *ORA37* gene, the *ORA37* ORF digested with BamHI was cloned into pIC-19R (Marsh et al., 1984) digested with BamHI such that the 5'-end of the *ORA37* ORF flanked the EcoRI restriction site. The *ORA37* insert was excised from pIC-19R with EcoRI/SalI and cloned into the pHANNIBAL vector (accession number AJ311872) to generate pHAN-ORA37as. To create an inverted repeat, the *ORA37* ORF was cloned as a BamHI fragment into pIC-20H (Marsh et al., 1984) digested with BglII. The *ORA37* ORF was excised from pIC-20H with HindIII and cloned into pHAN-ORA37as to generate a pHANNIBAL construct containing the *ORA37* ORF as an inverted repeat. For the construction of the RNAi control line (S-GUS #6), 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 (XVE), the *ORA37* open reading frame was excised from pBluescript SK+ with ApaI/SpeI and cloned into the binary vector pER8 (Zuo et al, 2000) digested with ApaI/SpeI.

The binary vectors pCAMBIA1300-ORA37 and pCAMBIA1301 were introduced into *Agrobacterium tumefaciens* strain LBA1115 (containing the Vir plasmid pSDM3010). The binary vectors pART27-ORA37 and pART27-GUS were introduced into *A. tumefaciens* strain LBA4404 while pER8-ORA37 was 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. Details for the construction of the XVE-*ORA59* plants are reported in Chapter 2.

Northern blot analyses

Total RNA was extracted from frozen tissue by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70 % ethanol, and resuspended in water. As described by Memelink et al. (1994), 10 µg RNA samples were subjected to electrophoresis on 1.5% 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 detailed in Chapter 3. Blots were exposed on X-ray films (Fuji, Tokyo, Japan).

DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5'- GGG GTA CCG GAT CCC AAC TTT TCT CGA GCT GAG TGA C- 3' and 5'- GCT CTA GAG AAT TCT AGA CAG GAC GCG ACA TCG G- 3' for *ORA37* (*At3g15210*); 5'- GGG GTA CCG GAT CCT CTT AAG TGG AGA GTT TTC C- 3' and 5'- GCT CTA GAC TCG AGG CCA CGG TGG CTT CTT TTC C- 3' for *ORA59* (*At1g06160*); 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'- TCA AAC TCT TCG GAT CTC AC- 3' and 5'- CCC AAG CGG TTA AAT GAT CG- 3' for *CYP79B2* (*At4g39950*); 5'- CTC CTT CTT CCT TGC AAA TGG- 3' and 5'- GTC CGT TTA GAT CCA ATC CC- 3' for *CYP79B3* (*At2g22330*); 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 ATT GCT TTC AAA TCC- 3' and 5'- TCA AAG AAG AGC AGA TTT AAG- 3' for *Tryptophan synthase α chain* (*TSAα, At3g06050*); 5'- AAT GAG CTC TCA TGG CTA AGT TTG CTT CC- 3' and 5'- AAT CCA TGG AAT ACA CAC GAT TTA GCA CC- 3' for *Plant defensin 1.2* (*PDF1.2, At5g44420*); 5'- GCT TCA GAC TAC TGT GAA CC- 3' and 5'- TCC ACC GTT AAT GAT GTT CG- 3' for *Chitinase B* (*ChiB, At3g12500*); 5'- CGG GAT CCA TAT GAA GAT CAG ACT TAG CAT AAC- 3' and 5'- CGG GAT CCT CAA ACG CGA TCA ATG GCC GAA AC- 3' for *Hevein-like* gene (*HEL, At3g04720*); 5'- ATG GCT TAT TCT AAG GTT GC- 3' and 5'- CTA GGC ACA TTT GAA ACC AG- 3' for *Protease Inhibitor* (*PI*, *At5g4890*); 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'- TAT AGC CGG TTT AGT AGC GGC- 3' and 5'- TGT GCC GTA CTC ATT GTA CC- 3' for *CYP83B1* (*At4g31500*); 5'- ATG GTG ATG GCT GGT GCT TC- 3' and 5'- TTA GAG AGG AAC GCT GTG CAA G- 3' for *Chalcone synthase* (*CHS*, *At5g13930*); 5'- ATG GGA CAC CTA ATT CCT C- 3' and 5'- CTA AGC CT TTT CGT TAA CTC- 3' for *Chatechol-O-methyl transferase* (*Cat-O-MT*, *At1g76790*); 5'- CAA TTC AAC CGG TCA AAC CG- 3' and 5'- CAT ATT GTC ATC TTC GTT CC- 3' for *ATR1* (*At5g60890*); 5'- ATG CCT GCT TTA GCT TGC G- 3' and 5'- TCA CGC AGA GAT GTA ATC G- 3' for *Arginine decarboxylase2* (*ADC2*, *At4g34710*); 5'- ATG GCG AAA AAG TTG GAA GC- 3' and 5'- TCA

TTT GGC TAT GGG CGC AAC- 3' for *Myrosinase-binding protein* (*MBP*, *At3g16470*); 5'- CAG GCA CCT CTG CTA CTT TC- 3' and 5'- ATA GCT CCA TGG AGG ACA CC- 3' for *Tryptophan synthase β subunit* (*TSB1*, *At5g54810*); 5'- ATG AAA GGA AGA ATT TTG ATT TTG- 3' and 5'- TTA CAA CAG TTT AGG CGG CC- 3' for *Thionin2.1* (*Thi2.1*, *At1g72260*); 5'- ATG GCG AAC GAG GTG ATT C- 3' and 5'- TTA CTC AGG TAC AAA TTT CTT CC- 3' for *Glutathions S-transferase8* (*GST8*, *At1g78380*); 5'- ATG GCG GCG ATA GAG GAC AG- 3' and 5'- CTA GAC GAA GAT ACC AGA AG- 3' for *Chlorophyllase1* (*CLH1*, *At5g43860*); 5'- ATG GAT CCT TAC AAG TAT CG- 3' and 5'- CTA GAT GCT TGG CCT CAC GTT C- 3' for *Catalase3* (*At1g20620*); 5'- ATG GCT CT TCA GCA GTG TC- 3' and 5'- TTA GTT GGT ATA GTT ACT TAT AAC- 3' for *Allene oxide cyclase3* (*AOC3*, *At3g25770*); 5'- ATG GAG AAG AAT ACT TCT CAA AC- 3' and 5'- CTA ATT GAC ACG TGA ACA ATC- 3' for *Peroxidase ATP8* (*PerATP8*, *At4g30170*); 5'- ATG AAA GAG ACT AAT TTT GG- 3' and 5'- TTA TAC GAA AAT TTT CAA ATA TTC- 3' for *Isoflavone reductase* (*IFR*, *At4g13660*); 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 ACG GCG GCA CAA GGG AAC- 3' and 5'- TCA GAG GCG GGA AGA AGG AG- 3' for *OPDA reductase3* (*OPR3*, *At2g06050*); 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*).

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Chapter 5

JA-responsive AP2/ERF-domain transcription factors have distinct roles in JA signaling in *Arabidopsis thaliana*

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Abstract

Jasmonic acid (JA) is an important plant hormone involved in defense responses against external threats. JA perception leads to the activation of specific set of defense genes. In Arabidopsis, the expression of several genes, named *ORA* genes, encoding members of the AP2/ERF-domain transcription factor family, is induced by JA. The role of ORA59, ORA47 and ORA37 in JA signaling was described in the previous chapters. In order to assess the functions of the remaining ORA transcription factors and to address the question of functional redundancy, transgenic plants overexpressing individual *ORA* genes under the control of an inducible promoter were constructed. Several JA-responsive genes showed high transcript levels in plants overexpressing a specific *ORA,* indicating that these genes are regulated by a unique AP2/ERF-domain transcription factor within the set tested. In contrast, a number of JA-responsive genes were upregulated in several *ORA*-overexpressing plants, suggesting functional redundancy among these ORAs.

Introduction

Plants have multiple defense mechanisms to fight against external stress, including wounding and attack by insects and microbial pathogens. Some of these defense mechanisms involve preformed chemical and physical barriers, which impede access to the host plant, whereas others are stimulated in response to the attack and subsequently limit further damage to the plant. Pathogen or herbivore challenge activates a number of signaling pathways that coordinately regulate expression of many genes encoding various
transcriptional regulators, enzymes functioning in the synthesis of phytoalexins and other secondary metabolites, pathogenesis-related proteins, and a number of other antimicrobial molecules (Schenk et al., 2000). At least three signal molecules are known to regulate the signaling pathways associated with plant defense responses. These are salicylic acid (SA), jasmonic acid (JA) and ethylene. Substantial cross talk occurs among these signaling pathways to mount a defense response that is adapted to the type of challenge (Turner et al., 2002; Thomma et al., 2001; Kunkel and Brooks, 2002).

JA and its methyl ester, methyl jasmonate (MeJA), are cyclopentanone derivatives which are synthesized from linolenic acid *via* the octadecanoid pathway (Turner et al., 2002). JA synthesis is induced by a range of biotic and abiotic stresses, including osmotic stress, wounding, drought, exposure to elicitors, insect attack and pathogen infection (Creelman and Mullet 1995; Turner et al., 2002). Exogenous application of (Me)JA results in major

reprogramming of gene expression. A number of genes that are known to be involved in plant stress responses are induced by JA treatment. For example, JA induces the expression of genes encoding antimicrobial proteins including plant defensin (PDF1.2; Penninckx et al., 1996) and thionin (THI2.1; Epple et al., 1995), and genes encoding biosynthetic enzymes involved in primary and secondary metabolism (Turner et al., 2002; Atallah and Memelink, 2004; Pauw and Memelink, 2005).

These genes are differentially expressed depending on the nature of the stress perceived by the plant, suggesting that the transcription of these genes is controlled in a specific manner. How JA signaling activates the expression of specific genes is largely unknown.

In plants, a number of transcription factors have been implicated in the regulation of stress-related JA responses (Pauw and Memelink, 2005).

