Jasmonate-responsive AP2-domain transcription factors in Arabidopsis

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D.D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 24 februari 2005
klokke 14:15 uur

door

Mirna Atallah

geboren te Byblos (Libanon)
in 1975

Promotiecommissie

Promotor:	Prof. dr. J.W. Kijne
Co-promotor:	Dr. J. Memelink
Referent:	Prof. dr. C.M.J. Pieterse (Universiteit Utrecht)
Overige leden:	Prof. dr. P.J.J. Hooykaas
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A ma mère,

Xander et Melissa

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Chapter 1

General introduction

The role of the jasmonate signal transduction pathway in the response of plants to stress.

Mirna Atallah and Johan Memelink

Modified from: Encyclopedia of Plant & Crop Science, RM Goodman, ed, Marcel Dekker Inc, New York *2004*, pp 1006-1009

Introduction

Plants differentially activate distinct defense pathways in response to stress. Depending on the type of stress, plants synthesize the signaling molecules jasmonic acid (JA), salicylic acid (SA), or ethylene, which regulate the defense response.

Jasmonates (JAs) are fatty acid derivatives synthesized via the octadecanoid (ODA) pathway (Mueller, 1997). They play pivotal roles in wound and defense responses, and in anther and pollen development (Creelman and Mullet, 1997; Turner *et al.*, 2002). The defense JA pathway comprises several signal transduction events: the perception of the primary stress stimulus and transduction of the signal locally and systemically; the perception of this signal and induction of JA biosynthesis; the perception of JA and expression of responsive genes; and finally, integration of JA signaling with outputs from other signaling pathways.

Stress-induced JA-biosynthesis

How stress signals affect JA biosynthesis is largely unknown. In *Catharanthus roseus* cells, elicitor-induced JA biosynthesis depends on an increase in cytoplasmic Ca^{2+} concentration and protein phosphorylation (Memelink *et al.*, 2001) (Fig. 1). In tobacco, wound-induced JA biosynthesis depends on the mitogen-activated protein kinase WIPK (Seo *et al.*, 1995; 1999; Turner *et al.*, 2002).

More is known about the JA biosynthetic pathway itself (Turner *et al.*, 2002). The biosynthesis of JAs, which include the biologically active intermediates in the ODA pathway and derivatives of jasmonic acid, begins in the plastids with phospholipase (PL)-mediated release of α-linolenic acid (LA) from membrane lipids (Turner *et al.*, 2002). LA is then converted by lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) into the intermediate 12-oxo-phytodienoic acid (OPDA). This compound is converted in the peroxisomes into JA by OPDA reductase 3 (OPR3), and by three rounds of β-oxidation. JA can be methylated in the cytoplasm to its volatile derivative methyl-jasmonate (MeJA) by S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase (JMT) (Fig. 2).

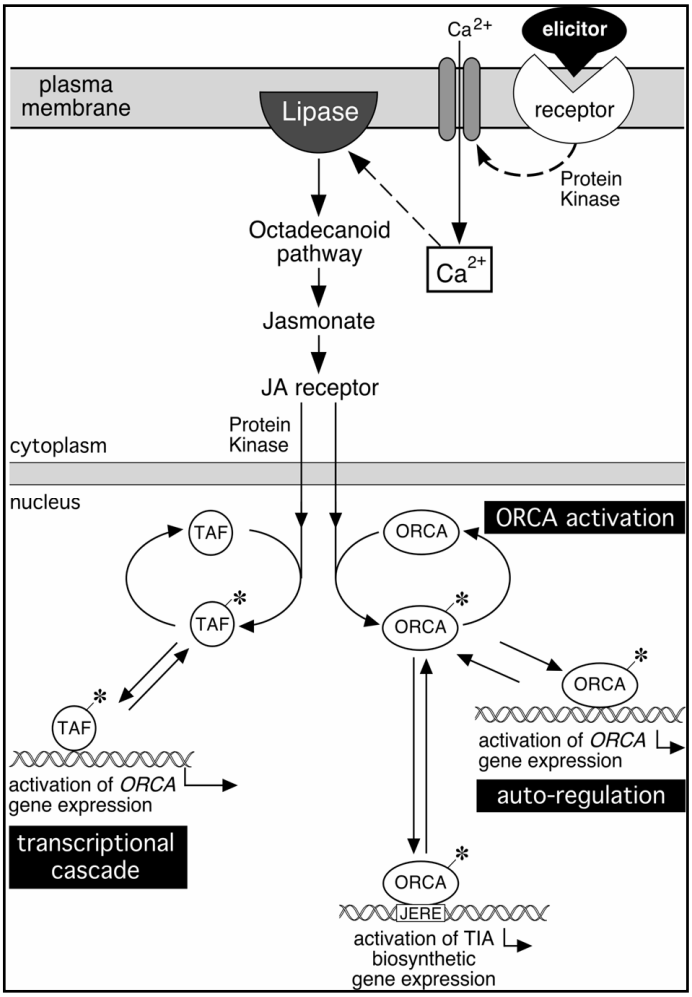

Figure 1. Model for elicitor signal transduction leading to TIA biosynthetic gene expression in *C. roseus*.

Wounding induces the expression of several JA biosynthesis genes. Therefore, one possible mechanism for stress-induced JA biosynthesis is *de novo* synthesis of biosynthetic enzymes. In addition, the expression of JA biosynthesis genes is induced by JAs themselves, indicating that JA signaling is amplified by a positive feedback mechanism.

Several mutants in *Arabidopsis thaliana* affected in JA biosynthesis have been isolated (Turner *et al.*, 2002). The *fad3-2fad7-2fad8* triple mutant, lacking the fatty acid desaturases necessary to synthesize the JA precursor linolenate, contains negligible amounts of LA and JAs (McConn and Browse, 1996). The *opr3*

mutant (also known as *dde1: delayed dehiscence1*) lacks the OPDA reductase isoform required for JA biosynthesis, but accumulates OPDA when wounded (Sanders *et al.*, 2000; Stintzi and Browse, 2000; Stintzi *et al.*, 2001).

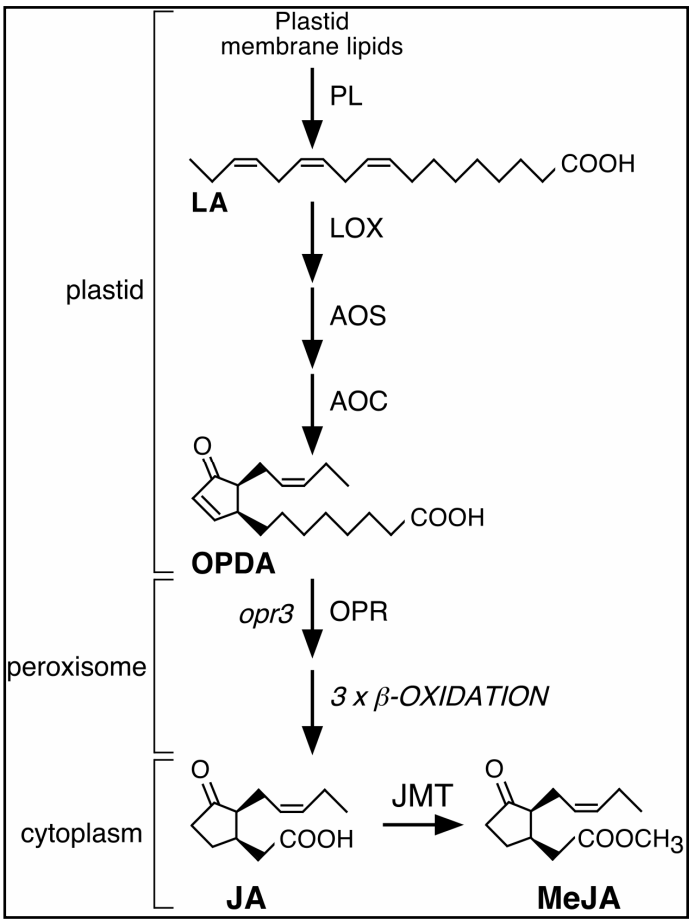

Figure 2. Schematic representation of the JA biosynthetic pathway. A mutant blocked in a biosynthesis step is in italics.

JA signal transduction

How JAs are perceived by plant cells is unknown. The mechanisms whereby JA signaling triggers gene expression are just starting to be elucidated. A JA- and elicitor-responsive element (JERE) in the promoter of the terpenoid indole alkaloid (TIA) biosynthetic gene *Strictosidine synthase* (*Str*) from *C. roseus* interacts with two transcription factors called Octadecanoid-Responsive *Catharanthus* AP2/ERF-

domain proteins (ORCAs) (Menke *et al.*, 1999b; van der Fits and Memelink, 2001). ORCA2 was isolated by yeast one hybrid screening using the JERE as bait (Menke *et al.*, 1999b) and ORCA3 was isolated by a genetic T-DNA activation tagging approach (Memelink *et al.*, 2001). Both belong to the AP2/ERF family of transcription factors, which are not present in animals and are characterized by the AP2/ERF DNA-binding domain.

Significantly, *ORCA* gene expression is rapidly induced by MeJA. In addition, cycloheximide did not inhibit JA-induced target gene expression suggesting that JA activates pre-existing ORCA transcription factors by inducing a post-translational modification, for example phosphorylation (Menke *et al.*, 1999a; van der Fits and Memelink, 2001). Activated ORCA proteins may auto-regulate *ORCA* gene expression as well as regulating TIA biosynthetic gene expression. Alternatively, JA-induced *ORCA* gene expression can occur via a transcriptional cascade, including a yet unidentified transcription-activating factor (TAF), which is activated via post-translational modification (Fig. 1).

In Arabidopsis, the AP2/ERF-domain transcription factor ETHYLENE RESPONSE FACTOR 1 (ERF1) was shown to be involved in JA signal transduction as well as in ethylene signaling (Lorenzo *et al.*, 2003). Constitutive expression of ERF1 leads to increased expression levels of defense-related genes that are synergistically induced by a combination of ethylene and JA, including *PDF1.2*, and confers resistance to several necrotrophic fungi (Lorenzo *et al.*, 2003; and references therein). Therefore, it appears that Arabidopsis also uses a subset of its 126 AP2/ERF-domain transcription factors, including ERF1, to regulate JA-responsive gene expression.

Several JA-insensitive Arabidopsis mutants have been found by screening for a reduction in the inhibition of root growth caused by MeJA or by the bacterial toxin coronatine, a structural analogue of JA and OPDA (Turner *et al.*, 2002). The *coronatine-insensitive 1* (*coi1*) mutant is affected in a gene encoding a protein with 16 leucine-rich repeats and an F-box motif. The COI1 F-box protein associates with Skp1-like proteins (S) and cullin (C) to form SCF^{COI1} ubiquitin-ligase complexes (Xu *et al.*, 2002). F-box proteins are the components of SCF complexes, which recognize substrate proteins and target them for degradation via the ubiquitin-proteasome pathway. Therefore, COI1 seems to recruit one or more repressors of JA responses for degradation (Turner *et al.*, 2002; Xu *et al.*,

2002). The JA-insensitive mutant *mpk4* was identified by its dwarf phenotype, and is affected in the gene encoding the mitogen-activated protein kinase 4 (Petersen *et al.*, 2000).

JA responses

A key role for JAs in defense of tomato against insect herbivores and microbial pathogens was proposed by Farmer and Ryan in 1992, who showed that intermediates and end products of the octadecanoid pathway, but not other closely related lipids, induced proteinase inhibitors that deter insect feeding (Turner *et al.*, 2002). JA is the physiological signal for several wound- and pathogen-induced responses in plants, and it is essential for pollen development in Arabidopsis (Turner *et al.*, 2002). Exogenously applied (Me)JA results in major reprogramming of gene expression, including defense-related genes that are activated by wounding and pathogen attack. The JA-responsive *PDF1.2* and *THI2.1* genes encode anti-microbial plant defensin and thionin proteins, respectively (Penninckx *et al.*, 1996; Epple *et al.*, 1995). JAs also induce the expression of biosynthesis genes leading to the accumulation of anti-microbial secondary metabolites, including alkaloids, terpenoids, flavonoids, anthraquinones and glucosinolates, in different plant species (Memelink *et al.*, 2001; Blechert *et al.*, 1995).

