ORA EST : functional analysis of jasmonate-responsive AP2/ERF-domain transcription factors in Arabidopsis thaliana

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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;

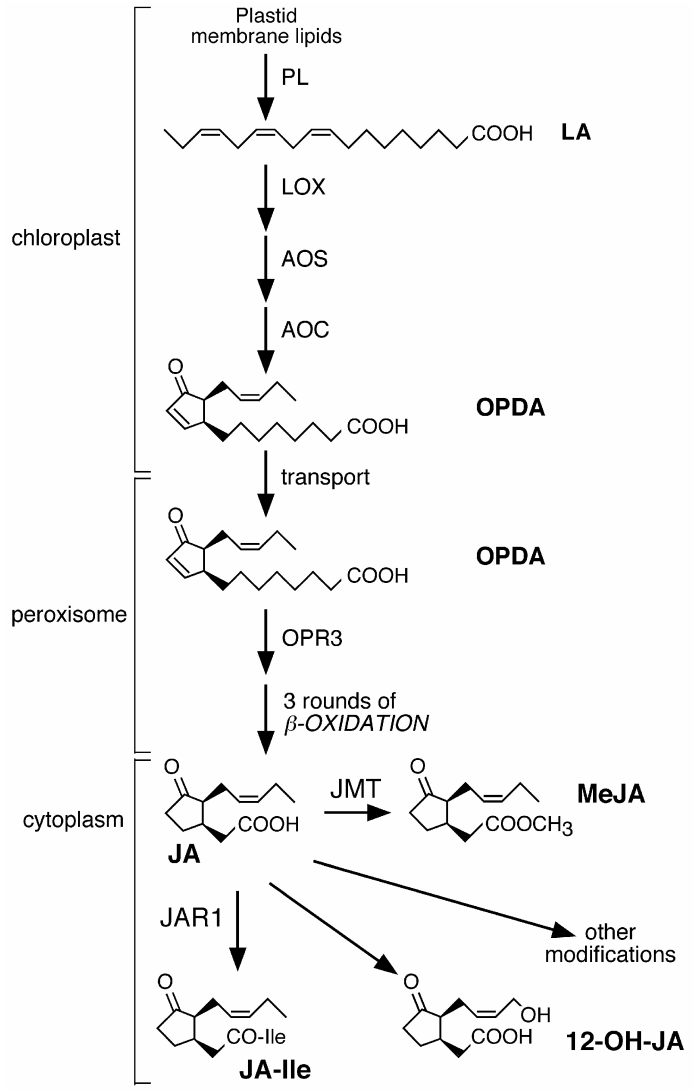

Figure 1. Schematic representation of the octadecanoid pathway leading to jasmonic acid biosynthesis. 12-OH-JA, 12-hydroxy-jasmonic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; JA, jasmonic acid; JAR1, enzyme responsible for the conjugation of JA with isoleucine (JA-Ile); JMT, *S*-adenosyl-L-methionine:jasmonic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

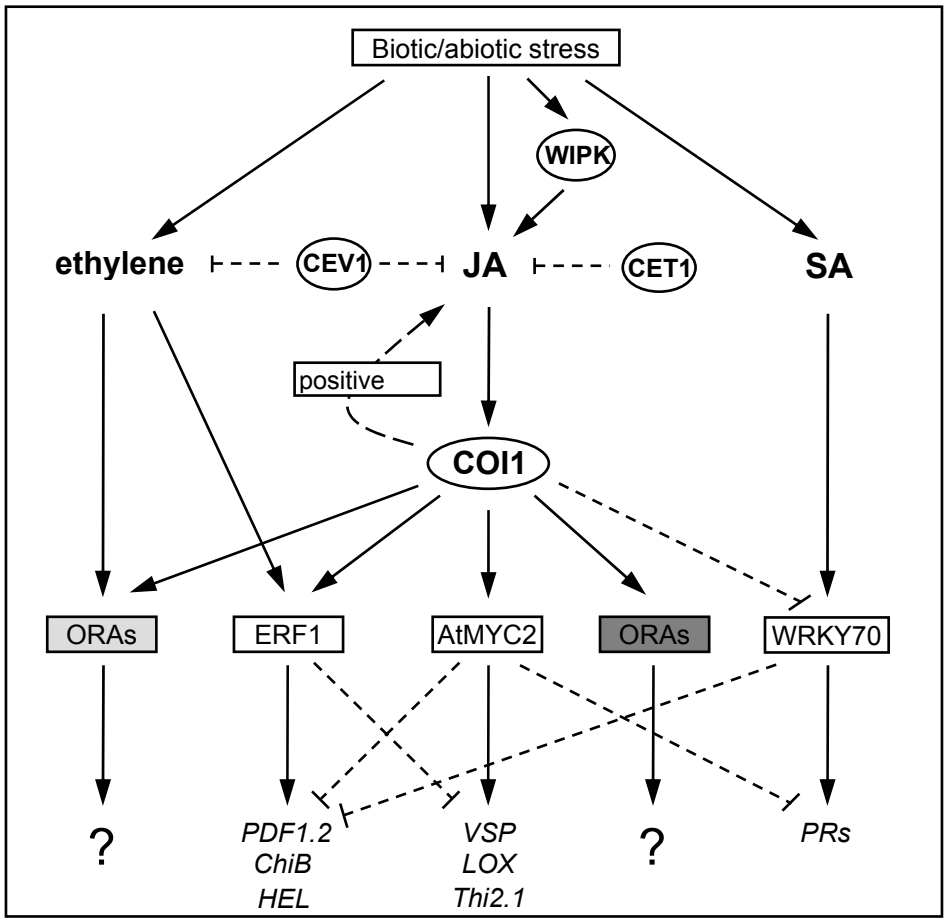

Figure 2. Stress-responsive network connecting the jasmonic acid (JA), ethylene and salicylic acid (SA) signaling pathways in Arabidopsis. Regulatory proteins are circled. Transcription factors are boxed. Arrows indicate induction of gene expression or positive interaction. Dashed lines indicate repression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