The AP2/ERF-domain transcription factors ORCA2 and ORCA3 from *Catharanthus roseus* were shown to regulate the JA-responsive expression of several genes encoding biosynthetic enzymes involved in the production of defense-related secondary metabolites. *ORCA* gene expression was rapidly induced after treatment with MeJA (Menke et al., 1999; van der Fits and Memelink, 2000 and 2001). In Arabidopsis, the *ERF1* gene, encoding an AP2/ERF-domain transcription factor, is induced by JA or ethylene. Overexpression of *ERF1* upregulates the expression of a large number of JA- and ethylene-responsive genes involved in defense (Lorenzo et al., 2003). Therefore, the JA-responsive expression of several genes is mediated by specific AP2/ERF-domain transcription factors.

In Arabidopsis, the AP2/ERF-domain transcription factor family comprises 124 proteins. In a family-wide screening, Atallah (2005) previously characterized 14 genes encoding Arabidopsis AP2/ERF-domain proteins, which were rapidly induced by JA treatment in 10-days-old seedlings. The JA-induced expression of these genes, named *Octadecanoid-Responsive Arabidopsis AP2/ERF* (*ORA*) genes, was severely reduced in the JA-insensitive *coi1* mutant, consistent with a possible role in JA signaling.

Atallah (2005) used the conserved DNA-binding AP2 domains from all the members of the AP2/ERF-domain protein family to construct a phylogenetic tree. Results revealed that the 14 ORA proteins did not cluster together. Close relationships were only observed between the AP2 domains from ORA1, ORA2 and ORA4, and from ORA63, ORA68 and ORA71. This indicates that, although sharing common features, the amino acid sequences of most of the ORA AP2 domains show some divergence. A transcription factor controls the expression of its target genes by sequence-specific binding to their promoter region. The sequence divergence between the ORA AP2 domains suggests that the ORA transcription factors might regulate different JA-responsive target gene sets. Moreover, the 14 *ORA* genes showed

differential expression kinetics in response to JA (Atallah, 2005), suggesting that the ORA proteins play distinct roles in the JA signaling cascade.

In this study, we investigated the role of the different ORA transcription factors in the JA response using a gain-of-function approach. Our goal was to identify genes showing increased or reduced expression in transgenic plants overexpressing the *ORA* genes. In the most common form of the gain-of-function strategy, the gene of interest is overexpressed using a strong constitutive promoter, such as the cauliflower mosaic virus 35S promoter. However, there are disadvantages to constitutive overexpression of transcription factors. Altering the expression of transcription factors involved in defense has wide-ranging consequences on the plant host, including on plant development (Whalen, 2005). Plants constitutively overexpressing genes coding for transcription factors, including *ORA59* (Chapter 2), *ERF1* (Solano et al., 1998), *AtWRKY6* (Robatzek and Somssich, 2002) and *AtWRKY18* (Chen and Chen, 2002), often exhibit growth retardation and stunted phenotypes, among others. This plant dwarfism is likely to reflect a general stress condition that may lead to the expression of stress-responsive genes that are not direct targets of the transcription factor. Moreover, constitutive expression leads to the expression of both direct target genes and target genes of downstream transcription factors. In addition, due to its abundance or inappropriate expression in time and space, the overexpressed regulatory protein could activate genes that it does not in the wild-type (Zhang, 2003). For example, constitutive overexpression of the *AtERF1* gene led to high expression of the defense gene *PDF1.2*, whereas transient or inducible overexpression of *AtERF1* did not result in *PDF1.2* expression (Chapter 2). In conclusion, genes switched on by constitutive overexpression of a transcription factor are not necessarily true target genes.

In our experimental set-up, we used transgenic plants overexpressing the *ORA* genes under the control of an estradiol-inducible promoter relying on a chimeric XVE transcription factor (Zuo et al., 2000a). Shortly, in the absence of estradiol, the chimeric XVE transcription factor is restricted to the cytoplasm and there is no expression of the *ORA* gene. When the inducer is present, the XVE regulatory protein localizes to the nucleus and binds to the artificial promoter that controls *ORA* gene expression resulting in a high transcript level. We speculated that, by inducing *ORA* expression for a relatively short period of time, this approach would prevent the expression of late or unrelated genes in response to *ORA* overexpression and would allow us to identify with a higher probability direct target genes of ORA transcription factors. Although the period in which the cells are exposed to high amounts of ORA transcription factors is limited, one cannot exclude that production of ORA proteins to levels in excess of those found under physiological conditions might result in

induction of genes that would not be target genes in normal conditions. However, this possibility is far less likely than when using a constitutive overexpression approach.
Only ten *ORA* genes (*ORA1*, *ORA2*, *ORA4*, *ORA19*, *ORA31*, *ORA33*, *ORA37*, *ORA44*, *ORA47* and *ORA59*) were included in this study, since the other 4 *ORAs* (*ORA63*, *ORA68*, *ORA71* and *ORA91*) were identified at a later stage after this study was initiated.

Results

The XVE-inducible system as a powerful tool for transient gene overexpression

The open reading frames of all ten *ORAs*, as well as the *GFP* and *GUS* control genes, were inserted into the target expression cassette of the XVE module in the pER8 vector (Zuo et al., 2000a). The pER8-derived constructs were used to transform Arabidopsis. Ten to twenty primary transformants per construct were selected and allowed to self-pollinate for analyses of the subsequent T2 generation for expression of the *ORA, GUS* and *GFP* genes.

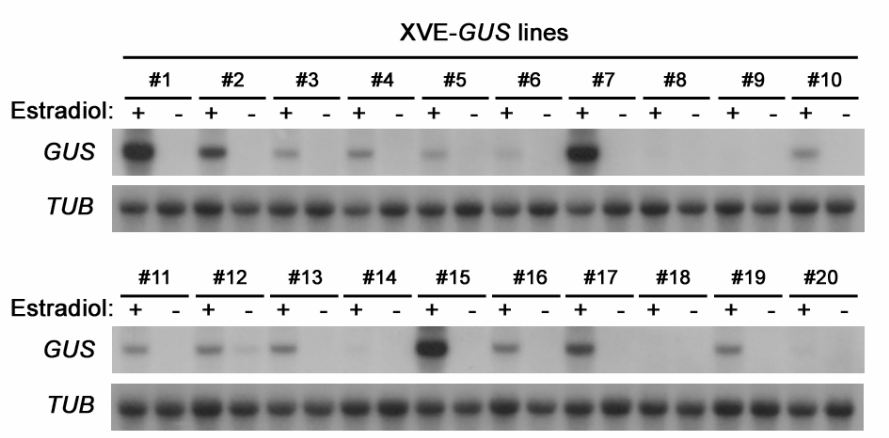

Figure 1. The *GUS* gene is induced by estradiol in the majority of independent transgenic XVE-*GUS* lines. Ten-days-old seedlings from 20 independent transgenic lines containing the *GUS* gene under the control of the XVE module were treated for 24 hours with 2 µM estradiol (+) or with the solvent DMSO (-). The RNA gel blot was hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

Transgenic control lines carrying the *GUS* gene under the control of the XVE expression module were first analyzed to optimize the induction procedure. Independent transgenic XVE-*GUS* lines of the T2 generation were first screened for their ability to express the *GUS* transgene in the presence or absence of the inducer estradiol. Based on the results obtained

by Zuo et al. (2000a), a treatment with 2 µM estradiol for 24 hours was used as the condition for initial screening. RNA gel blot analyses performed with treated- and untreated-two-weeks-old XVE-*GUS* seedlings showed that 14 out of 20 lines expressed the *GUS* gene in the presence of the inducer (Figure 1). Variable *GUS* transcript levels were observed between independent transgenic lines. Except for line #12, no expression of the *GUS* gene was observed in non-induced transgenic lines, indicating that the XVE system is tightly controlled. The XVE-*GUS* line #15, which was highly expressing the *GUS* gene in the presence of estradiol, was selected for further analyses. RNA gel blot analyses of several independent XVE-*GFP* lines were also performed with similar results (data not shown).

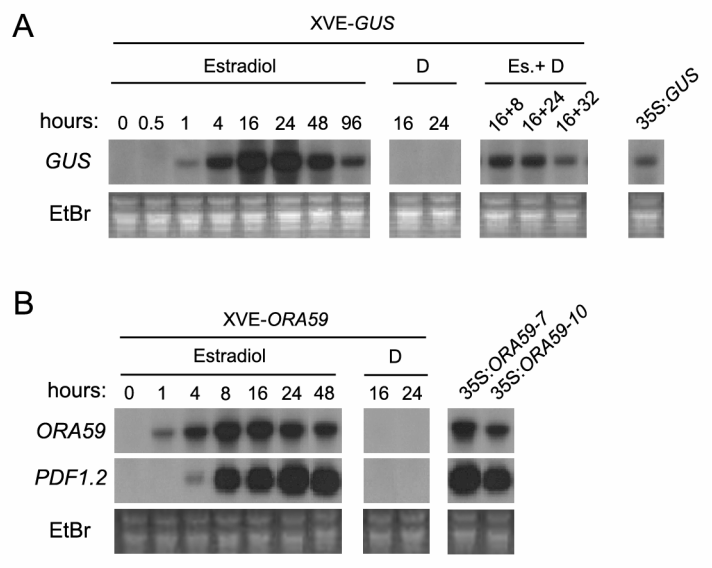

Figure 2. Time course analysis of XVE-controlled transgene expression in response to estradiol.
(A). Two-weeks-old XVE-*GUS* (line 15) seedlings were treated for the number of hours indicated with 2 µM estradiol (Es) or the solvent DMSO (D). **(B).** Two-weeks-old XVE-*ORA59* (line 6) seedlings were treated for the number of hours indicated with 2 µM estradiol or the solvent DMSO (D). The RNA gel blots were hybridized with the indicated probes. Equal loading and RNA integrity was verified by ethidium bromide staining of the gel (EtBr) prior to blotting.