Arabidopsis mutants defective in JA biosynthesis or perception are deficient in certain defense responses and are often male sterile. The *coi1* mutant is defective in its resistance to certain insects and pathogens, and fails to express JA-regulated genes, including *PDF1.2* and *THI2.1* (Turner *et al.*, 2002). A single amino acid substitution in COI1, which disrupts SCF^{COI1} complex formation, results in loss of the JA response (Xu *et al.*, 2002). The *mpk4* mutant is blocked in the induction of JA-inducible *PDF1.2* and *THI2.1* genes and has reduced fertility (Petersen *et al.*, 2000) (Fig. 3). The *fad* triple mutant shows enhanced sensitivity to the fungus *Pythium irregulare* and the dipteran insect *Bradysia impatiens* (Turner *et al.*, 2002). The *opr3* mutant is male sterile, indicating that pollen development uniquely requires JA and not OPDA. Fertility is restored in the *opr3* mutant and the *fad* triple mutant by application of JA. OPDA plays a major role as a stress signal, since its synthesis in the *opr3* mutant is sufficient to trigger a

defense response against *B. impatiens* and the fungus *Alternaria brassicicola* (Stintzi *et al.*, 2001).

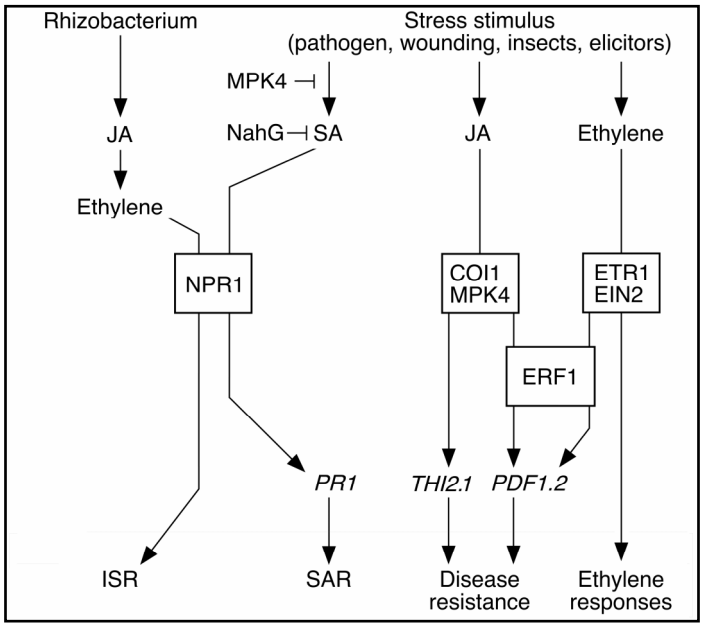

Figure 3. Model showing signaling in stress responses in Arabidopsis.

JAs play an important role in ISR, a form of induced systemic resistance elicited by non-pathogenic strains of the root-colonizing bacterium *Pseudomonas fluorescens* (Pieterse and van Loon, 1999) (Fig. 3).

Cross-talk between defense signaling pathways

The JA signaling pathway interacts cooperatively and antagonistically with the ethylene and SA pathways in a variety of responses, leading to fine-tuning of the complex defense response. Together with JA, ethylene plays a crucial role in defense against necrotrophic microbes, in expression of *PDF1.2* and other defense genes, and in ISR (Fig. 3). Mutants affected in ethylene signal transduction, including the ethylene receptor mutant *ethylene-resistant1* (*etr1*) and the *ethylene-insensitive2* (*ein2*) mutant, also have reduced expression of certain JA-responsive genes (Lorenzo *et al.*, 2003), are more susceptible to certain microbial pathogens, and cannot mount ISR (Fig. 3). A subset of AP2/ERF-domain

transcription factors, including Arabidopsis ERF1 (Lorenzo *et al.*, 2003), may serve as the platform to integrate the input from the JA and ethylene signaling pathways (Fig. 3).

Systemic acquired resistance (SAR) is a defense response in which, in contrast to ISR, SA is the key regulatory signal. Transgenic Arabidopsis NahG plants expressing the bacterial SA-degrading enzyme salicylate hydroxylase cannot mount SAR. SAR provides protection in uninfected plant parts against pathogens and is correlated with the expression of pathogenesis-related (PR) proteins with anti-microbial activity (Fig. 3). The NPR1 (Nonexpressor of PR genes 1) protein has a dual role in systemic resistance mechanisms mediated by either SA (SAR) or JA and ethylene (ISR) (Turner *et al.*, 2002; Pieterse and van Loon, 1999) (Fig. 3). The *mpk4* mutant, blocked in JA signaling, exhibits elevated levels of SA and constitutive SAR (Petersen *et al.*, 2000).

Conclusion

The roles of JAs in development, defense responses and gene expression are currently being delineated through the analysis of additional gain-of-function and loss-of-function Arabidopsis mutants (Turner *et al.*, 2002; Berger, 2002), and through the analysis of JA-responsive promoters and transcription factors. Future work will focus on the regulation of JA synthesis, the identification of JA receptors, the identification of JA-responsive transcription factors in different plant species and of other signal transduction steps that regulate transcription factor activity, and on the mechanisms of cross-talk between different defense signaling pathways.

Outline of the thesis

The studies described in this thesis are focused on the molecular mode of action of the important plant stress hormone JA in gene expression in Arabidopsis. JA-responsive gene expression forms an important part of the plant defense response. It has been shown previously that JA-responsive gene expression in *C. roseus* is mediated via ORCA transcription factors (Menke *et al.*, 1999b; van der Fits and Memelink, 2000; 2001), which belong to the class of AP2-domain transcription factors. The expression of the *ORCA* genes themselves is JA-responsive. Based on these observations, the working hypothesis that stood

at the basis of the research described in this thesis was formulated: JA-responsive gene expression in Arabidopsis is also mediated by members of the AP2-domain transcription factor family, and the corresponding genes are also expressed in a JA-responsive manner. The goal of the thesis work was to find JA-responsive members of the large AP2-domain transcription factor gene family in Arabidopsis (so-called ORA transcription factors), to clarify their role in the JA signal transduction network, and to attempt to establish that these members are indeed involved in JA-responsive gene expression.

In **Chapter 2**, the identification of 14 JA-responsive genes encoding AP2-domain transcription factors (ORAs) from Arabidopsis is described. Further analysis of their response to JA in different Arabidopsis mutants shows that expression of all these *ORA* genes depends on the central JA signal transduction protein COI1, and the expression of a subset of five *ORA* genes depends additionally on the ethylene signaling components ETR1 and EIN2. In **Chapter 3**, the results showed that constitutive overexpression of ORA1, ORA2 and ORA4 resulted in the expression of several JA-responsive defense-related genes, suggesting their involvement in plant defense responses. ORA1, ORA2 and ORA4 were also shown to interact in a sequence-specific manner with the previously identified JA-responsive JERE element from Catharanthus *in vitro* (Menke *et al.*, 1999b). In **Chapter 4**, studies on the expression of the *ORA* genes in response to SA are described. The results show that the expression of two *ORA* genes is negatively affected by SA, while another subset is induced. Further analysis provided strong indications that the phenolic structure of SA induces *ORA* gene expression via an NPR1-independent pathway. Finally, in **Chapter 5** a summary and general discussion of the results are presented.

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Chapter 2

RNA expression profiling of the AP2-domain family of Arabidopsis transcription factors in response to jasmonic acid.

Mirna Atallah, Martial Pré, and Johan Memelink

Abstract

Jasmonic acid (JA) is a plant signaling molecule that plays a key role in defense against certain pathogens and insects. JA does so by inducing 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 mechanisms whereby JA signaling results in gene expression. In *Catharanthus roseus*, JA-responsive expression of alkaloid biosynthesis genes is regulated by AP2-domain transcription factors. Therefore, we focused our attention on this family of transcription factors in our efforts to identify JA-responsive transcription factors in *Arabidopsis thaliana*. The Arabidopsis genome encodes 126 proteins with a single DNA-binding domain of AP2-domain–like structure. Expression profiling of this gene family resulted in the identification of fourteen members called *ORA* genes, which show increased expression in response to JA within 4 hours in Arabidopsis seedlings. Several *ORA* genes were also induced by salicylic acid (SA) or ethylene, however this response was not as fast as for JA. JA-responsive *ORA* gene expression depends on COI1, a central component of the JA signal transduction pathway. Induction of JA-responsive *ORA* gene expression was not affected by a mutation in the SA pathway component NPR1. The expression of several *ORA* genes was induced synergistically by JA in combination with ethylene. This *ORA* gene subset showed reduced JA responsiveness in Arabidopsis mutants affected in ethylene signal transduction, suggesting that the encoded ORA proteins may play key roles in the integration of both signals to activate JA- and ethylene-dependent responses.

Introduction

In response to pathogen or insect attack, plants produce secondary stress signals such as jasmonic acid (JA), salicylic acid (SA), and ethylene. These molecules activate signal transduction pathways, which interact synergistically and antagonistically resulting in the induction of specific defense gene sets (Glazebrook, 2001; Rojo *et al.*, 2003).

JA and its volatile derivative methyljasmonate (MeJA), collectively called jasmonates, are fatty acid derivatives synthesized from linolenic acid via the octadecanoid pathway (Turner *et al.*, 2002). JA is an important plant hormone that regulates developmental processes including root growth, pollen

development, and fruit ripening (Turner *et al.*, 2002; Atallah and Memelink, 2004). In addition, in response to stress, wounding, UV irradiation, insect or pathogen attack, JA induces the expression of a large number of defense-related genes, including genes encoding the plant defensins PDF1.2 and thionin, proteinase inhibitors, and enzymes involved in the biosynthesis of protective secondary metabolites (Turner *et al.*, 2002; Atallah and Memelink, 2004).

How JA signaling activates the expression of specific genes is largely unknown. In *Catharanthus roseus*, JA-responsive expression of alkaloid biosynthesis genes is regulated by AP2-domain transcription factors called ORCAs (Menke *et al*., 1999; van der Fits and Memelink, 2000).

The AP2 domain is a DNA-binding domain of around 60 amino acids, which was first recognized as a tandemly repeated motif in the Arabidopsis APETALA (AP2) protein (Jofuku *et al.*, 1994). However, the ORCA proteins possess a single AP2 domain. *ORCA* gene expression is rapidly induced by MeJA (Menke *et al.*, 1999; van der Fits and Memelink, 2001). To study how JA regulates gene expression, we switched to the model plant species *Arabidopsis thaliana*, which possesses several advantages such as a completely sequenced genome, insertion element-tagged plant collections, and the availability of a number of interesting mutants. Some of these mutants are affected in defense signaling pathways and are powerful tools in studying regulation of defense gene expression, such as the JA-insensitive *coi1-1* mutant (Feys *et al.*, 1994), the SA-insensitive *npr1-1* mutant (Cao *et al*., 1994) and ethylene-insensitive mutants including *ein2-1* and *etr1-1* (Guzman and Ecker, 1990; Bleecker *et al*., 1988). Based on our discovery of the ORCA transcription factors, we focused our attention on the AP2-domain transcription factor family.