To investigate the induction kinetics, 2-weeks-old XVE-*GUS#15* seedlings were treated for varying time periods with estradiol (Figure 2A). Expression of the *GUS* gene was detectable after 1 hour in the presence of the inducer and a peak of expression was obtained after 16-24 hours of treatment. After this peak, the *GUS* transcript level gradually declined. After 16 hours of induction, *GUS* transcript level was about 4- to 5-fold higher than that of a representative line carrying the *GUS* gene under the control of the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter. To determine the persistence of the induction treatment, transgenic plants induced for 16 hours with estradiol were transferred to non-inducing medium and *GUS*

expression was measured over time. As shown in Figure 2A, although reduced, *GUS* expression was still detectable after 32 hours in the absence of inducer. Untreated transgenic plants showed undetectable *GUS* expression.

We performed similar analyses with plants containing the XVE-*ORA59* module. Overexpression of the *ORA59* gene was previously shown to induce the expression of the defense-related *PDF1.2* gene (Chapter 2). Expression of the *PDF1.2* gene was assessed in induced XVE-*ORA59* plants to determine the optimal time points for the identification of ORA target genes. Similar to the XVE-*GUS* lines, screening of the XVE-*ORA59* lines showed that 9 out of 10 independent lines had induced expression of the *ORA59* gene in response to estradiol (data not shown). The representative XVE-*ORA59* line number 6 was chosen for further analyses.

Two-weeks-old XVE-*ORA59-6* seedlings were treated for varying time periods with estradiol. In accordance with the results obtained with the XVE-*GUS* plants (Figure 2A), expression of the *ORA59* gene was detectable after 1 hour in the presence of the inducer and a peak of expression was obtained after 8-16 hours of treatment (Figure 2B). Estradiol-induced expression of the *PDF1.2* was detectable after 4 hours with a peak of expression after 24 hours. At this time point, the *PDF1.2* transcript level was at least as high as that observed in two independent transgenic plants constitutively overexpressing the *ORA59* gene (35S:*ORA59*-7 and -10). No expression of the *PDF1.2* gene was observed in untreated XVE-*ORA59* plants. Based on our findings, a treatment for 24 hours with 2 µM estradiol was considered as the optimal condition for the identification of ORA-regulated genes in the different XVE-*ORA* transgenic lines.

For the other *ORAs,* the screening for the identification of XVE-*ORA* lines was performed as described before. For each construct, at least 50 % of the independent lines tested showed high expression of the transgene after 24 hours of treatment with the inducer (data not shown). All transgenic XVE-inducible plants growing under normal conditions displayed no visible aberrant phenotype compared to wild-type plants, indicating that insertion of the XVE expression module in the plant genome, as well as constitutive expression of the XVE transcription factor, had no detectable effect on plant development.

Identification of ORA-regulated genes

The *ORA* genes are induced in response to JA (Atallah, 2005). For a number of these *ORA* genes, the respective encoded proteins were shown to bind *in vitro* to a GCC-box *cis*-acting element and to activate transcription *in vivo* in a transient assay via this element (Atallah,

2005; Adel Zarei, personal communication). Together, these observations strongly suggest that the ORA transcription factors are terminal components of the JA signal transduction pathway regulating defense gene expression. In order to test this hypothesis, RNA was extracted from the different transgenic lines grown in the presence or absence of estradiol during 24 hours.

A number of putative candidate target genes (Table 1), which are known to be responsive to JA and/or ethylene, were selected and their expression was measured in the different transgenic lines (Figure 3 and Table 1). These genes encode proteins involved in defense against biotic or abiotic stress, JA biosynthesis or primary and secondary metabolism.

In each transgenic line, expression of the respective XVE-*ORA* gene was highly increased in response to the inducer estradiol (Figure 3, top panels). The expression level of the *ORA* transgenes was similar among the lines.

For a large number of the tested genes, such as *TSAα*, *FST*, *BG1* and *HEL* genes, we observed a slight increase in transcript level in the induced XVE-*ORA1* line, suggesting that *ORA1* overexpression positively regulates the expression of these genes. In the non-induced XVE-*ORA2* transgenic line, the basal expression of a large number of genes, including the *CYP83B1, TSAα*, *CLH1* and *HEL* genes, was significantly higher than in the XVE-*GUS* control line. However, expression of these genes was similar in induced and non-induced XVE-*ORA2* plants.

Gene expression profiling in the different induced XVE-*ORA* lines allowed us to cluster the putative target genes in four groups. Expression of the genes belonging to group I such as *ASA1*, *TSAα*, and *FST*, was induced in transgenic lines overexpressing the *ORA1*, *ORA33*, *ORA47* or *ORA59* genes. Increase in gene expression was most significant in induced XVE-*ORA47* and XVE-*ORA59* lines. Genes from group II, including the *ADC2* and *IFR* genes, were induced in transgenic lines overexpressing *ORA1*, *ORA33* and *ORA47*.

Group III, containing most of the tested genes (Table 1), represents genes that are only induced in the XVE-*ORA47* line. Finally, expression of the genes from group IV, including *PDF1.2* and *HEL*, was strongly induced in the XVE-*ORA59* line, whereas a slight but significant induction of the *HEL* gene was also observed in the XVE-*ORA1* line. For all the genes tested, no significant induction of expression could be detected in lines inducibly expressing the *ORA2*, *ORA4*, *ORA19*, *ORA31*, *ORA37* and *ORA44* genes, demonstrating that overexpression of these *ORAs* did not alter the expression of the tested genes.

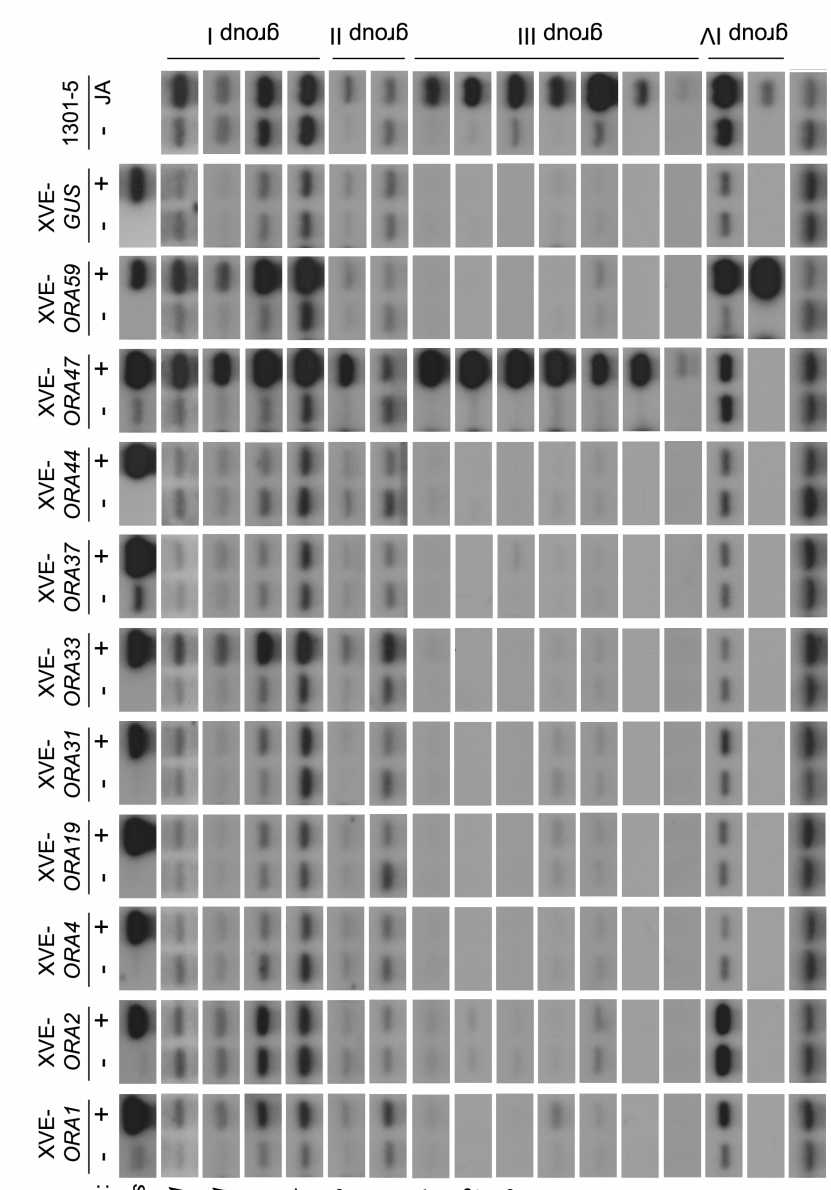

Figure 3. Expression of selected JA-responsive genes in the different *ORA*-overexpressing lines. Two-weeks-old Arabidopsis seedlings from transgenic lines carrying the *ORA1, ORA2, ORA4, ORA19, ORA31, ORA33, ORA37, ORA44, ORA47, ORA59* or *GUS* gene in the XVE module, were treated for 24 hours with estradiol (+) or the solvent DMSO (-). The top panels show the expression of the individual *ORA* transgene in response to estradiol for each line. The expression levels of the transgenes were similar among the lines. Two-weeks-old control plants, carrying a 35S:*GUS* construct (line 1301-5), were treated for 8 hours with 50 µM JA or with 0.1% of the solvent DMSO (-). The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

Analysis of the expression of the selected genes in a control line treated for 8 hours with JA showed that most of the genes were induced by JA under our growth and treatment conditions (Figure 3 and Table 1). For each *ORA,* target gene expression profiling was performed simultaneously in a second independent transgenic XVE-*ORA* line with identical results.

Description	AGI	JA	XVE-*ORA1*	XVE-*ORA2*	XVE-*ORA4*	XVE-*ORA19*	XVE-*ORA31*	XVE-*ORA33*	XVE-*ORA37*	XVE-*ORA44*	XVE-*ORA47*	XVE-*ORA59*
JA biosynthesis												
Allene oxide synthase (AOS)	At5g42650	+									+	
Allene oxide cyclase 2 (AOC2)	At3g25770	+									+	
Lipoxygenase 2 (LOX2)	At3g45140	+							+/-		+	
12-oxo-phytodienoate reductase (OPR3)	At2g06050	+									+	
Defense												
β-glucosidase homolog (BG1)	At1g52400	+									+	
Hevein-like gene (HEL)	At3g04720	+	+/-									+
Vegetative storage protein 1 (VSP1)	At5g24780	+									+	
Plant defensin (PDF1.2)	At5g44420	+										+
Thionin (Thi2.1)	At1g72260	+									+	
AvrRPto-induced gene (AIG2)	At3g28930	+/-										+
Receptor-like protein kinase (ATR1)	At5g60890	+	+/-					+/-			+	
MAP Kinase 3 (MAPK3)	At3g45640		+/-					+/-				+/-
Primary metabolism												
Anthranilate synthase alpha subunit 1 (ASA1)	At5g05730	+						+/-			+	+
Tryptophan synthase beta subunit 1 (TSB1)	At5g54810										+	+
Tryptophan synthase alpha subunit (TSAα)	At3g06050	+	+					+			+	+
Arginine decarboxylase 2 (ADC2)	At4g34710	+						+			+	
Secondary metabolism												
Putative Catechol-O-methyl transferase	At1g76790	+									+	
Chalcone Synthase (CHS)	At5g13930										+	
Cytochrome P450 CYP83B1	At4g31500		+					+			+	+
Cytochrome P450 CYP79B2	At4g39950	+	+/-					+/-			+	
Cytochrome P450 CYP79B3	At2g22330	+	+/-					+/-			+	
Isoflavone reductase-like protein (IFR)	At4g13660		+/-					+				
Myrosinase-binding protein-like	At3g16470	+	+/-								+	
Putative flavonol sulfotransferase (FST)	At1g74100		+/-					+			+	+
Others												
Chlorophyllase 1 (CLH1)	At5g43860	+									+	
Glutathione S-transferase (GST8)	At1g78380	+	+/-					+/-	+/-		+/-	+
Peroxidase ATP8 (PerATP8)	At4g30170		+/-					+/-				
Cytochrome P450	At4g22710		+/-					+/-				+

Table 1. List of genes tested for expression in induced XVE-*ORA* lines. The AGI gene codes are indicated. The XVE inducible lines were treated for 24 hours with 2 µM estradiol. (+) indicates increased expression level in the induced XVE line relative to expression in non-induced XVE line revealed by RNA gel blot analysis. (+/-) indicates a weak but significant increase in the expression level (see Figure 3). Blank boxes indicate that the expression was not changed following induction of *ORA* expression by application of estradiol for 24 hours. The induced expression in response to JA treatment in a control line is also indicated by (+ and +/-).

Discussion

In this chapter, we show that the XVE inducible system is a useful tool for the characterization of genes regulated by the ORA transcription factors. Using this system, we identified defense-related JA-responsive genes that were upregulated in Arabidopsis plants overexpressing either one or several *ORA*(*s*). Our data suggest that ORA1, ORA33, ORA47 and ORA59 play a role in regulating JA responses. For the other ORAs, we did not find upregulated genes with the selected set of putative target genes. Although this remains to be proven, we speculate that the JA-responsive genes identified in this study are direct target genes of the ORAs.

The XVE system has been used for expression of a number of genes in transgenic Arabidopsis plants and in tobacco BY2 and *Catharanthus roseus* cell suspensions (Abe and Hashimoto, 2005; Zuo et al., 2000b; Pauw, 2004). Our results are in accordance with the data from Zuo et al. (2000a) showing that the XVE system is tightly regulated and highly inducible without detectable toxicity. In contrast to the dwarf phenotype exhibited by plants constitutively overexpressing several *ORA* genes, including *ORA59* (Chapter 2), *ORA47* (Chapter 3) and *ORA37* (Chapter 4), we could not detect an aberrant phenotype in any of the XVE lines that we constructed compared to wild-type plants. Furthermore, RNA gel blot analyses showed that expression of the transgene stayed silent in the XVE-inducible lines in the absence of the inducer estradiol (Figure 1 and 2). However, several transgenic XVE-*ORA* lines, such as the XVE-*ORA37* and XVE-*ORA47* lines (Figure 3), displayed a low but above background expression of the *ORA* transgene in the absence of inducer, indicating leaky expression of the transgene. As tested with the *GUS* and *ORA59* genes, the transgene mRNA in induced XVE lines accumulated to a level similar or superior to the mRNA level in representative plants overexpressing the gene from the constitutive 35S promoter (Figure 1), demonstrating that the XVE module is a strong expression system.

The screening performed by Atallah (2005) with the whole family of AP2/ERF genes identified 14 *ORA* genes expressed within 4 hours in response to JA in 10-days-old Arabidopsis seedlings grown in liquid culture. One can speculate that other AP2/ERF genes are likely to be JA-responsive as their JA-induced expression might occur at later time points in seedlings, or only in specific tissues present at low abundance or absent in seedlings, or at later stages of development. This is illustrated by the fact that microarray analysis performed with whole genome chips allowed us to identify a number of genes encoding AP2/ERF-domain transcription factors that were induced by JA after 8 and/or 24 hours (data not shown). These include several *ORA* genes as well as a number of other AP2/ERF genes. Furthermore, the screening strategy of Atallah did not identify AP2/ERF genes which are only

responsive to JA in combination with another signaling molecule. The gene encoding the AP2/ERF-domain transcription factor ERF1 was shown to be responsive to a combination of JA and ethylene (Lorenzo et al. 2003). Although the authors also show *ERF1* induction in response to JA alone, the *ERF1* gene was not identified as a JA-responsive *ORA* gene in Atallah's screen (2005). As a result, we did not include the *ERF1* gene in our study for the identification of JA-responsive target genes. However, the relationship between ERF1 and other ORA AP2/ERF-domain transcription factors is discussed in Chapter 2. This study was performed with 10 out of 14 previously identified *ORA* genes. Extending this study with the four *ORA* genes, together with other AP2/ERF genes, such as *ERF1* or the newly identified JA-responsive genes, will lead to a better understanding of the role of each ORA and putative functional redundancy.

In this study, several JA-responsive genes were tested for changes in expression in induced XVE-*ORA* lines. Most of these genes were induced in plants treated for 8 hours with JA (Figure 3; Table 1). In contrast, a number of genes, such as *IFR*, *FST*, or *CYP83B1*, showed similar expression in JA-treated plants compared to untreated plants (Figure 3; Table 1). However, it is possible that these genes are induced by JA at a later or earlier time point than 8 hours. It is also possible that these genes are not JA-responsive in young seedlings or that they are expressed in response to a combination of JA with a second signal. All genes tested were upregulated in at least one XVE-*ORA* line in response to estradiol (Table 1).

Genes from group I showed increased expression in estradiol-treated XVE-*ORA1*, XVE-*ORA33*, XVE-*ORA47* and XVE-*ORA59* lines, suggesting that the corresponding ORAs positively regulate the expression of the group I genes. These results also indicate that several ORA proteins can regulate the same genes, suggesting a possible partial functional redundancy between ORA1, ORA33, ORA47 and ORA59 transcription factors. However, some specificity exists between these four ORAs. Genes from group II were not induced in the XVE-*ORA59* line, whereas their expression was increased in response to *ORA1*, *ORA33* and *ORA47*. Similarly, genes from group III were exclusively induced in the XVE-*ORA47* line, and genes from group IV showed increased transcript levels only in the XVE-*ORA59* line, with the exception of the *HEL* gene which was also induced in the XVE-*ORA1* line (Figure 3). These multiple groups of genes with differential expression profiles might suggest that ORA1, ORA33, ORA47 and ORA59 regulate distinct but overlapping sets of JA-responsive genes.

The ORA1, ORA33, ORA47 and ORA59 transcription factors are very likely to act at different levels in the JA signaling pathway. Functional analysis of ORA47 demonstrated that overexpression of *ORA47* resulted in elevated level of the jasmonic acid precursor OPDA via activation of the JA biosynthetic genes (Chapter 3; Figure 3 and Table 1). As discussed in Chapter 3, induction of a large number of genes in the XVE-*ORA47* line is likely to be due to

gene activation in response to ORA47-mediated OPDA production rather than to direct binding of ORA47 to the promoters of these genes.

Functional analysis of ORA59 demonstrated that ORA59 was involved in the regulation of a subset of JA- and ethylene-responsive genes, including the *PDF1.2* and *HEL* genes from group IV and the tryptophan biosynthetic genes from group I (Table 1; Chapter 2). Plants showing post-transcriptional *ORA59* gene silencing failed to induce the expression of the *PDF1.2* and *HEL* genes in response to JA and/or ethylene, demonstrating the strict requirement for ORA59 for the regulation of these genes by JA and/or ethylene. Expression of several genes from group I was still induced in RNAi-*ORA59* plants in response to JA (data not shown), albeit at a reduced level, suggesting that other transcription factors than ORA59, presumably ORA1 or ORA33, are responsible for part of the JA-induced expression of the group I genes. Target gene expression in double/triple knock-out mutant plants is required to assess a putative functional redundancy between ORA1, ORA33 and ORA59.

In contrast, none of the selected JA-responsive genes were induced in the XVE-*ORA2*, XVE-*ORA4*, XVE-*ORA19*, XVE-*ORA31*, XVE-*ORA37* and XVE-*ORA44* lines. Except for ORA37, of which the role in JA signaling pathway is demonstrated in Chapter 4, one explanation of our results is that ORA2, ORA4, ORA19, ORA31 and ORA44 do not participate in JA signaling. However, it is possible that these transcription factors regulate JA-responsive genes that were not tested in our screening. This can be studied by performing genome-wide microarray analyses using plants overexpressing these *ORA* genes. It is also possible, although less likely, that ORA2, ORA4, ORA19, ORA31 and ORA44 have lost their capacity to activate JA-responsive genes during evolution.