In Arabidopsis, this family of transcription factors is reported to comprise 144 proteins and was divided into 3 subfamilies (Riechmann *et al*., 2000). The AP2-like subfamily with 14 proteins has two AP2 domains and its members function mainly in development. This group includes the founding member APETALA2 (AP2), which is involved in flower development (Jofuku *et al.*, 1994). The ERF-like subfamily was reported to comprise 124 proteins having one AP2 domain. Multiple members function in stress responses, such as DREB1/CBF1 and DREB2 proteins, which interact with the DRE (dehydration-responsive element) and are involved in low-temperature- and dehydration-/ high salinity-responsive

gene expression respectively (Liu *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998). The *AtERF1-5* genes are responsive to abiotic stress and ethylene (Fujimoto *et al.*, 2000). Several other members, including TINY (Wilson *et al.*, 1996) and LEAFY PETIOLE (LEP; van der Graaff *et al.*, 2000) are involved in development. Lastly, the RAV-like subfamily with 6 proteins possesses two distinct DNA-binding domains, an AP2 domain and a B3 domain, which is found also in the transcription factors VP1 from maize and ABI3 from Arabidopsis (Riechmann *et al.*, 2000; Kagaya *et al.*, 1999).

When we started this research, the ORCA proteins from Catharanthus were the first and only transcription factors demonstrated to regulate JA-responsive gene expression. When we set out to identify JA-responsive members of the Arabidopsis AP2-domain family, our working hypothesis was that the corresponding genes are also expressed in a JA-responsive manner as observed for the *ORCA* genes. This assumption was supported by the recent finding of ERF1 as a component of the signaling pathway mediating crosstalk between ethylene and JA and the observation that *ERF1* gene expression is responsive to JA (Lorenzo *et al.*, 2003). To identify AP2-domain transcription factor gene family members that show JA-responsive expression, our strategy was to amplify all genes encoding transcription factors with a single DNA-binding domain of the AP2 type from Arabidopsis by PCR, and to use the genes as probes in Northern blot hybridisations to study their expression after exposure of seedlings to JA. This strategy resulted in the identification of fourteen JA-responsive genes encoding AP2-domain transcription factors that we called ORA (Octadecanoid-Responsive Arabidopsis AP2 domain). Analysis of their expression in Arabidopsis mutants shows that the JA-responsive expression of all *ORA* genes depends on COI1. The JA-responsive expression of a subset of five *ORA* genes depends additionally on ETR1 and EIN2. The expression of this subset was also found to be synergistically induced by a combination of JA and the ethylene-releasing agent ethephon, suggesting that the encoded ORA proteins integrate JA and ethylene signal inputs to coordinate the appropriate gene expression response.

Materials and Methods

Database search

To identify members of the Arabidopsis AP2-domain protein family, a database search was performed using the AP2 domain of ORCA2 from *Catharanthus roseus* (Menke *et al*., 1999) as a query sequence in the pblast program against the Arabidopsis proteome database available at the Munich information center for protein sequences (http://mips.gsf.de; Schoof *et al*., 2002). The last update was done in April 2004.

Tree building

The phylogenetic tree was constructed with 126 AP2-domains of around 60 amino acids derived from all AP2-domain proteins with a single DNA-binding domain using ClustalW at the DDBJ server (hypernig.nig.ac.jp) with the default settings, including 1000 bootstraps. The tree was displayed using Treeview (Page, 1996; taxonomy.zoology.gla.ac.uk/rod/treeview.html), with APETALA2 AP-domain repeat 1 defined as an outgroup.

Plant material, growth conditions, and treatments

The Arabidopsis wild type (WT), mutant (*npr1-1*, *ein2-1*, *etr1-1*, *coi1-1*) and transgenic (NahG) plants used were Columbia (Col-0) ecotype. Seeds were surface-sterilized by incubation for 1 minute in 70 % ethanol, 15 minutes in 50% bleach, and five rinses with sterile water. Per treatment 3 mg corresponding to around 150 seeds were added to 50 ml of MA medium (Masson and Paszkowski, 1992) in a 250 ml widemouth Erlenmeyer flask capped with aluminium foil and stratified for 3 days at 4 °C. Following 10 days of incubation in a growth chamber (16 h light/8 h dark, 4000 lux) at 21 °C on a shaker at 120 rpm, seedlings were treated for different times with 50 μM JA (Sigma) dissolved in dimethylsulfoxide (DMSO; 0.1 % final concentration in the culture volume), 1 mM salicylic acid (Sigma) or 1 mM of the ethylene-releasing agent ethephon (Sigma) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration in the culture volume). Control seedlings were treated with final concentrations of 0.1 % DMSO or 0.5 mM sodium phosphate. Seedlings were harvested in liquid nitrogen, and stored at −80 °C until RNA isolation.

To obtain *coi1-1* mutant seedlings, a mixture of COI1/COI1, COI1/*coi1-1*, and *coi1-1*/ *coi1-1* seeds were surface-sterilized and plated on solid MA medium

supplemented with 50 μM JA for 4 days. Per treatment 50 mutant *coi1-1* seedlings, which did not show anthocyanin production and inhibition of root growth in the presence of JA, were transferred to 50 ml of liquid MA medium in a 250 ml Erlenmeyer and used 10 d after germination for treatment and RNA extraction.

RNA extraction and Northern blotting

Total RNA was isolated by extraction with 2 ml/g of tissue of phenol buffer (1:1 mixture of phenol and 100 mM LiCl, 10 mM EDTA, 1% SDS, 100 mM Tris) heated to 80 ^{o}C and 1 ml of chloroform. The mixture was centrifuged at 4600 rpm, and the aqueous phase was extracted with one volume of chloroform. After addition of one-third volume of 8 M LiCl, the RNA was precipitated overnight at 4°C. The RNA was collected by centrifugation at 10000 rpm for 30 min, washed twice with 70% ethanol, dried under vacuum, dissolved in water and stored at –20°C. Total RNA samples of 10 μg were dried in a speedvac, dissolved in 10 μl of sample buffer and electrophoretically separated on 1.5% agarose/1% formaldehyde gels as described (Memelink *et al*., 1994). Gels were blotted as described (Memelink *et al*., 1994) to GeneScreen nylon membranes (PerkinElmer Life Sciences Inc.). Blots were prehybridized in 1 M NaCl/ 10 % dextran sulfate sodium salt (Sigma)/ 1% SDS/ 50 μg/ml denatured salmon sperm DNA at 65^{o}C for 3 hours, and hybridized overnight to ^{32}P-labeled probes prepared as described (Memelink *et al*., 1994). Blots were washed as described (Memelink *et al*., 1994) and exposed to X-ray films (Fuji RX).

Full-length open reading frames for all AP2-like genes with one DNA binding domain were generated via PCR amplification with gene-specific primers. All genes were amplified from Arabidopsis genomic DNA except for *At1g15360*, *At1g22190*, *At1g25470*, *At1g43160*, *At2g40350*, *At2g41710*, *At3g14230*, *At3g54990*, *At4g13040*, *At4g31060*, *At5g07310*, *At5g11190*, *At5g25190*, *At5g25390*, *At5g50080*, *At5g51190*, *At5g61890* and *At5g64750*, which were amplified from a cDNA library prepared from above-ground parts of mature flowering plants. PCR fragments were cloned in pBluescript II SK+, pIC-20R, or pIC-20H as BamHI, BamHI/BglII, or BglII fragments respectively. Fragments were re-excised from the vectors and used as probes. Otherwise PCR products were run on gel and purified before being used as probes. In the case of the *ORA*

genes, specificity of hybridization signals was verified using non-conserved parts of the coding regions outside of the conserved AP2 domains.

DNA fragments corresponding to the open reading frames of genes encoding β-tubulin (*TUB*, *At5g44340*), hevein-like protein (*HEL*, *At3g04720*), vegetative storage protein 1 (*VSP1*, *At5g24780*), basic chitinase (*CHIB*, *At3g12500*), pathogenesis related-1 protein (*PR-1*, *At2g19990*), plant defensin (*PDF1.2*, *At5g44420*) and a 300 bp fragment at the 3' end of lipoxygenase 2 (*LOX2*, *At3g45140*) were amplified by PCR from Arabidopsis genomic DNA. The PCR primer sets used were (*TUB*) 5'-CGGAATTCATGAGAGAGATCCTTCATATC-3' and 5'-CCCTCGAGTTAAGTCTCGTACTCCTCTTC-3'; (*HEL*) 5'-CGGGATCCATATGAAGATCAG ACTTAGCATAAC-3' and 5'-CGGGATCCTCAAACGCGATCAATGGCCGAAAC-3'; (*VSP1*) 5'-CGGGATCCATGAAAATCCTCTCACTTT-3' and 5'-CCCTCGAGTTAAGAAGGTACGTA GTAGAG-3'; (*CHIB*) 5'-GCTTCAGACTACTGTGAACC-3' and 5'-TCCACCGTTAATGAT GTTCG-3'; (*PR-1*) 5'-GTAGGTGCTCTTGTTCTTCC-3' and 5'-TTCACATAATTCCCACG AGG-3'; (*PDF1.2*) 5'-AATGAGCTCTCATGGCTAAGTTTGCTTCC-3' and 5'-AATCCATG GAATACACACGATTTAGCACC-3'; (*LOX2*) 5'-CGGGATCCGTGCGGAACATAGGCCACG G-3' and 5'-CGGGATCCGGAACACCCATTCCGGTAAC-3'.

Results

Identification of JA-responsive members of the Arabidopsis AP2-domain transcription factor gene family

To identify the members of the Arabidopsis AP2-domain family, a BLAST (Basic Local Alignment Search Tool) search of the Arabidopsis proteome database was performed using the AP2 domain of the JA-responsive transcription factor ORCA2 from *Catharanthus roseus* (Menke *et al*., 1999) as a query sequence.

With this search we identified 146 proteins (Table 1, supplemental data) possessing an AP2 domain instead of 144 as reported by Riechmann *et al.* (2000), 145 as reported by Sakuma *et al.* (2002), or 141 as reported by Alonso *et al*. (2003). Of these 146, 126 proteins belong to the subfamily with a single DNA-binding domain of the AP2 type previously reported to include 124 (Riechmann *et al*., 2000) or 125 members (Sakuma *et al*., 2002). Fourteen proteins have two AP2 domains (At1g16060, At1g51190, At1g72570, At1g79700, At2g28550, At3g20840, At3g54320, At4g36920, At4g37750, At5g10510, At5g17430, At5g57390, At5g65510, At5g67180) and 6 proteins belong to the

RAV-like subfamily (At1g13260, At1g25560, At1g50680, At1g51120, At1g68840, At3g25730).

To identify JA-responsive members of the Arabidopsis gene family encoding proteins with a single AP2 DNA-binding domain, our strategy was to amplify all 126 genes by PCR and to use these genes as probes in Northern blot hybridisations to study their expression after exposure of seedlings to JA. Ten-days old seedlings grown in liquid culture were used to have controlled growth conditions and even exposure to a fixed concentration of JA. Seedlings were treated with 50 μM JA for 4 hours instead of its volatile derivative methyljasmonate (MeJA) to limit variations in the concentration due to volatilisation. Gene expression in seedlings treated with SA and the ethylene-releasing agent ethephon was analysed to determine the specificity of the gene expression response. The genes *LOX2*, *PR-1*, and *HEL*, which are responsive to JA, SA, and ethylene respectively, were used as controls to verify that the hormone treatments were effective and specific.