It should be kept in mind that our screening for ORA-regulated has limitations. For example, recently, transient assays showed that *ORA47* overexpression activated the *AOC3* promoter in *Catharanthus roseus* suspension cells in the absence of JA, whereas *ORA33* overexpression activated the *AOC3* promoter only when the *C. roseus* cells were incubated in the presence of JA (Adel Zarei, personal communication). In addition, in Arabidopsis protoplasts, an ORA59-GFP fusion protein is stabilized and localizes to the nucleus in response to JA (Antony Champion, personal communication). These observations show that JA not only activates the expression of the *ORA* genes, but may also regulate ORA protein activity. Therefore, it is possible that we missed target genes for several ORAs because the activity of these ORAs depends on JA signaling. ORA activity could also be limited by the concomitant activation (or expression) of a JA-responsive co-factor or of a second signal. Further work on the JA-mediated post-translational signals regulating the activity of the different ORAs is likely to reveal additional new aspects of the roles of the ORA transcription factors in JA-related plant defense mechanisms.

Materials and Methods

Biological materials, growth conditions and treatments

Arabidopsis thaliana ecotype Col-0 is the genetic background for wild-type plants and all transgenic 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 the T2 generation were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin. Following stratification for 3 days at 4ºC, seeds were incubated at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days, after which 15 to 20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml MA medium and were incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated for different time periods with 2 µM estradiol (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.2% final concentration). As control, seedlings were treated with 0.2% DMSO. Treatments with JA were performed by adding 50 µM JA (Sigma) dissolved in DMSO (0.1% final concentration) to the liquid medium. As controls, seedlings were treated with 0.1% DMSO.

Binary constructs and plant transformation

The full-length open reading frames (ORF) for *ORA4* (*At2g44840*), *ORA19* (*At2g22200*), *ORA31* (*At5g47230*) and *ORA33* (*At4g34410*) were PCR-amplified from Arabidopsis genomic DNA using the primer sets: 5'-GAA GAT CTC ATA TGA GCT CAT CTG ATT CCG-3' and 5'-GAA GAT CTT TAT ATC CGA TTA TCA GAA TAA G-3' for *ORA4*; 5'-CGG GAT CCA TAT GGA AAC TGC TTC TCT TTC TTT C-3' and 5'-GAA GAT CTT TAA GAA TTG GCC AGT TTA C-3' for *ORA19*; 5'-CGG GAT CCA TAT GGC GAC TCC TAA CGA AGT ATC-3' and 5'-CGG GAT CCT CAA ACA ACG GTC AAC TGG-3' for *ORA31*; 5'-CGG GAT CCA TAT GCA TTA TCC TAA CAA CAG AAC C-3' an 5'-CGG GAT CCT CAC TGG AAC ATA TCA GCA ATT G-3' for *ORA33*. The *ORA44* (*At1g43160*) ORF was PCR amplified from an Arabidopsis cDNA library prepared from above-ground parts of mature flowering plants using the primer set 5'-CGG GAT CCA TAT GGT GTC TAT GCT GAC TAA TG-3' and 5'-CGG GAT CCA CAA GAC TTT GAT CAC AAA TT-3'. PCR fragments were digested with BamHI (ORA31, ORA33 and ORA44), BglII (ORA4) or BamHI/BglII (ORA19) and inserted in pBluescript SK+ (Stratagene, La Jolla, CA) digested with BamHI and plasmid clones containing the PCR fragments oriented such that the 5'-end of the *ORA* ORFs flanked the EcoRI site were selected.

For the construction of plants with estradiol-responsive transgene expression (XVE), the *ORA4*, *ORA19* and *ORA44* open reading frames were excised from pBluescript SK+ with XhoI/XbaI and cloned into the binary vector pER8 (Zuo et al, 2000a) digested with XhoI/SpeI. The *ORA33* and *ORA31* open reading frames were excised from pBluescript SK+ with ApaI/XbaI and ApaI/SpeI , respectively, and cloned into pER8 digested with ApaI/SpeI. The *GFP* ORF was transferred as a SalI/PstI fragment from plasmid

35Somega-sGFP(S65T) (Chiu et al., 1996) to pBluescript II SK$^+$, from where it was transferred as an ApaI/SpeI fragment into pER8. Details for the construction of the XVE-*ORA59*, XVE-*ORA1* (also called *AtERF1*), XVE-*ORA2* (also called *AtERF2*), XVE-*GUS* and 35S:*ORA59* transgenic plants are described in Chapter 2, whereas details for the construction of the XVE-*ORA37* (also called *AtERF4*) and 35S:*GUS* (line 1301-5), and the XVE-*ORA47* plants are described in Chapters 4 and 3, respectively.

The different pER8-ORA vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 except pER8-ORA31 which was introduced into *A. tumefaciens* strain LBA4404. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic T1 plants were selected on MA medium containing 100 mg/L timentin and 20 mg/L hygromycin. The following T2 lines were used for RNA gel blot analyses: XVE-*ORA1* lines #1 and #3, XVE-*ORA2* lines #1 and #7, XVE-*ORA4* lines #1 and #9, XVE-*ORA19* lines #4 and #9, XVE-*ORA31* lines #1 and #9, XVE-*ORA33* lines #9 and #17, XVE-*ORA37* lines #10 and #16, XVE-*ORA44* lines #8 and #9, XVE-*ORA47* lines #20 and #21, XVE-*ORA59* lines #6 and #10, and XVE-*GUS* lines #7 and #15. Figure 3 shows the RNA gel blot analyses data from the underlined lines only.

Northern blot analyses

Total RNA was extracted from frozen tissue by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70 % ethanol, and resuspended in water. As described by Memelink et al. (1994), 10 µg RNA samples were subjected to electrophoresis on 1.5% 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 (Chapter 3). Blots were exposed on X-ray films (Fuji, Tokyo, Japan).

Hybridization with the specific *ORAs* was performed using PCR-amplified DNA fragments corresponding to non-conserved parts of the coding regions outside of the conserved AP2 domains. For the preparation of probes of the target genes, DNA fragments corresponding to the full genomic sequence were PCR amplified from Arabidopsis genomic DNA (see Materials and Methods from previous chapters).

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Chapter 6

Summary and general discussion

Plants respond to environmental stress or pathogen attack by producing a number of endogenous secondary signaling molecules including jasmonic acid (JA), ethylene and salicylic acid. The biosynthesis of one or a combination of these hormones and subsequent transduction of the signals triggers dramatic modifications in transcription leading to adapted defense responses. In response to certain pathogens, JA and ethylene cooperate to synergistically induce the expression of a large number of defense genes and such a concerted action of JA and ethylene is though to be responsible for the establishment of a fine-tuned complex defense response.

Jasmonic acid and related compounds, collectively referred to as jasmonates, form a family of cyclopentanone derivatives synthesized from linolenic acid via the octadecanoid pathway (Turner et al., 2002; Atallah and Memelink, 2004). These molecules regulate several aspects of plant growth and development as well as responses to many biotic and abiotic stresses (Turner et al., 2002). In recent years, several lines of evidence have shown that several members of the jasmonate family, including the JA-precursor OPDA, are biologically active and can have overlapping as well as distinct roles as signal molecules.

At present, it is largely unknown what are the molecular players regulating JA production in response to stress signals. In contrast, the mechanisms whereby JA signaling triggers gene expression are becoming well documented. In *Catharanthus roseus*, JA induces the expression of two genes encoding transcription factors called Octadecanoid-Responsive Catharanthus AP2/ERF-domain (ORCA) proteins (Menke et al., 1999; van der Fits and Memelink, 2000 and 2001), which belong to the AP2/ERF-domain protein family. The ORCAs mediate the JA response by positively regulating the expression of several JA-responsive genes via direct binding to a JA-responsive element in the promoter of these genes (Menke et al., 1999; van der Fits and Memelink, 2001). Based on these observations, it was postulated that JA-responsive expression of a subset of genes in the model plant *Arabidopsis thaliana* is also mediated by specific members of the AP2/ERF-domain transcription factor family, and that the corresponding genes are also expressed in a JA-responsive manner. In Arabidopsis, the AP2/ERF-domain transcription factor family was reported to comprise 124 proteins. In a family-wide screening, Atallah (2005) previously characterized an initial set of 10 genes, called *Octadecanoid-Responsive Arabidopsis AP2/ERF* (*ORA*) genes, which were rapidly induced by JA treatment in young seedlings, and later on found 4 additional *ORA* genes.

The studies described in this thesis are focused on the functional characterization of the 10 ORA proteins identified initially with emphasis on their role in JA-responsive gene expression and in the JA signaling network. **Chapter 2** describes the role of the transcription

factor ORA59 in the network involving JA and ethylene defense signaling pathways. *ORA59* gene expression was induced by JA or ethylene, and synergistically induced by both hormones (Atallah, 2005). Analysis of the JA-insensitive (*coi1-1*) and ethylene-insensitive (*etr1-1* and *ein2-1*) mutants revealed that expression of the *ORA59* gene in response to JA (Atallah, 2005) or ethylene (this study) required both JA and ethylene signaling pathways simultaneously. Genome-wide microarray analysis showed that overexpression of the *ORA59* gene resulted in increased expression of a large number of JA- and ethylene-responsive genes, including several genes involved in defense or in primary and secondary metabolism. Several defense genes, including *PDF1.2* and *HEL*, are expressed as a result of ORA59 overexpression even in a *coi1-1* mutant background, which is consistent with the hypothesis that ORA59 functions downstream from COI1. Plants overexpressing *ORA59* were more resistant to infection with the necrotrophic fungus *Botrytis cinerea* compared to infected wild-type plants. Plants overexpressing *ERF1*, a gene encoding a related member of the AP2/ERF family, were previously shown to induce *PDF1.2* and *HEL* gene expression (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, *ERF1* gene induction by JA and/or ethylene was dependent on both JA and ethylene signaling pathways (Lorenzo et al., 2003). These similarities in gene expression patterns and in putative target genes, as well as the fact that they are the closest mutual homologues in the AP2/ERF-domain family, suggest that ORA59 and ERF1 have redundant functions in JA and ethylene signal transduction. However, the essential role of ORA59 as an integrator of the JA and ethylene signals leading to regulation of defense genes was demonstrated with plants silencing the *ORA59* gene via an RNAi approach. In response to JA and/or ethylene, or after infection with the fungi *B. cinerea* or *Alternaria brassicicola,* expression of *PDF1.2* and other defense genes was blocked in *ORA59*-silenced plants. Induction of the *ERF1* gene in the *ORA59*-silenced plants was identical to that in control plants in response to JA and/or ethylene, showing that RNAi-mediated silencing was specific for *ORA59*. 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 ethylene signal inputs to coordinate the appropriate gene expression response directed against pathogen attack and thereby provide new insight in the nature of the molecular components involved in the crosstalk between these two hormones.

The data presented in Chapter 2 also emphasize the necessity of interpreting results obtained by constitutive overexpression of a gene in conjunction with data from other complementary approaches, such as inducible overexpression, as well as gene silencing by RNAi or insertional knock-out. Constitutive overexpression of *AtERF1* (Atallah, 2005),

AtERF2 (Brown et al., 2003; Atallah, 2005), *ERF1* (Solano et al., 1998) and *ORA59* (this study) in Arabidopsis all resulted in increased *PDF1.2* expression. However, using two different approaches, we found that only ORA59 and ERF1 were able to function as transcriptional activators of *PDF1.2* gene expression, indicating that *PDF1.2* is not a direct target gene of the other AP2/ERF-domain family members, but is likely to be transcriptionally upregulated as a result of general stress due to overexpression. 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.

Chapter 3 describes the functional characterization of the transcription factor ORA47. Constitutive overexpression of the *ORA47* gene in Arabidopsis resulted in an extreme dwarf phenotype with production of anthocyanins at the shoot apex. This phenotype was similar to that of wild-type plants grown in the presence of JA. Therefore we speculated that plants overexpressing *ORA47* exhibited constitutive JA responses. To be able to study the function of ORA47, we switched to an inducible overexpression system. Induced overexpression of *ORA47* led to the activation of a large number of genes encoding JA biosynthetic enzymes, including AOC3, AOS and LOX2. Consistent with this finding, induced plants overexpressing *ORA47* exhibited a 2- to 4-fold higher level of the JA-precursor 12-oxo-phytodienoic acid (OPDA) compared to induced control plants. Surprisingly, JA content in *ORA47*-overexpressing plants remained similar to that observed in the control plants. The first steps of JA biosynthesis occur in the chloroplasts and lead to the formation of OPDA (Turner et al., 2002). Subsequently, OPDA needs to be targeted to the peroxisome to undergo further enzymatic modifications to yield JA. Overexpression of *ORA47* led to induced expression of the genes encoding biosynthesis enzymes, including OPR3 and the β-oxidation enzymes, involved in the conversion of OPDA to JA. It is not clear why elevated amounts of OPDA, together with the activation of genes coding for downstream JA biosynthesis enzymes, did not lead to higher levels of JA in the transgenic plants. We speculate that the OPDA produced in *ORA47*-overexpressing plants failed to be converted to JA due to a lack of transport of OPDA from the chloroplasts to the peroxisomes.

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 OPDA production. We speculate that ORA47 controls oxylipin biosynthesis via direct transcriptional regulation of the JA biosynthesis genes, although this remains to be demonstrated. Overexpression of *ORA47* in the *coi1-1* mutant background did not result in activation of the JA biosynthesis genes *AOC3*, *AOS* and *LOX2*. It is possible that the activity of the ORA47 protein requires post-translational modifications or protein-protein interactions that depend on JA signaling via COI1.

The expression of all JA biosynthesis genes, including *LOX2*, *AOS* and *AOC*, was shown to be induced by treatment with exogenous JA or MeJA (Turner et al., 2002). These results indicate the existence of a positive feedback regulatory mechanism for oxylipin biosynthesis. The results described in this chapter indicate that it is likely that ORA47 is involved in the regulation of this auto-stimulatory loop. It is also possible that, ORA47 not only functions in JA-responsive oxylipin biosynthesis within an auto-stimulatory loop, but also in the initial oxylipin biosynthesis in response to the primary stress signal. This however remains to be investigated. In any case the AP2/ERF-domain protein ORA47 is the first transcription factor shown to control the biosynthesis of regulatory oxylipin signals. ORA47 does not appear to have a unique function, since in plants impaired in *ORA47* expression, the JA-induced expression of the JA biosynthesis genes was comparable to the expression in wild-type plants, indicating that (an) additional transcription factor(s) regulate(s) the expression of the JA biosynthesis genes in response to JA.

In **Chapter 4**, the attention was focused on ORA37. 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), ethylene (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 ethylene treatment, *ORA37*-overexpressing plants showed significantly lower induction of a subset of JA- and ethylene-responsive genes, including the defense genes *PDF1.2, HEL* and *ChiB,* compared to control plants treated similarly. On the other hand, plants in which *ORA37* expression was silenced via RNAi showed increased *PDF1.2, HEL* and *ChiB* transcript levels after JA- and/or ethylene-treatment compared to control plants, corroborating the complementary results obtained with *ORA37*-overexpressing plants. This demonstrates that ORA37 plays a role in JA and ethylene signaling by repressing the expression of a number of genes in response to JA and/or ethylene. The same genes were shown to be positively regulated by ORA59 (Chapter 2). Overexpression or silencing of the *ORA37* gene had no effect on the expression of *ORA59* in response to JA and/or ethylene, indicating that ORA37 does not regulate *ORA59* expression. We speculate that ORA37 and ORA59 act antagonistically on the regulation of expression of a same subset of JA- and ethylene-responsive genes.

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*. 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 ethylene 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 ethylene-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 bHLHZIP-type transcription factor AtMYC2 (Figure 1; Lorenzo et al., 2004). It was therefore suggested that the negative regulation of the *VSP2* gene by ethylene 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 ethylene 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. Unraveling the mechanisms whereby ORA37 operates is now a great challenge.

Therefore, JA and ethylene 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 ethylene remains unclear. The balance between AP2/ERF-domain activators and repressors on common target promoters may provide a mechanism for switch-like transcriptional control.

Based on the results obtained from research conducted in *Catharanthus roseus* with ORCA transcription factors (Menke et al., 1999; van der Fits and Memelink, 2000; 2001), the working hypotheses underlying the thesis studies was that JA-responsive gene expression in Arabidopsis is also mediated by members of the AP2/ERF-domain transcription factor family, and that the corresponding genes are also induced by JA. In *Arabidopsis thaliana,* 14 *ORA* genes were shown to be induced by JA in young seedlings (Atallah, 2005). This raised the questions whether ORA proteins were indeed involved in the JA signal transduction network and whether ORA proteins were having identical or distinct functions, if any, in JA signaling. Some answers to these questions have been provided in the previous chapters. The results demonstrated that ORA37, ORA47 and ORA59 transcription factors are important molecular players regulating discrete responses in the JA signaling network. In **Chapter 5**, attempts to assign functions to the other ORA transcription factors and to address the question of putative functional redundancy between ORAs are described. The ten *ORA* genes which

were initially identified were individually overexpressed in plants. To be able to identify direct ORA target genes and to avoid non-specific gene activation that may occur as a result of constitutive overexpression, transgenic plants overexpressing the *ORA* genes under the control of an estradiol-inducible promoter (XVE system; Zuo et al., 2000) were constructed. Analyses of transgene expression in the presence or absence of inducer demonstrated that the XVE system was tightly controlled. Moreover, transgene expression in induced XVE plants reached transcript levels that were similar to or even higher than those observed in transgenic plants in which the transgene is controlled by the strong constitutive CaMV 35S promoter.

A number of putative candidate target genes, which are known to be responsive to JA and/or ethylene, were selected and their expression was measured in the different transgenic lines overexpressing individual *ORAs*. These genes encode proteins involved in defense against biotic or abiotic stress, JA biosynthesis or primary and secondary metabolism. Gene expression profiling in the different XVE-*ORA* lines allowed clustering of the putative target genes in four groups. Genes from group I were induced in XVE-*ORA1*, XVE-*ORA33*, XVE-*ORA47* and XVE-*ORA59* transgenic lines. Genes from group II were induced in XVE-*ORA1*, XVE-*ORA33* and XVE-*ORA47* transgenic lines. Genes from group III were exclusively induced in XVE-*ORA47* transgenic lines, whereas genes from group IV were exclusively induced in XVE-*ORA59* transgenic lines. In addition, there was a group of tested genes which were not induced in any of the ORA lines. These results indicated that several ORA proteins can regulate the same set of genes (e.g. genes from group I and II), suggesting a possible functional redundancy between ORA1, ORA33, ORA47 and ORA59. However, up-regulation of the majority of genes in XVE-*ORA47* plants is likely to be caused indirectly as a result of oxylipin production. Therefore, ORA47 is likely to play a role other than those of ORA1, ORA33 and ORA59 in JA signaling.

Despite the possibility of similar functions among ORAs, the presence of multiple groups of genes with differential expression profiles suggests that ORA1, ORA33 and ORA59 regulate distinct sets of JA-responsive genes although some overlap occurs among ORA-regulated genes.