Fourteen AP2-like genes were found to be responsive to JA (Fig. 1). They were called *ORA* genes for Octadecanoid-Responsive Arabidopsis AP2-domain. The *ORA* genes showed four different types of expression kinetics. Rapid transient expression was observed with genes *ORA1* (*At4g17500*), *ORA2* (*At5g47220*) and *ORA47* (*At1g74930*). The expression of these *ORA* genes was rapidly induced by JA with a peak at 15 min of treatment, and returned to basal levels within 4 hours of exposure to JA. The genes *ORA4* (*At2g44840*), *ORA31* (*At5g47230*), *ORA33* (*At4g34410*), *ORA37* (*At3g15210*) and *ORA59* (*At1g06160*), showed intermediately transient kinetics of expression. JA induced these genes within a short interval of time, starting after 15-30 min and peaking after 30 min of treatment. The expression of the genes *ORA63* (*At5g61890*) and *ORA71* (*At5g07310*) was extremely transient, and was detectable only after 1 hour of treatment, but not after 30 min or 2 hours. The genes *ORA19* (*At2g22200*), *ORA44* (*At1g43160*), *ORA68* (*At5g13330*) and *ORA91* (*At1g12630*) were characterized by a more prolonged expression in response to JA up to the longest time point of 4 hours. *ORA19* and *ORA44* were induced within 15 min of treatment, whereas *ORA68* and *ORA91* showed elevated expression from 1 hour onward. SA and ethephon treatments did not have rapid or strong effects on *ORA* gene expression. However, a weak induction was observed with the genes *ORA1*,

ORA2, and *ORA68* after 2 to 4 hours of SA or ethylene treatment. *ORA59* gene expression was repressed after 2 hours of exposure to SA (Fig. 2). The *LOX2* gene used as a JA control was induced after 30 min of JA addition and mRNA continued to accumulate up to 4 h (Fig. 1). SA and ethylene induced the control genes *PR-1* and *HEL* respectively after 2 and 4 h of treatment. Hybridisation with the *TUB* gene showed equal loading of RNA (Figs. 1 and 2).

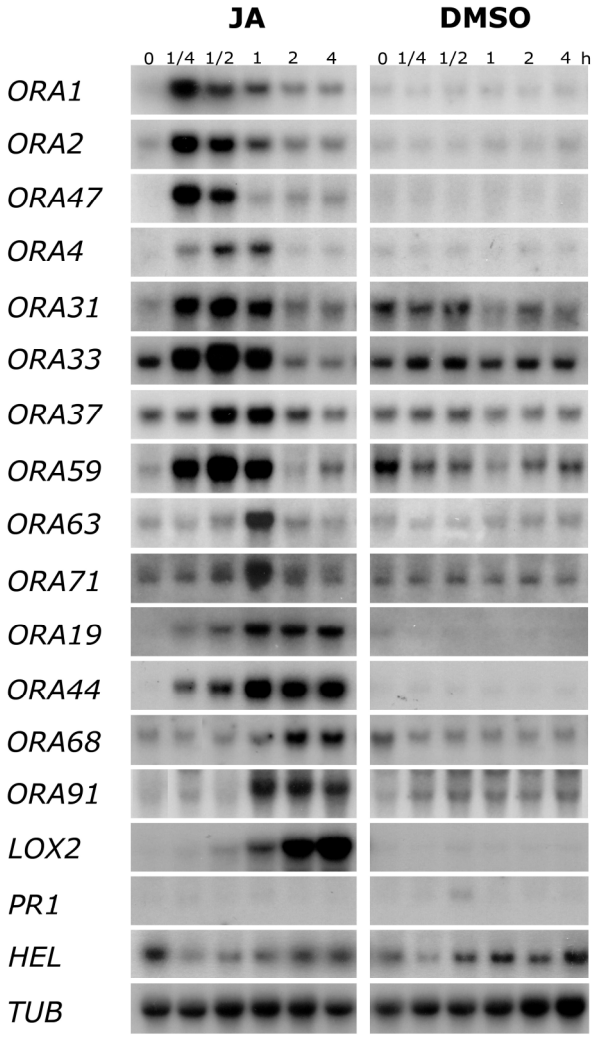


Figure 1. Kinetics of *ORA* transcription factor gene expression in response to JA. RNA gel blot analysis of *ORA* gene expression in 10 days old Arabidopsis seedlings grown in liquid culture after treatment with 50 μM JA or the solvent DMSO at 0.1% final concentration for the number of hours indicated. The complete ORFs of the *ORA* genes were used as probes. *LOX2*, *PR1*, *HEL* and *TUB* probes were used to verify specificity of the treatment and RNA loading respectively.

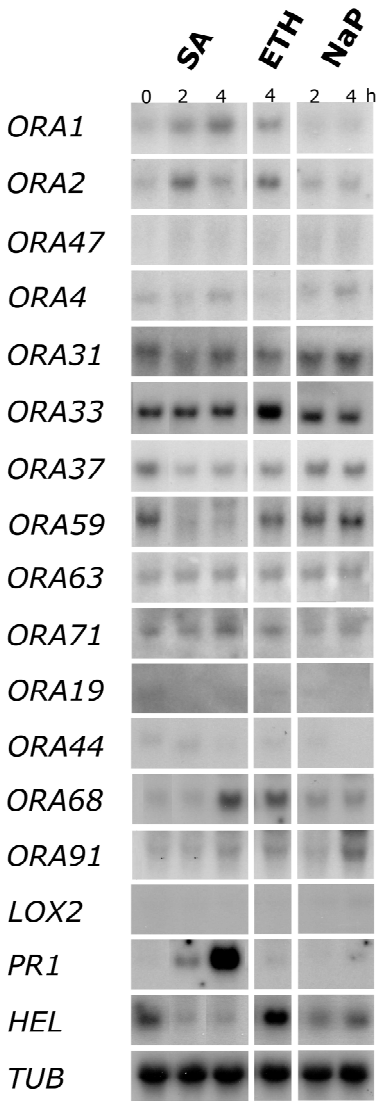


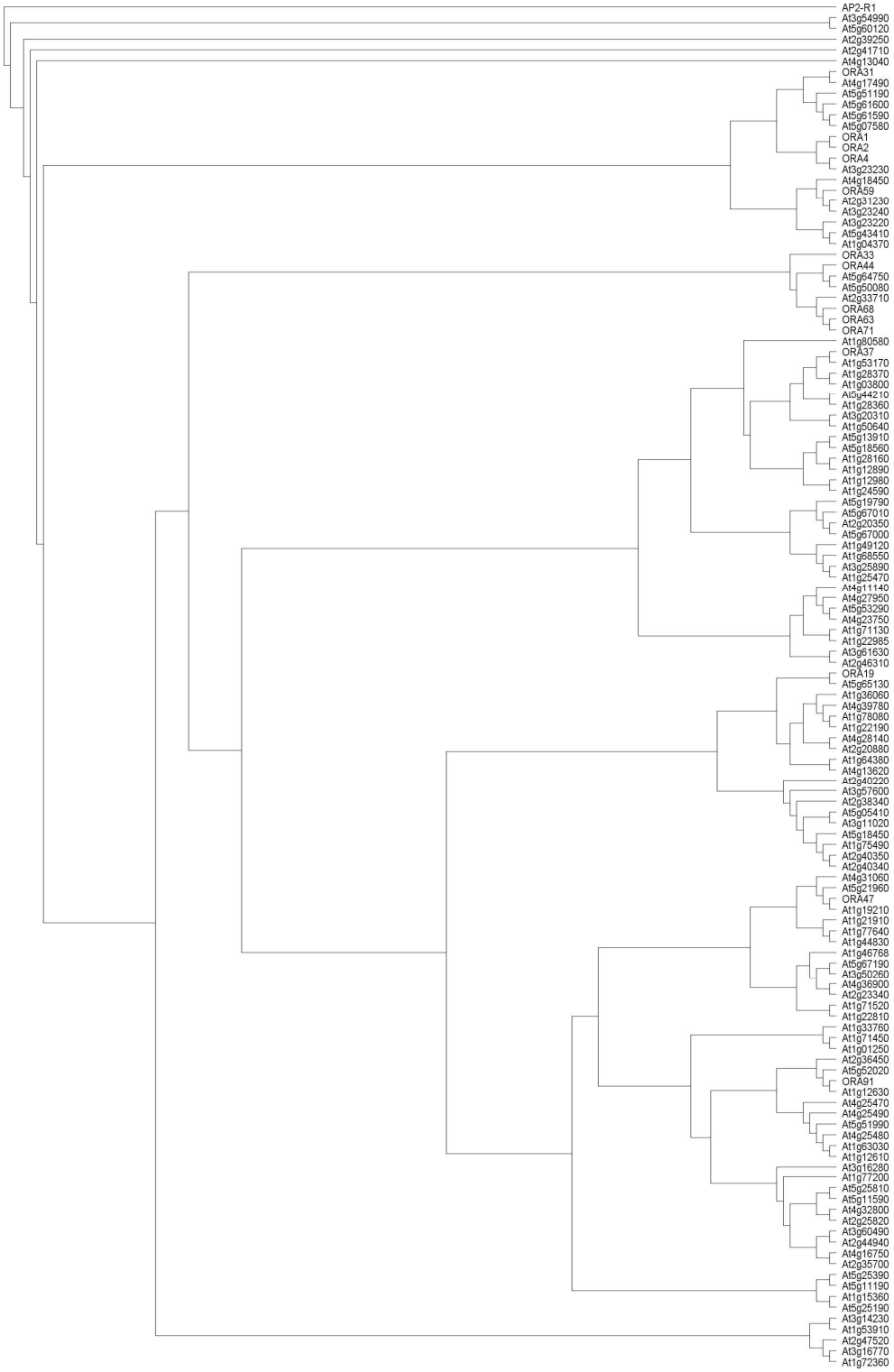
Figure 2. Expression of *ORA* transcription factor genes in response to SA and ethephon. RNA gel blot analysis of *ORA* gene expression in 10 days old Arabidopsis seedlings grown in liquid culture after treatment with 1 mM SA, 1 mM ethephon or the solvent Na-phosphate for the number of hours indicated. The complete ORFs of the *ORA* genes were used as probes. *LOX2*, *PR1*, *HEL* and *TUB* probes were used to verify specificity of the treatments and RNA loading respectively. Panels in Figs. 1 and 2 were hybridised with each probe on the same blots and were exposed for the same times, allowing direct comparison of expression levels between treatments.

Phylogenetic classification of the *ORA* proteins

The conserved AP2 domains from all the members of the subfamily with a single DNA-binding domain including the ORA proteins were used to construct a phylogenetic tree. The amino acid sequences of the AP2 domains were aligned in the CLUSTALW program for multiple alignments, and used to construct the phylogenetic tree (Fig. 3). The tree was rooted to the N-terminal DNA-binding domain of APETALA2 (At4g36920). A bootstrap analysis with 1000 replicates was

conducted. A bootstrap value equal to or higher than 70% is considered as statistically significant. As noted before, the At4g13040 domain falls apart from the other AP2 domains (Sakuma *et al*., 2002), and the AP2 domains of At2g39250, At2g41710, At3g54990 and At5g60120 are also distantly related to the others, and are more related to the APETALA2-like group with 2 domains (Sakuma *et al*., 2002; Alonso *et al*., 2003). Although the other domains are highly conserved, the tree has multiple branches with little statistically significant clustering. The ORA AP2 domains are scattered over the tree in different subgroups. A few ORA AP2 domains are clustered. The AP2 domains of ORA1 and ORA2 are highly related, and the corresponding genes have similar expression kinetics (Fig. 1). Clustered in the same group with a non-significant bootstrap value is ORA4. ORA59 clusters with ERF1 (At3g23240), and the corresponding genes have similar expression kinetics (Figs. 1 and 7; Lorenzo *et al*., 2003). Although the *ERF1* gene was not induced by JA under our experimental conditions, we found similar expression patterns for *ORA59* and *ERF1* in response to ethephon, and to a combination of ethephon and JA (Fig.7 and data not shown). The AP2 domains of ORA63 and ORA71 clustered closely together, and the corresponding genes had similar expression kinetics in response to JA. In the same cluster is ORA68. The ORA37 domain clustered together with 7 other AP2 domains. All the corresponding proteins contain a C-terminal LxLxLx repression domain, also called ERF-associated amphiphilic repression (EAR) domain (Ohta *et al.*, 2001). These are the only AP2-domain family members containing this repression domain. The fact that the LxLxLx-domain-containing proteins are grouped based on their AP2-domain, indicates that there are stringent functional constraints determining co-evolution of these two domains.