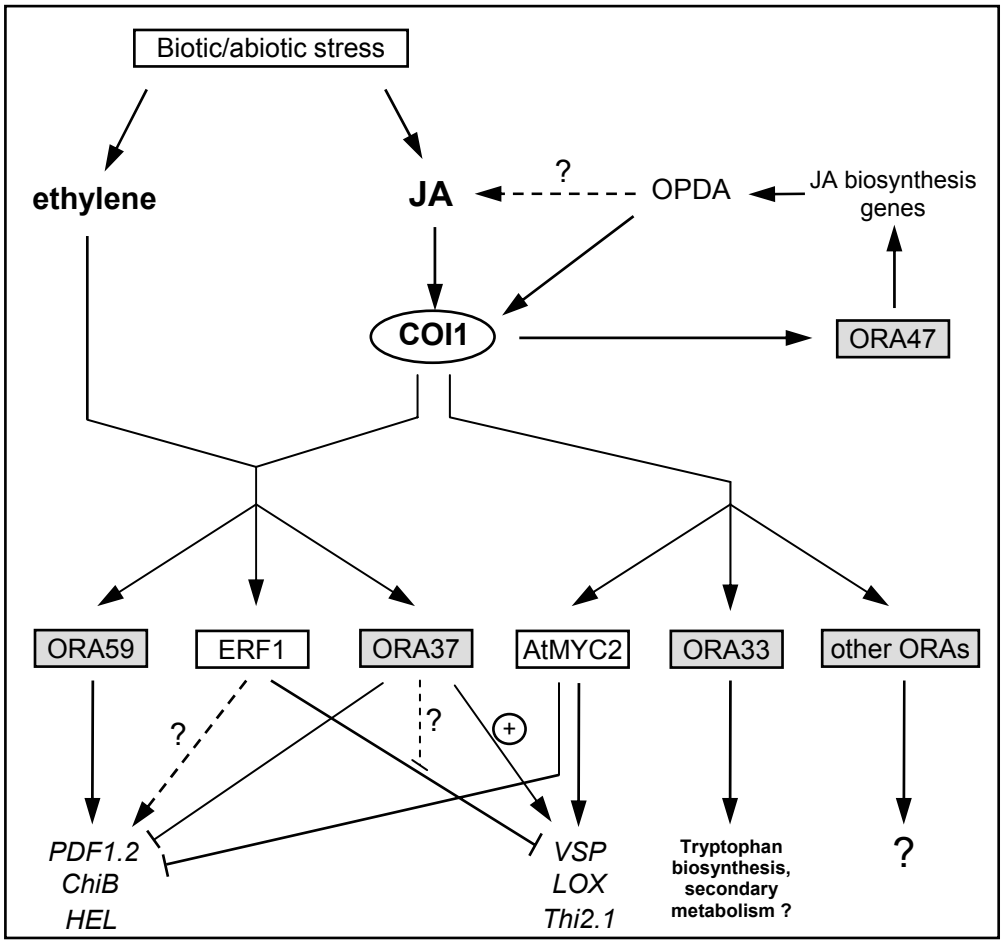

Figure 1. Role of the ORA transcription factors in the stress-responsive network involving the JA and ethylene signaling pathways. Different types of biotic or abiotic stress, such as infection with certain pathogens or wounding, induce the synthesis and subsequent activation of the JA and ethylene signaling pathways. JA (and/or related oxylipins) activates the expression of several transcription factors, including ORAs, via COI1, a central regulator of all JA-dependent responses. The transcription factor ORA47 acts as a regulator of the positive feed-back loop of JA. ORA47 activates JA biosynthesis genes, resulting in production of OPDA, a bioactive precursor of JA. This results in the amplification of the signal initiated by JA. The putative conversion of OPDA to JA is indicated by a question mark. The transcription factor ORA33 activates genes involved in tryptophan biosynthesis and secondary metabolism. The bHLHZIP-type transcription factor AtMYC2 positively regulates the expression of wound-responsive genes (i.e. *VSP*, *LOX* and *Thi2.1*) and represses other genes, including *PDF1.2*, *ChiB* and *HEL*. The JA and ethylene signals cooperate to activate the transcription factors ORA59, ERF1 and ORA37. ORA59 is the key regulator of the *PDF1.2*, *ChiB* and *HEL* genes in response to ethylene 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*, *ChiB* and *HEL* genes in response to JA and/or ethylene. ORA37 also enhances the JA-induced expression of *VSP* genes (circled plus), presumably by repressing the negative effect of ethylene operated through ERF1 (dashed bar line). The functions of other ORAs remain to be characterized.

In contrast, none of the putative JA-responsive target genes tested was found to be induced in the *ORA2*, *ORA4*, *ORA19*, *ORA31*, *ORA37* and *ORA44* lines. Except for ORA37, of which the role in JA signaling has been described in Chapter 4, the results can be interpreted to indicate that the ORA2, ORA4, ORA19, ORA31 and ORA44 proteins do not participate in JA signaling. However, it is more likely that these transcription factors regulate JA-responsive genes that were not tested in our screening. It is also possible that the activity of these ORAs requires JA-dependent post-translational modifications, such as protein phosphorylation or ubiquitination, or JA-dependent changes in sub-cellular localization or protein-protein interactions. If this is the case, the conducted screen would have been ineffective in the identification of target genes.

The aim of the thesis was to unravel the function of JA-responsive AP2/ERF-domain transcription factors, named ORAs, in the model plant species *Arabidopsis thaliana* by modulating their expression levels. The studies described in this thesis have led to the functional characterization of several ORAs, although the role of many ORAs in the JA signaling pathway remains unclear. The ORA33, ORA37, ORA47 and ORA59 proteins act as terminal components of the JA signal transduction pathway by regulating defense gene expression in response to JA. In addition, ORA59 and ORA37 not only integrate signals from JA but also from ethylene. ORA59 and ORA37 act antagonistically on the same subset of target genes, further increasing the complexity of the cross-talk between JA and ethylene. Figure 1 shows a model of the JA and ethylene signaling pathways and the role of ORAs, as well as other transcription factors, in these regulatory networks. In response to a specific stimulus, production of JA, as well as ethylene, leads to activation of several AP2/ERF-domain transcription factors, which, in turn, regulate the expression of specific genes resulting in defense. This work contributed to a better understanding of the molecular mode of action of JA in regulating gene expression in Arabidopsis. For instance, the results obtained with ORA59 caused significant changes in the existing view on the molecular components involved in JA-responsive gene expression and in the cross-talk between JA and ethylene (Figure 1; see also Figure 2 from chapter 1). The conclusions presented here essentially rely on data obtained from transcript analyses. In addition, based on several lines of evidence (discussed above), we speculate that follow-up research focused on ORA proteins and post-transcriptional regulation of ORAs might enlarge the present understanding of the role of ORAs in gene regulation and in JA signaling. Future challenges remain in unraveling the role of JA on the activity or stability of the ORA proteins and in identifying putative regulatory protein partners. Characterization of the transcription factors that regulate *ORA* gene expression is also of major importance to understand how JA orchestrates the complex defense response.

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Samenvatting

Planten reageren op stressomstandigheden in hun omgeving of op infectie met pathogene organismen met de productie van een aantal endogene signaalmoleculen, waaronder jasmonzuur (JA), ethyleen en salicylzuur. De biosynthese van één of een combinatie van deze hormonen en daaropvolgende signaaltransductieprocessen leiden tot grote verschuivingen in de transcriptie van genen, hetgeen leidt tot een doeltreffende verdedigingsrespons. In antwoord op bepaalde stressomstandigheden of pathogenen werken JA en ethyleen samen om de expressie van een groot aantal verdedigingsgenen te stimuleren.

JA en verwante stoffen, gezamenlijk aangeduid als jasmonaten, vormen een familie van cyclopentanoonafgeleiden, welke gemaakt worden van linoleenzuur via de zogenaamde octadecanoïed route. Deze moleculen regelen ook verscheidene aspecten van groei en ontwikkeling, maar zijn vooral betrokken bij verdediging tegen (a)biotische stress. Recentelijk is gebleken dat naast JA ook andere jasmonaten, zoals de JA precursor 12-oxo-phytodienoic acid (OPDA), actief zijn als signaalmoleculen.

Op dit moment is het nog grotendeels onbekend hoe de productie van JA op moleculair niveau aangezet wordt door stresssignalen. Daarentegen is het inzicht in hoe JA de expressie van genen betrokken bij verdediging aanschakelt groeiende. In de roze maagdenpalm (*Catharanthus roseus*) induceert JA de expressie van twee genen voor transcriptiefactoren die Octadecanoïed-Responsieve Catharanthus AP2/ERF-domein (ORCA) eiwitten genoemd zijn. Deze behoren tot de AP2/ERF-domein familie van transcriptiefactoreiwitten. Onder invloed van JA schakelen de ORCA eiwitten een aantal JA-gevoelige genen aan door direct te binden aan het promoter DNA van deze genen en hun transcriptie te stimuleren. Gebaseerd op deze bevindingen werd gepostuleerd dat in de zandraket (*Arabidopsis thaliana*) de expressie van een subset van genen onder invloed van JA ook geregeld wordt door leden van de AP2/ERF-domein familie. Atallah heeft in haar proefschrift 10 genen beschreven van de AP2/ERF familie, *Octadecanoïed-Responsieve Arabidopsis AP2/ERF* (*ORA*) genen genaamd, welke zeer snel door JA aangeschakeld worden in jonge zaailingen.

Het onderzoek beschreven in dit proefschrift had tot doel om de functies van deze 10 genen op te helderen, en in het bijzonder hun rol in JA-gevoelige genexpressie. De strategie die hierbij gevolgd werd was om de expressie van deze *ORA* genen te verlagen of te verhogen, en te kijken welk effect dat heeft op genexpressie of het aanzien van de plant.