Apart from the clustered proteins mentioned, there was no link between the phylogenetic relationship between ORAs and the expression kinetics of the corresponding genes. Another tree was constructed using the full-length protein sequences (data not shown). This tree did not lead to a different clustering of the ORA proteins, indicating that there is no sequence relationship outside the conserved AP2 domain.


AP2-R1
At3g54990
At5g60120
At2g39250
At2g41710
At4g13040
ORA31
At4g17490
At5g51190
At5g61600
At5g61590
At5g07580
ORA1
ORA2
ORA4
At3g23230
At4g18450
ORA59
At2g31230
At3g23240
At3g23220
At5g43410
At1g04370
ORA33
ORA44
At5g64750
At5g50080
At2g33710
ORA68
ORA63
ORA71
At1g80580
ORA37
At1g53170
At1g28370
At1g03800
At5g44210
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At1g71130
At1g22985
At3g61630
At2g46310
ORA19
At5g65130
At1g36060
At4g39780
At1g78080
At1g22190
At4g28140
At2g20880
At1g64380
At4g13620
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At3g11020
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At1g75490
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At4g31060
At5g21960
ORA47
At1g19210
At1g21910
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At3g50260
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At2g23340
At1g71520
At1g22810
At1g33760
At1g71450
At1g01250
At2g36450
At5g52020
ORA91
At1g12630
At4g25470
At4g25490
At5g51990
At4g25480
At1g63030
At1g12610
At3g16280
At1g77200
At5g25810
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At4g32800
At2g25820
At3g60490
At2g44940
At4g16750
At2g35700
At5g25390
At5g11190
At1g15360
At5g25190
At3g14230
At1g53910
At2g47520
At3g16770
At1g72360

Figure 3. Neighbour-joining phylogenetic tree of the single AP2–domain protein family. The tree was created by the bootstrap option of the CLUSTAL X multiple alignment package and the Neighbour-joining method using the AP2 domain sequences. The lengths of the branches are proportional to the evolutionary distances between the sequences. The tree, rooted to APETALA2 protein At4g36920, contains 126 Arabidopsis AP2 domains.

ORA expression patterns in Arabidopsis mutants

In order to determine how JA controls *ORA* gene expression, we analysed the induction of *ORA* gene expression by JA in mutants affected in JA, SA and ethylene responses.

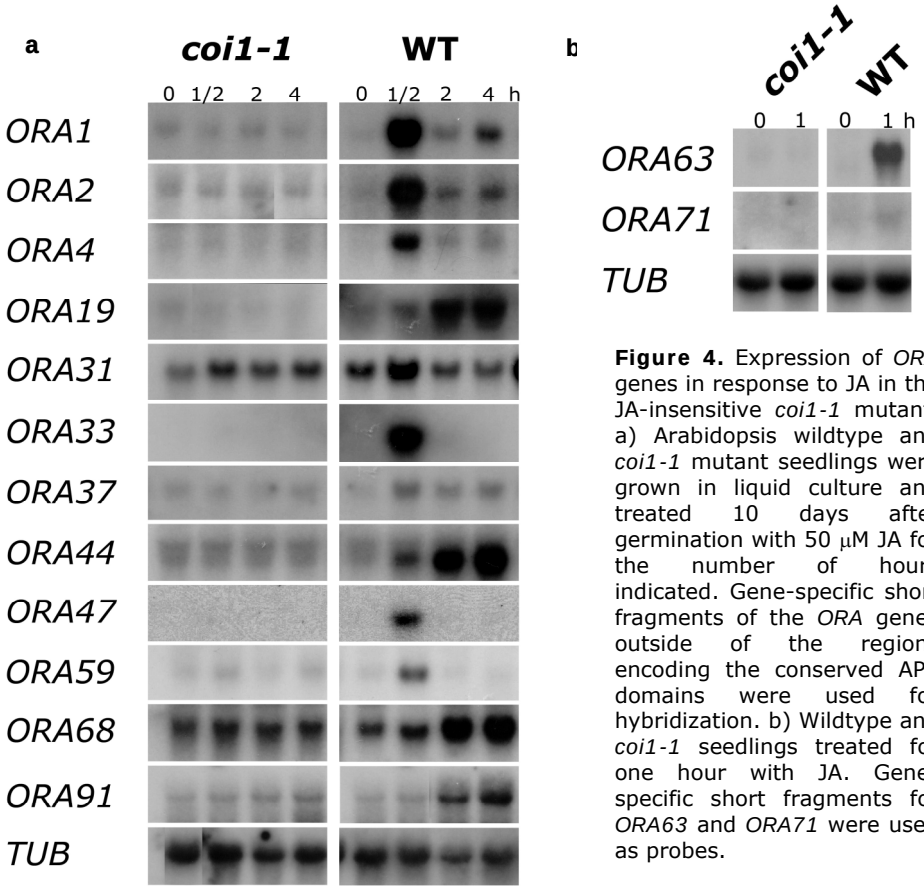

Figure 4. Expression of *ORA* genes in response to JA in the JA-insensitive *coi1-1* mutant. a) Arabidopsis wildtype and *coi1-1* mutant seedlings were grown in liquid culture and treated 10 days after germination with 50 μM JA for the number of hours indicated. Gene-specific short fragments of the *ORA* genes outside of the regions encoding the conserved AP2 domains were used for hybridization. b) Wildtype and *coi1-1* seedlings treated for one hour with JA. Gene-specific short fragments for *ORA63* and *ORA71* were used as probes.

The *coi1-1* (coronatin-insensitive) mutant is JA-insensitive and is unable to express the defense-related genes *THI2.1*, *PDF1.2*, and *VSP1* in response to JA

(Feys *et al.*, 1994). The induction of the *ORA* genes by JA was completely inhibited by the *coi1-1* mutation (Fig. 4).

We also studied the response of the *ORA* genes to JA in plants with a mutation in the *NPR1* gene. The *npr1-1* mutant (nonexpressor of PR genes) is impaired in the expression of *PR* genes in response to SA and in development of systemic acquired resistance in response to pathogen attack or application of SA (Cao *et al.*, 1994). The *NPR1* gene is also required for rhizobacteria-mediated induced systemic resistance (ISR), which is also dependent on an intact JA signal transduction pathway (Glazebrook, 2001; Pieterse and van Loon, 1999). The *npr1-1* mutation had no effect on *ORA* gene expression after JA treatment, demonstrating that the induction of *ORA* genes by JA is not dependent on nor inhibited by NPR1 (Fig. 5a, b). A notable exception was the *ORA91* gene, which showed reduced induction by JA in the *npr1-1* mutant. To further substantiate that JA-responsive expression of the majority of the *ORA* genes is independent of SA, transgenic NahG plants were used, which are unable to accumulate SA because of the expression of a SA-metabolizing bacterial salicylate hydroxylase enzyme (Delaney *et al.*, 1994) (Fig. 5a, b). The *NahG* transgene did not affect the expression of the *ORA* genes including *ORA91* in response to JA, which corroborates the notion that SA signal transduction is not required for the induction of *ORA* gene expression by JA. Analysis of the *TUB* mRNA level showed the equal loading of total RNA.

The expression of certain defense-related genes, including *PDF1.2*, *HEL*, and *CHIB*, upon pathogen attack requires the concomitant activation of both the JA and ethylene signaling pathways (Penninckx *et al.*, 1998). Therefore the expression of the *ORA* genes in response to JA was analysed in the ethylene-insensitive mutants *etr1-1* and *ein2-1*. The effect of the mutations on the expression of the *ORA* genes in response to JA in these mutants divided the *ORA* genes in two groups. One group consisting of *ORA1*, *2*, *4*, *19*, *33*, *47*, *63*, *71* and *91* showed a wild-type responsiveness to JA (Fig. 6a, b), while the second group consisting of *ORA31*, *37*, *44*, *59* and *68* showed a reduced response to JA (Fig. 6a, b). Equal amounts of RNA were loaded on the gel as shown by the *TUB* mRNA level.

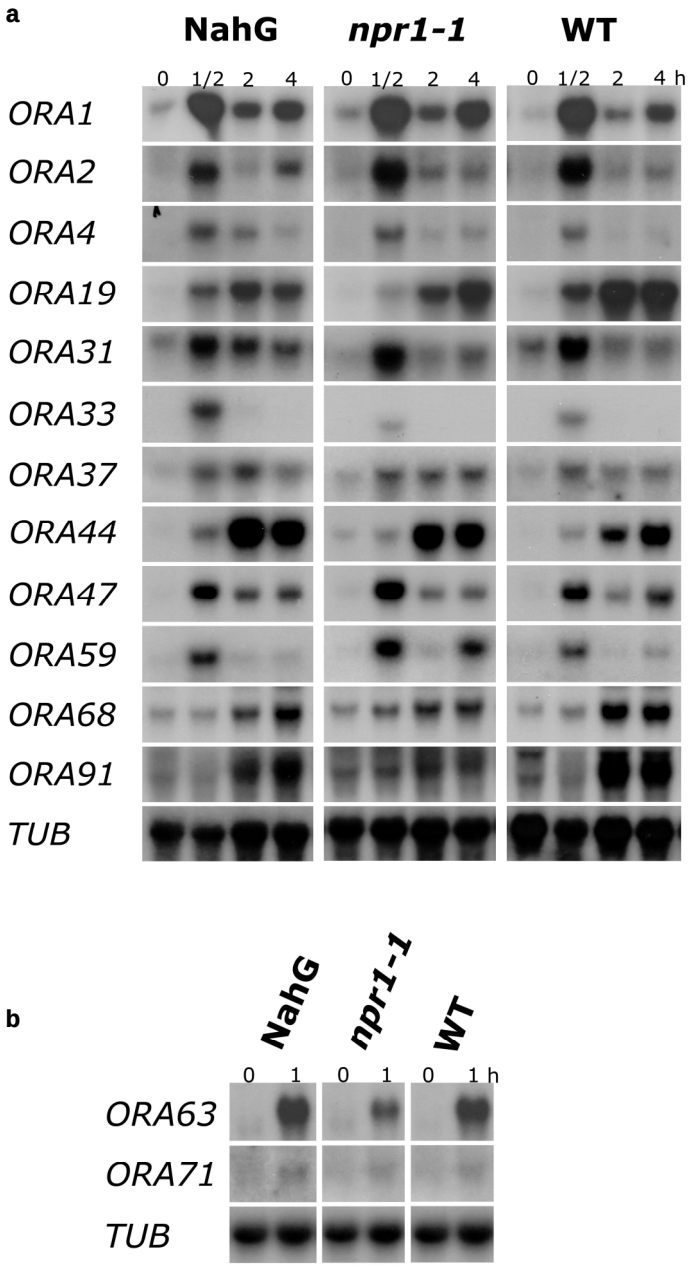

Figure 5. Expression of *ORA* genes in response to JA in the SA-insensitive *npr1-1* mutant and transgenic NahG seedlings. a) Arabidopsis wildtype, *npr1-1* mutant and NahG transgenic seedlings were grown in liquid culture and treated 10 days after germination with 50 μM JA for the number of hours indicated. Gene-specific short fragments of the *ORA* genes outside of the regions encoding the conserved AP2 domains were used for hybridization. b) Seedlings as in (a) were treated for one hour with JA. Gene-specific short fragments for *ORA63* and *ORA71* were used as probes.

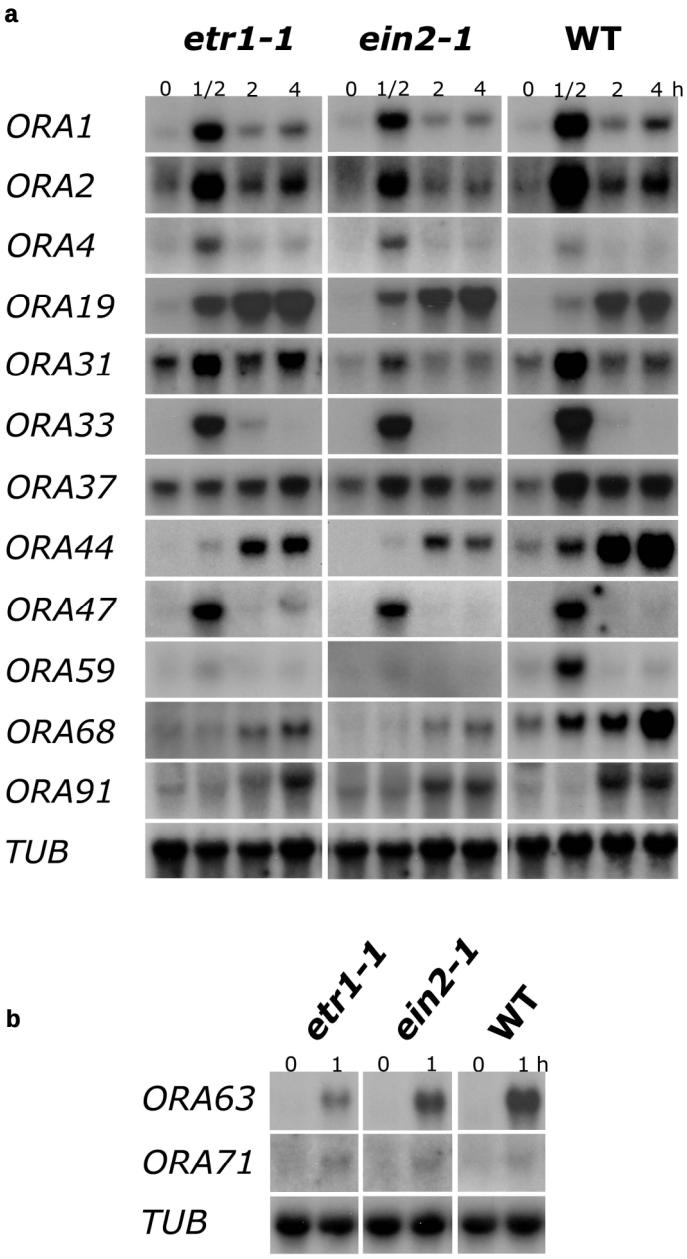

Figure 6. Expression of *ORA* genes in response to JA in the ethylene-insensitive *ein2-1* and *etr1-1* mutants. a) Arabidopsis wildtype, *etr1-1* and *ein2-1* mutants seedlings were grown in liquid culture and treated 10 days after germination with 50 μM JA for the number of hours indicated. Gene-specific short fragments of the *ORA* genes outside of the regions encoding the conserved AP2 domains were used for hybridization. b) Seedlings as in (a) were treated for one hour with JA. Gene-specific short fragments for *ORA63* and *ORA71* were used as probes.

***ORA* expression patterns in response to a combination of JA and ethylene**

Several studies showed positive interactions between the JA and ethylene signaling pathways on defense responses (Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003). Therefore, seedlings were treated with a combination of JA and ethylene in order to test the effect on *ORA* gene expression. Northern blot analysis showed that the combined treatment led to a long lasting super-induction of *ORA31*, *37*, *44*, *59* and *68* gene expression. The combined treatment induced these *ORA* genes at the same early time point as the JA treatment, but the maximum expression level was higher and the expression was more prolonged (Fig. 7). Interestingly, this same *ORA* gene subset also showed reduced JA-responsive expression in Arabidopsis mutants impaired in ethylene signaling (Fig. 6a). The other *ORA* genes showed similar levels and kinetics of expression following combined treatment and treatment with JA alone (Fig. 7). In accordance, the expression of these *ORA* genes was not affected by mutations in ethylene signal transduction components (Fig. 6). A notable exception was the *ORA19* gene, which showed reduced expression in response to the combination of hormones in comparison to the treatment with JA alone (Fig. 7). Separate treatments with JA or ethephon resulted in weak induction of the expression of *PDF1.2*, *CHIB*, and *HEL*. All three genes showed superinduction of the expression level in response to the combined treatment. In contrast, the expression of the JA-responsive genes *LOX2* and *VSP1* genes were strongly induced by JA alone, showed no response to ethephon, and their JA-responsive expression was dramatically decreased in the presence of ethephon (Fig. 7).

Discussion

We have previously shown that JA-responsive gene expression in *Catharanthus roseus* is mediated via ORCA transcription factors (Menke *et al.*, 1999; van der Fits and Memelink, 2000), which belong to the class of AP2-domain transcription factors. The expression of the *ORCA* genes themselves is JA-responsive. Based on these observations, we speculated that JA-responsive gene expression in *Arabidopsis* is also mediated by members of the AP2-domain transcription factor family, and that the corresponding genes are also expressed in a JA-responsive manner. The goals of this study were to find JA-responsive members (encoding so-called ORA transcription factors) of the single AP2-domain

transcription factor gene subfamily in Arabidopsis, to establish whether these members respond specifically to JA, and to determine whether they form part of the established JA signal transduction pathway involving COI1. Here we demonstrate that JA induces the expression of fourteen Arabidopsis genes encoding AP2-domain transcription factors in a COI1-dependent manner. We found differences in the kinetics of *ORA* gene expression in response to JA. Some *ORA* genes showed a faster and/or more transient induction than others.

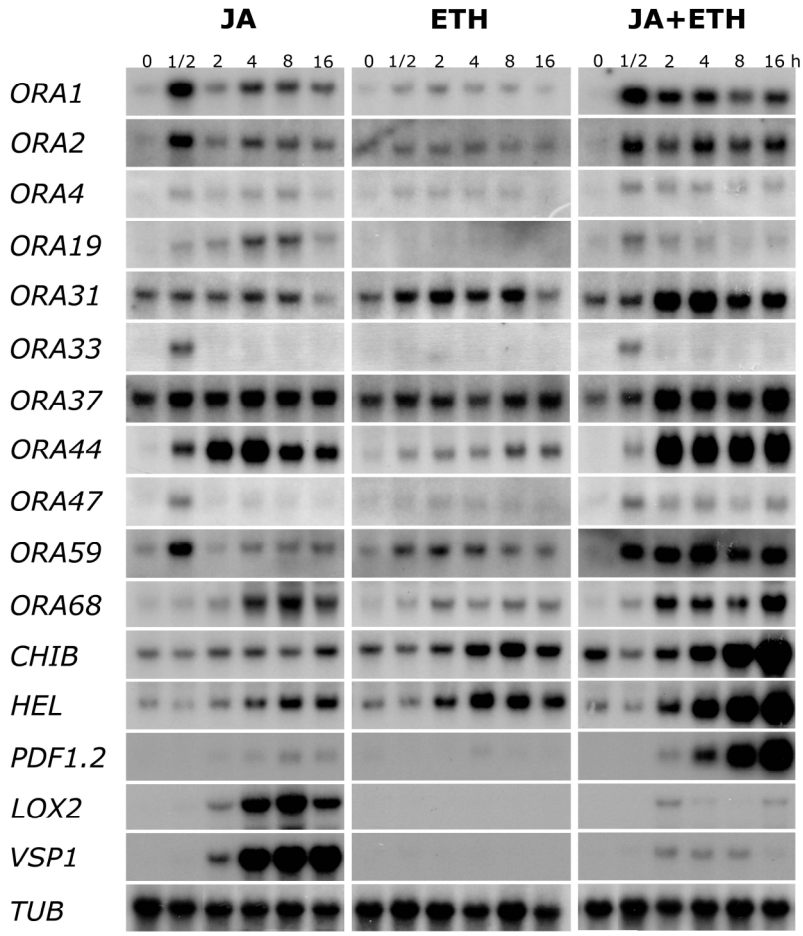

Figure 7. Expression of *ORA* genes in response to a combined treatment with JA and ethephon. Ten days old wild type Arabidopsis seedlings grown in liquid culture were treated with 50 μM JA or 1 mM ethephon or with both for the number of hours indicated. For the *ORA* genes gene-specific probes were used.

Our screening strategy based on Northern blot analysis using multiple time points of treatment probably detected most or all AP2-like genes expressed within 4 hours in response to JA in 10-days old Arabidopsis seedlings grown in liquid culture.

Our screen would miss JA-responsive AP2-like genes expressed only at later time points in seedlings, or expressed only in specific tissues present at low abundance or absent in seedlings, or expressed only at later stages of development. Moreover, AP2-domain transcription factor genes that are responsive only to a combination of JA and another signaling molecule would not be identified in our screen. For example, the gene encoding the AP2-domain transcription factor ERF1, which was reported to be responsive to a combination of JA and ethylene (Lorenzo *et al.*, 2003), was not identified in our screen as a JA-responsive gene. And finally, our strategy of screening for increased mRNA levels will miss all genes, which encode AP2-domain transcription factors involved in JA-responsive gene expression that are post-translationally regulated by signal transduction pathways initiated by JA.

Six of the *ORA* genes were previously functionally characterized. These encode ORA1, 2, 4, 31, 37 and 44, which were previously called AtERF1, AtERF2, AtERF13, AtERF5, AtERF4 and Rap2.6 respectively (Fujimoto *et al.*, 2000; Onate-Sanchez and Singh, 2002; Chen *et al.*, 2002). These *ORA* genes were shown to be induced by ethylene, wounding, pathogens and virus attack. However, their response to JA occurs much more quickly than to the previously studied signals, which had response times of 3 hours or more (Fujimoto *et al.*, 2000; Onate-Sanchez and Singh, 2002; Chen *et al.*, 2002).

The AP2 domains of the fourteen ORA proteins did not cluster together in the phylogenetic tree. Close relationships were only observed between ORA1, 2 and 4 and between ORA63, 68 and 71 suggesting that these genes have most likely arisen from recent genomic duplication events. Therefore these genes can be functionally redundant, which can be studied by generating single, double and triple mutant plants. The expression kinetics of the different *ORA* genes did not correlate with the sequence relationship of the encoded proteins. This raises the question, what the functional importance is of the difference in their expression kinetics. It is possible that late *ORA* genes may function later in the induction of JA-responsive genes, or different ORA groups regulate different JA-responsive

target gene sets. Another possibility could be that ORAs encoded by early response genes regulate the expression of intermediately and late responding *ORA* genes.

The expression of the *ORA* genes was not induced by JA treatment in the JA-insensitive *coi1-1* mutant, demonstrating that JA-responsive expression of the *ORA* genes is dependent on a functional COI1 protein. The induction of *ORA* genes by JA in *npr1-1* and NahG plants impaired in SA signaling was normal except for *ORA91*, demonstrating that SA and NPR1 are not necessary for JA-responsive expression of the majority of the *ORA* genes.

However, several *ORA* genes showed a different response to JA in plants impaired in the ethylene signaling pathway. Nine *ORA* genes showed a wild-type response to JA in ethylene-insensitive mutant plants, while the five other genes, *ORA31*, *37*, *44*, *59* and *68*, showed a reduction or total inhibition of the JA response. The expression of the latter 5 *ORA* genes was superinduced by a combined treatment with JA and ethylene. Super-induction of gene expression was also shown for some defense-related genes including *PDF1.2*, *CHIB* and *ERF1* (Penninckx *et al.*, 1998; Lorenzo *et al*., 2003). Together these results suggest that crosstalk between the JA and ethylene signaling pathways occurs at the level of multiple AP2-domain transcription factors. Our results suggest that ORA31, 37, 44, 59 and 68 integrate inputs from the JA and ethylene signaling pathways, in addition to the previously identified ERF1 transcription factor (Lorenzo *et al.*, 2003).