Hoofdstuk 2 beschrijft de rol van de transcriptiefactor ORA59. De expressie van het *ORA59* gen wordt aangeschakeld door JA en ethyleen afzonderlijk, terwijl deze hormonen samen een zeer sterke stimulatie van de expressie van dit gen teweegbrengen. Analyse van de expressie van alle Arabidopsis genen met behulp van micro-arrays toonde aan dat

verhoogde expressie van het *ORA59* gen leidt tot een verhoging van de expressie van een groot aantal genen die ook aangeschakeld worden door de combinatie van JA en ethyleen. Deze groep bevatte meerdere genen waarvan bekend is dat ze zijn betrokken bij verdediging (zoals *PDF1.2* en *HEL*) of bij het primaire of het secundaire metabolisme. Planten met een verhoogde expressie van ORA59 zijn ook meer resistent tegen infectie met de schimmel *Botrytis cinerea*. Door anderen is eerder aangetoond dat een verhoogde expressie van ERF1, een transcriptiefactor die sterk verwant is aan ORA59, ook leidt tot verhoogde expressie van de genen *PDF1.2* en *HEL*, en ook meer resistent zijn tegen *B. cinerea*. Dit suggereert dat ORA59 en ERF1 vergelijkbare en mogelijk uitwisselbare functies hebben. Echter, zoals in Hoofdstuk 2 beschreven leidde volledige en specifieke uitschakeling van de expressie van het *ORA59* gen tot planten die genen betrokken bij verdediging, inclusief *PDF1.2* en *HEL*, niet meer tot expressie kunnen brengen in respons op schimmelinfectie of in respons op een combinatie van JA en ethyleen. Deze planten zijn ook veel gevoeliger voor schimmelinfectie. Deze experimenten tonen voor het eerst aan dat ORA59 een unieke en essentiële functie heeft in het aanschakelen van genen door JA en ethyleen, en in schimmelresistentie.

Hoofdstuk 3 beschrijft de functies van ORA47. Continu verhoogde expressie van ORA47 leidt tot dwerggroei en de productie van anthocyanen, een fenotype dat lijkt op het effect van JA. Dit leidde tot de hypothese dat verhoogde expressie van ORA47 leidt tot de productie van JA en daardoor tot een JA fenotype. Om de functie van ORA47 te kunnen bestuderen werd gebruik gemaakt van een induceerbaar expressiesysteem. Verhoogde expressie van ORA47 na inductie leidde tot een toename van de expressie van genen betrokken bij de biosynthese van JA, evenals tot een 2- tot 4-voudige stijging van het niveau van de JA precursor OPDA. Een verrassing was dat het niveau van JA niet gestegen was.

Naast het effect op JA biosynthesegenen, leidde inductie van ORA47 expressie ook tot verhoogde expressie van verscheidene genen betrokken bij verdediging, wat waarschijnlijk een indirect effect is als gevolg van de verhoogde productie van jasmonaten zoals OPDA. De verwachting is dat de genen betrokken bij JA biosynthese wel direct door ORA47 aangeschakeld worden, hoewel dat nog experimenteel bevestigd moet worden.

Door anderen is aangetoond, en dat is in dit proefschrift ook bevestigd, dat de expressie van alle JA biosynthesegenen door JA aangeschakeld wordt. De resultaten in dit hoofdstuk geven voor het eerst aan dat ORA47 betrokken is bij deze zelfstimulerende regulatoire lus, die waarschijnlijk dient om het signaal te versterken.

In **Hoofdstuk 4** is de aandacht gericht op ORA37. ORA37 verschilt van alle andere AP2/ERF-domein transcriptiefactoren beschreven in dit proefschrift door de aanwezigheid van een zogenaamd ERF-geassocieerd repressie (EAR) domein in het C-terminale uiteinde

van het eiwit. Dit EAR domein heeft een sterk onderdrukkend effect op de transcriptie van genen. Het *ORA37* gen, ook aangeduid met *AtERF4*, wordt aangeschakeld door JA, ethyleen of verwonding. Verhoogde expressie van ORA37 had geen effect op het basale expressieniveau van JA-gevoelige genen in onbehandelde planten. Echter na behandeling met JA en/of ethyleen werd een significant lager expressieniveau waargenomen voor een subset van JA- en ethyleen-gevoelige genen, waaronder ook de genen *PDF1.2, HEL* en *ChiB*. Het tegengestelde werd waargenomen na verlaging van het expressieniveau van ORA37. *PDF1.2*, *HEL* en *ChiB* hadden dan een hoger expressieniveau na behandeling met JA en/of ethyleen dan in controle planten. Dit toont aan dat ORA37 een rol speelt in de signaaltransductie van JA en ethyleen als negatieve regulator van een set van verdedigingsgenen. ORA37 en ORA59 werken dus tegengesteld op deze subset van JA- en ethyleen-gevoelige genen.

Er werd nog een effect waargenomen van verhoogde expressie van ORA37, namelijk een verhoogd expressieniveau van een andere subset van JA-gevoelige genen, waaronder *VSP1* en *CYP79B2,* na behandeling met JA. Dit geeft aan dat ORA37 ook positief kan werken op genexpressie. Aangenomen dat ORA37 door de aanwezigheid van het EAR domein altijd als een remmer werkt, dan kan het positieve effect verklaard worden doordat ORA37 een negatief effect heeft op de expressie van een remmer van deze genen onder het motto twee keer negatief is ook positief. De uitdaging is nu om uit te zoeken of dit inderdaad zo werkt en welke andere remmer hierbij betrokken is.

Een merkwaardige constatering is nu dat JA en ethyleen zowel activatoren (zoals ORA59 en ERF1) als remmers (zoals ORA37) van de expressie van dezelfde subset van genen induceren. Het nut hiervan is nog onduidelijk. Het kan zijn dat een balans tussen positieve en negatieve regulatie-eiwitten leidt tot een alles of niets effect op genexpressie in respons op JA en ethyleen.

In **Hoofdstuk 5** worden pogingen beschreven om functies in genexpressie toe te kennen aan de overige ORA transcriptiefactoren. Tevens werd onderzocht of sommige ORA eiwitten gedeeltelijk dezelfde sets van genen reguleren. Elk van de 10 ORA eiwitten werd verhoogd tot expressie gebracht in een induceerbaar systeem dat aangeschakeld kan worden door toevoeging van de plantvreemde stof estradiol. Van een aantal kandidaat-genen werd het expressieniveau gemeten in ieder van de planten met verhoogde expressie van één bepaalde ORA. Deze genen werden geselecteerd omdat bekend is dat ze reageren op JA, en omdat ze betrokken zijn bij verschillende processen zoals verdediging tegen biotische of abiotische stress, biosynthese van JA, of primair of secundair metabolisme. De genexpressieprofielen die werden gevonden gaven aanleiding om de genen te onderverdelen in verschillende groepen. Sommige groepen werden aangeschakeld door een verhoogd

expressieniveau van meer dan één ORA, wat aangeeft dat deze ORA eiwitten althans gedeeltelijk dezelfde functie hebben. Voor vijf ORA eiwitten werden geen genen gevonden met een veranderd expressieniveau binnen de gekozen set. Dit zou geïnterpreteerd kunnen worden door aan te nemen dat ze geen functie hebben in JA-gevoelige genexpressie. Waarschijnlijker is echter dat de activiteit van deze eiwitten afhangt van een JA-afhankelijke activeringsstap op eiwitniveau.

Het doel van het onderzoek beschreven in dit proefschrift was om de functies op te helderen van de ORA transcriptiefactoren in JA-gevoelige genexpressie in de zandraket. De aanpak was om het expressieniveau te veranderen en te kijken wat voor een effect dat heeft op genexpressie en het aanzien van de planten. Voor enkele ORAs is dit goed gelukt. Het is duidelijk geworden dat ORA33, ORA37, ORA47 en ORA59 inderdaad de expressie van bepaalde subsets van genen regelen in respons op JA (Figuur 1; zie ook Figuur 2 in hoofdstuk 1). Deze ontdekkingen zijn nieuw, en in het geval van ORA59 in tegenspraak met conclusies getrokken door anderen. ORA47 is hiermee ook de eerst ontdekte transcriptionele regulator van de biosynthese van een plantenhormoon.

De conclusies over de functies van de ORA transcriptiefactoren getrokken in dit proefschrift berusten voornamelijk op de analyse van genexpressie op mRNA niveau. Maar de verwachting is dat verder onderzoek naar de regulatie van ORA activiteit op eiwitniveau zal leiden tot een veel beter beeld van de rol van de ORA transcriptiefactoren in JA signaaltransductie. Toekomstig onderzoek zal zich dan ook ongetwijfeld richten op de mechanismen waardoor JA via covalente modificaties en eiwit-eiwit interacties de activiteit van de ORA transcriptiefactoren reguleert. Een andere brandende vraag is hoe de JA-afhankelijke transcriptie van de *ORA* genen zelf is gereguleerd. Dit is namelijk geen Droste effect van een transcriptiefactor die de expressie van een ander transcriptiefactorgen reguleert enzovoorts ad infinitum, maar in den beginne start de signaaltransductieketen met een reeds aanwezige “meester” transcriptiefactor, die direct op eiwitniveau wordt geactiveerd onder invloed van JA. De onthulling van deze vooralsnog duistere meester is een andere belangrijke uitdaging.

Curriculum vitae

Martial Pré was born on November 14, 1974 in Tours, France. He attended high school at Lycée Choiseul, Tours. In September 1994, he started the study of Biology at the Faculté de Sciences et Techniques, Université François Rabelais, Tours. From February 1998 until August 1998, he dedicated himself to a research training in plant molecular biology at the Institute of Biology, Leiden University, The Netherlands, under the supervision of Prof. Dr. Johan Memelink. From September 1998 untill September 1999, he was involved in ongoing research work at the Department of Plant Physiology (E.A. 2106 Biomolécules et Biotechnologies Végétales), Université François Rabelais, Tours, under the supervision of Prof. Dr. Pascal Gantet and received his Diploma in Initiation of Scientific Research (DIRS). From October 1999 until July 2000, he fulfilled military service. From April 2001 to September 2005, he worked on his PhD research project at the Institute of Biology, Leiden University, under the supervision of Prof. Dr. Johan Memelink. Since October 2005, he is working as a post-doc in the group of Prof. Dr. Johan Memelink at the Institute of Biology Leiden.