The results from this work suggest that ORAs might be connecting JA synthesis and signaling initiated by pathogen attack and wounding to defense gene expression. This raises the questions which target genes are regulated by the ORA transcription factors, and whether each ORA regulates a distinct target gene set or whether there is functional overlap. Based on the similar superinducing effects of JA and ethylene on the expression of the *ORA31*, *37*, *44*, *59* and *68* genes and the defense genes *PDF1.2*, *HEL* and *CHIB*, one could speculate that ORA31, 37, 44, 59 and 68 regulate this set of defense genes. If so, they would have overlapping functions with ERF1 (Lorenzo *et al.*, 2003). The JA-responsive expression of *ORA19* was negatively affected by ethephon in a similar manner as the expression of *VSP1* and *LOX2*, suggesting that the latter genes are target genes of ORA19.

Our findings are summarized in the model in Fig. 8. The 14 *ORA* genes can be divided in two groups based on the dependence of their JA responsiveness on components of the JA and ethylene signal transduction pathways. One group was dependent only on COI1 and induced uniquely by JA and the other group was dependent on COI1, ETR1, and EIN2 and induced synergistically by JA and ethylene. Proteins encoded by members of each of these two groups might be regulating similar or different sets of target genes. The first group dependent only on COI1 might encode proteins regulating JA-specific response genes, the second group might encode proteins regulating genes that respond to a combination of JA and ethylene. It cannot be excluded that proteins encoded by members of both groups might be regulating common target genes.

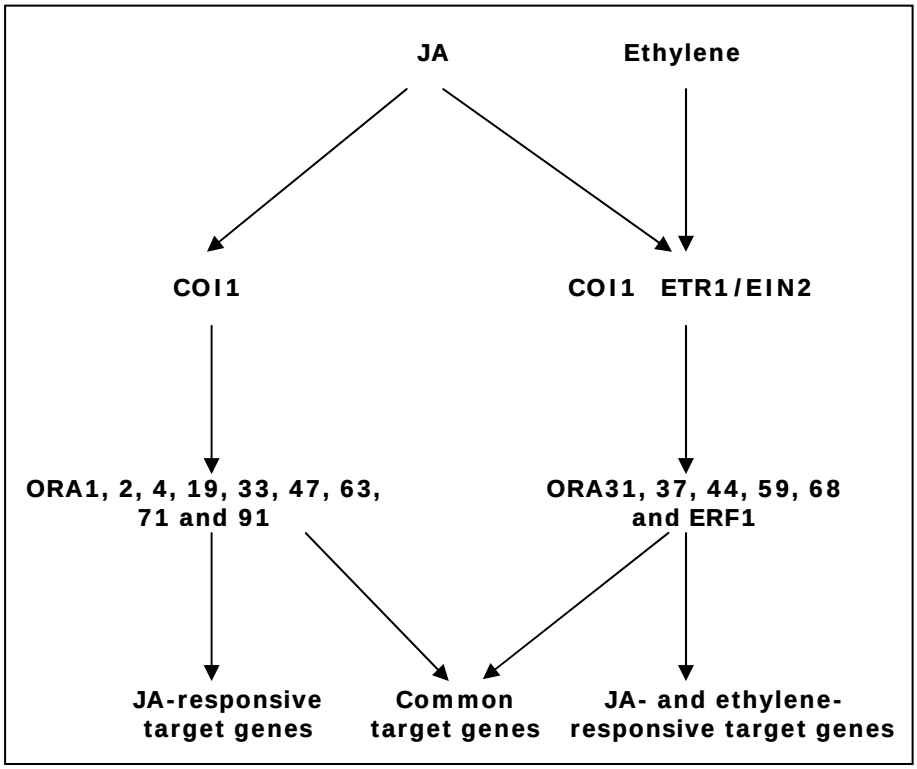

Figure 8. Model for the regulation of defense gene expression by the ORA transcription factors. *ORA* genes belong to one of two groups. One group is COI1-dependent and induced uniquely by JA, and the other group is COI1-, ETR1- and EIN2-dependent and induced synergistically by JA and ethylene. Proteins encoded by members of each group may regulate separate target genes or common ones.

Future challenging work remains in unravelling the specific roles of ORAs via identification of target genes and their roles in crosstalk between different signaling pathways.

Acknowledgments

We thank Ward de Winter for excellent technical assistance. Martial Pré was supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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Supplemental data

Table 1. List of the 126 AP2-like genes from *Arabidopsis thaliana* containing one DNA-binding domain. Important features of the expression patterns observed in wild type seedlings of ecotype Col-0 after treatment with 50 μM JA, 1 mM SA or 1 mM ethephon are listed. The column expression indicates whether mRNA levels were detectable at the growth conditions and hormone treatments used. The start time of induction (I) or repression (R) by each of the hormones is indicated. AP2 proteins possessing the EAR repression motif are marked with an asterisk in the AGI code column.

Nb	ORA	AGI code	Protein description	Expression	JA	SA	ETH
1	ORA1	At4g17500	ATERF1 (ethylene responsive element binding factor 1)	+	I>15 min	I>4 h	I>30 min
2	ORA2	At5g47220	ATERF2 (ethylene responsive element binding factor 2)	+	I>15 min	I>4 h	I>30 min
3	ORA4	At2g44840	ATERF13 (ethylene responsive element binding factor 13)	+	I>15 min		
4		At1g12980	hypothetical protein	+			
5		At4g11140	putative AP2 domain protein	+			
6		At2g46310	putative AP2 domain transcription factor	+		I>4 h	
7		At2g47520	putative AP2 domain transcription factor	+			
8		At4g39780	putative protein	+		R>1 h	
9		At3g11020	DREB2B transcription factor	Background		I> 16 h	
10		At5g05410	DREB2A transcription factor	+		I>1 h	
11		At4g25490	DREB1B/CBF1	Background			
12		At4g25481	DREB1A/CBF3	+			
13		At3g16770	AP2 domain containing protein RAP2.3/AtEBP	+	R>1 h		I>2 h
14		At2g40220	ABI4:abscisic acid-insensitive 4	Background			
15	ORA19	At2g22200	AP2 domain transcription factor	+	I>30 min		

16		At2g20350	putative AP2 domain transcription factor	+		I>2 h	
17		At1g28370*	ATERF11 (ethylene responsive element binding factor 11)	+			I>2 h
18		At1g28360	ATERF12 (ethylene responsive element binding factor 12)	+			
19		At1g68550	putative AP2 domain transcription factor	+			I>30 min
20		At1g53910	putative AP2 domain transcription factor	+		R>2 h	
21		At2g33710	putative AP2 domain transcription factor	+		I>2 h	
22		At1g71450	putative TINY	+			
23		At1g36060	putative AP2 domain containing protein RAP2.4	+	R>1 h	R>1 h	I>4 h
24		At3g23240	ERF1 (ethylene responsive factor 1)	+			I>2 h
25	ORA31	At5g47230	ATERF5 (ethylene responsive element binding factor 5)	+	I>15 min		I>8 h
26		At4g17490	ATERF6 (ethylene responsive element binding factor 6)	Background			
27	ORA33	At4g34410	putative protein	+	I>15 min		I>4 h
28		At2g31230	ATERF15 (ethylene responsive element binding factor 15)	+			
29		At1g04370	ATERF14 (ethylene responsive element binding factor 14)	-			

30	ORA37	At3g15210*	ATERF4 (ethylene responsive element binding factor 4)	+	I>30 min	R>2 h	
31		At1g03800	ATERF10 (ethylene responsive element binding factor 10)	+			
32		At3g61630	putative protein	-			
33		At1g78080	putative AP2 domain containing protein RAP2.4	+			
34		At1g50640*	ATERF3 (ethylene responsive element binding factor 3)	+		I>2 h	
35		At1g72360	putative AP2 domain transcription factor	+			I>16 h
36		At4g18450	EREBP-like protein	Background			
37	ORA44	At1g43160	AP2 domain protein RAP2.6	+	I>15 min	I>8 h	
38		At4g36900	TINY-like protein	+			
39	ORA47	At1g74930	putative AP2 domain transcription factor	+	I>15 min		
40		At4g27950	putative protein	+			
41		At2g20880	AP2 domain transcription factor	+		I>2 h	
42		At5g25810	transcription factor TINY	Background			
43		At1g80580	unknown protein	+			
44		At3g25890	unknown protein	+			
45		At3g23230	ethylene responsive element binding protein, putative	Background			
46		At5g53290	putative protein	Background			
47		At5g61600	DNA binding protein-like	+			
48		At5g61590	ethylene responsive element binding factor-like	+			

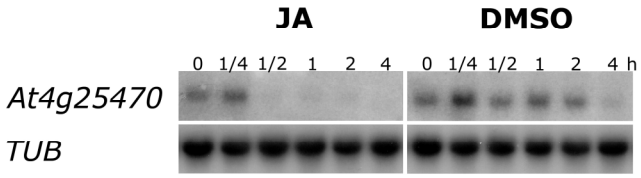
49		At5g13910	AP2/EREBP-like transcription factor LEAFY PETIOLE	+			
50	ORA59	At1g06160	ethylene responsive factor, putative	+	I>15 min	R>2 h	I>8 h
51		At1g71130	hypothetical protein	-			
52		At5g07580	transcription factor-like protein	+			
53		At5g51190	putative protein	Background			
54	ORA63	At5g61890	putative protein	+	I>1 h		
55		At5g43410	Nicotiana EREBP-3 like	+			
56		At2g39250	putative AP2 domain transcription factor	+		I>4 h	I>8 h
57		At4g23750	putative AP2 domain protein	+			
58	ORA68	At5g13330	putative protein	+	I>2 h	I>4 h	I>8 h
59		At1g64380	AP2-containing DNA-binding protein	+			
60		At3g23220	ehtylene responsive element binding protein, putative	+			
61	ORA71	At5g07310	putative transcription factor	+	I>1 h		
62		At3g14230	DNA-binding protein	+			I>16 h
63		At5g50080	putative protein	+			
64		At3g20310*	ATERF7 (ethylene responsive element binding factor 7)	+			
65		At5g44210	DNA binding protein EREBP-3-like protein	+			
66		At5g65130	putative protein	+			
67		At5g64750	putative protein	+			
68		At1g22190	hypothetical protein	+			
69		At4g28140*	ATERF9 (ethylene responsive element binding factor 9)	+			
70		At1g24590	hypothetical protein	+			

71		At1g53170*	ATERF8 (ethylene responsive element binding factor 8)	+			
72		At1g33760	TINY-like porotein	+			
73		At5g18560	AP2 domain -like protein	+			
74		At1g28160	AP2domain transcription factor, putative	+			
75		At2g23340	putative AP2 domain transcription factor	+			
76		At5g67190	TINY-like protein	Background			
77		At1g01250	transcription factor TINY, putative	+			
78		At1g77200	hypothetical protein	+			
79		At3g50260	putative protein	+			
80		At1g12610	hypothetical protein	+			
81	ORA91	At1g12630	hypothetical protein	+	I>1 h		I>8 h
82		At1g12890	hypothetical protein	-			
83		At1g15360	putative ethylene responsive element	+			
84		At1g19210	hypothetical protein	+			
85		At1g21910	TINY-like protein	+	R>15 min		I>2 h
86		At1g22810	TINY-like transcription factor	+			
87		At1g25470	hypothetical protein	-			
88		At1g44830	transcription factor, putative	+			
89		At1g49120	similar to AP2 domain containing protein RAP2.2	+			
90		At1g63030	transcription factor DREB1A, putative	+			
91		At1g63040	transcription factor DREB1A, putative	+			
92		At1g71520	hypothetical protein	+		I>1 h	

93		At1g75490	transcription factor DREB2A, putative	+			
94		At1g77640	hypothetical protein	+	R>30 min	I>2 h	
95		At2g25820	TINY-like AP2 domain transcription factor	+		I>8 h	
96		At2g35700	putative AP2 domain transcription factor	+			
97		At2g36450	putative AP2 domain transcription factor	+			
98		At2g38340	DREB-like AP2 domain transcription factor	+			
99		At2g40340	AP2 domain transcription factor	+		I>2 h	I> 4h
100		At2g40350	AP2 domain transcription factor	+		I>4 h	
101		At2g41710	putative AP2 domain transcription factor	+			
102		At2g44940	putative AP2 domain transcription factor	+			
103		At3g16280	putative AP2 domain transcription factor	+			
104		At3g54990	APETALA2-like protein	+			
105		At3g57600	AP2 transcription factor-like protein	+			
106		At3g60490	transcription factor-like protein	+			
107		At4g13040	hypothetical protein	+			
108		At4g13620	putative protein	+			
109		At4g16750	apetala2 domain TINY like protein	+			
110		At4g25470	DREB1C/CBF2	+	R>30 min		
111		At4g31060	putative protein	-			

112		At4g32800	transcription factor TINY homolog	+		R>4 h	
113		At5g11190	putative protein	+			
114		At5g11590	transcription factor like protein	+			
115		At5g18450	AP2 domain DNA-binding protein-like	-			
116		At5g19790	AP2 domain containing protein RAP2.11	+			
117		At5g21960	putative protein	+			
118		At5g25190	ethylene-responsive element-like protein	+			
119		At5g25390	AP2 domain containing protein	+			
120		At5g51990	AP2 domain transcription factor-like protein	+			
121		At5g52020	putative protein	+		I>8 h	
122		At5g60120	APETALA2 protein-like	+			
123		At5g67000	AP2 domain transcription factor-like	+			
124		At5g67010	AP2 domain transcription factor-like	Background			
125		At1g46768	AP2 domain protein RAP2.1	+		I>8 h	I>8 h
126		At1g22985	putative AP2 domain transcription factor	+			

a



b

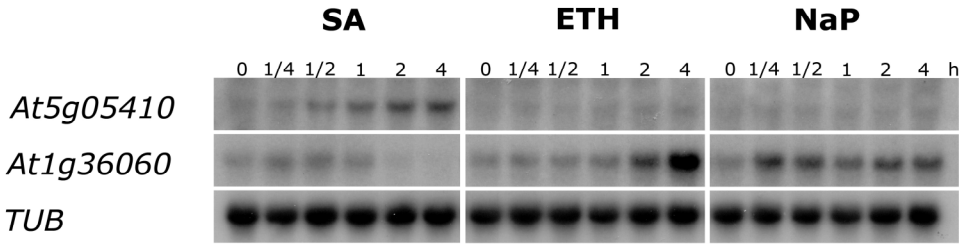


Figure 9. Examples of expression patterns of AP2-like genes in 10 days old Arabidopsis seedlings treated with 50 μM JA, 1 mM SA, or 1 mM ethephon for the number of hours indicated. a) Expression patterns of the JA-repressible At4g25470 gene after treatment with JA. b) Expression patterns of the SA-inducible At5g05410 gene and the SA-repressible and ethephon-inducible At1g36060 gene after treatment with SA or ethephon. *TUB* probe was used to verify the RNA loading.

Chapter 3

Constitutive expression of three JA-responsive AP2-domain transcription factor genes in Arabidopsis increases the expression of defense-related genes

Mirna Atallah, Maarten F. Sleutelberg, Antony Champion and Johan Memelink

Abstract

Jasmonic acid (JA) is an important plant hormone involved in defense responses. JA perception leads to the activation of a specific set of defense genes. In *Catharanthus roseus*, two AP2-domain transcription factors called ORCA2 and ORCA3 regulate the JA-responsive expression of the alkaloid biosynthetic gene *strictosidine synthase* (*STR*) via binding to a JA- and elicitor-responsive promoter element (JERE). In Arabidopsis, gene expression levels of 14 members called ORAs of the AP2-domain transcription factor family are increased by JA, suggesting that these ORA proteins regulate JA-responsive defense gene expression. Here we show that ORA1, ORA2 and ORA4 are transcriptional activators, which bind in a sequence-specific manner to the JERE. Overexpression of ORA1, ORA2 and ORA4 in transgenic Arabidopsis plants results in the activation of defense-related genes such as *PDF1.2*, *HEL*, *CHIB*, and *ADC2* and thus indicates the involvement of these ORAs in transcriptional regulation of the defense response.

Introduction

Jasmonic acid and its volatile derivative methyljasmonate (MeJA), collectively called jasmonates (JAs), are plant stress hormones that act as regulators of defense responses. 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 (Kramell *et al.*, 1995; Doares *et al.*, 1995; Parchmann *et al.*, 1997; Menke *et al.*, 1999; Penninckx *et al.*, 1996; Creelman and Mullet 1995). Compelling evidence for a key role of JA in plant defense comes from analysis of Arabidopsis mutants affected in JA biosynthesis or signaling, which have enhanced susceptibility to pests and pathogens (Staswick *et al.*, 1998; Vijayan *et al.*, 1998; Thomma *et al.*, 1998; Norman-Setterblad *et al.*, 2000).

Treatment of plants with JAs stimulates the expression of genes such as those encoding vegetative storage proteins (VSPs), thionin (THI2.1), hevein-like protein (HEL) and plant defensin (PDF1.2), which are also expressed in response to stress and pathogens (Turner *et al.*, 2002). Ethylene signaling is required in addition to JA signaling for pathogen-induced expression of *THI2.1*, *PDF1.2* and *HEL*, since expression of these genes is blocked in the ethylene-insensitive *ein2*

mutant (Norman-Setterblad *et al.*, 2000; Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003). It is largely unknown how the JA signal is transduced to affect gene expression.

In *Catharanthus roseus*, a JA- and elicitor-responsive element (JERE) was identified in the promoter of the terpenoid indole alkaloid (TIA) biosynthetic gene *strictosidine synthase* (*STR*) (Menke *et al.*, 1999). The JERE interacts with two JA-responsive transcription factors called ORCA2 and ORCA3. Significantly, *ORCA* gene expression was rapidly induced by MeJA, and ORCA proteins transactivate *STR* gene expression via specific binding to the JERE (Menke *et al.*, 1999; van der Fits and Memelink, 2001). Furthermore, overexpression of ORCA3 resulted in elevated expression levels of multiple JA-responsive genes, involved both in primary metabolism as well as in TIA metabolism (van der Fits and Memelink, 2000). These data demonstrate that specific AP2-domain proteins act as regulators of JA-responsive gene expression in Catharanthus.

In Arabidopsis, a number of AP2-domain transcription factors have been implicated in stress responses (Shinozaki and Yamaguchi-Shinozaki, 2000). The expression of the *CBF/DREB1B* genes is induced by cold stress (Xiong *et al.*, 2002). Ectopic overexpression of CBF1/DREB1B (Jaglo-Ottosen *et al.*, 1998), CBF3/DREB1A (Liu *et al.*, 1998) or CBF4 (Haake *et al.*, 2002) results in plants with enhanced expression of cold- and drought-inducible genes, thereby increasing freezing and drought tolerance. The *AtERF2* and *ERF1* genes are induced by ethylene and JA (Fujimoto *et al.*, 2000; Brown *et al.*, 2003; Solano *et al.*, 1998; Lorenzo *et al.*, 2003). Ectopic expression of AtERF2 (Brown *et al.*, 2003) or ERF1 (Lorenzo *et al.*, 2003) results in elevated expression levels of defense genes including *PDF1.2* and *CHIB*. The ERF1 plants were also shown to be more resistant to fungal infection (Berrocal-Lobo *et al.*, 2002).

In Arabidopsis, we identified 14 JA-responsive *ORA* genes encoding AP2-domain transcription factors (Chapter 2). *ORA* gene expression was rapidly induced by JA similarly to the *ORCA* genes in Catharanthus (Menke *et al.*, 1999; van der Fits and Memelink, 2001). Three of the ORA proteins (ORA1, 2 and 4) are more related to each other than to the other ORA proteins (Chapter 2), and they are also most related to the ORCA proteins. Therefore, we speculated that these ORA proteins have similar functions in regulating defense genes in response to JA. To test this assumption, the ORA proteins were constitutively expressed in

transgenic Arabidopsis plants, and the effect on the expression of JA-responsive defense genes was analyzed.

Materials and methods

Construction of *E.coli* expression plasmids

The *ORA1*, *ORA2*, and *ORA4* open reading frames were amplified by PCR from Arabidopsis genomic DNA using the following primer sets respectively: 5'-CGGGATCCATATGACGGCGGATTCTCAATC-3' and 5'-CGGGATCCTTATAAAACCAATA AACGATC-3'; 5'-CGGGATCCATATGTACGGACAGTGCAATATAG-3' and 5'-CGGGATC CTTATGAAACCAATAACTCATC -3'; 5'-GAAGATCTCATATGAGCTCATCTGATTCCG-3' and 5'-GAAGATCTTTATATCCGATTATCAGAATAAG -3'. *ORA1* and *ORA2* were cloned as BamHI fragments into pUC28 and pBluescript SK+ (Stratagene) respectively, whereas *ORA4* was cloned as a BglII fragment in pIC-20H (Marsh *et al*., 1984). The *ORA1* insert was excised from pUC28 with SmaI/HindIII and cloned in pGEX-KG (Guan and Dixon, 1991). The *ORA2* insert was excised from pBluescript SK+ with NdeI/BamHI and cloned into pUC28, and then cloned into pGEX4T-1 (Amersham Biosciences) as an EcoRI/SalI fragment. The *ORA4* insert was excised from pIC-20H with BglII and cloned into pGEX4T-1 digested with BamHI. The expression plasmids were introduced in *E.coli* strain BL21 (DE3) pLysS. Proteins were isolated by glutathione sepharose 4B affinity chromatography (Amersham Biosciences), and dialysed against EMSA binding buffer.

EMSA

Wildtype RV fragment from the *STR* promoter and mutant derivatives M2-M7 (Menke *et al*., 1999) were used as probes. DNA-binding reactions contained 0.1 ng of end-labelled DNA probe, 500 ng of poly(dAdT)-poly(dAdT), 1x binding buffer (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% glycerol), and protein extract in a 10 µl volume. Following addition of protein extract, reactions were incubated for 30 min at room temperature before loading on 5% acrylamide/bisacrylamide (37:1)-0.5x TBE gels under tension. After electrophoresis at 125 V for 1 h, gels were dried on Whatman DE81 paper and autoradiographed.

Plant transformation

The *ORA2* insert was excised from pBluescript SK+ with BamHI and cloned into pMOG183. The *ORA4* fragment was excised from pIC-20H with BglII and cloned into pMOG181 digested with BamHI. The pMOG vectors are pUC18 derivatives carrying a double-enhanced Cauliflower Mosaic Virus 35S promoter and the *nos* terminator separated by a BamHI site. The CaMV 35S cassette containing the *ORA* genes in sense orientation were excised with EcoRI/HindIII or SacI/HindIII from pMOG181 and pMOG183 respectively, and were introduced into the binary vector pCAMBIA1300 (accession number AF234296) containing the hygromycin resistance gene. *ORA1* was amplified by PCR on genomic DNA with the primer set 5'- GGGGTACCAAAATGTACCCATACGATGTTCCAGATTACGCTGGTTA CCCATACGATGTTCCAGATTACGCTGAGCTCATGACGGCGGATTCTCAATC–3' and 5'-C GGGATCCTTATAAAACCAATAAACGATC-3' and cloned in pGEM-T Easy (Promega). The resulting sequence encodes the ORA1 protein with a double haemagglutinin (HA) epitope tag at its N-terminal end. The *HA-ORA1* insert was excised with KpnI/BamHI and cloned into pRT101 (Töpfer *et al*., 1987). The CaMV 35S-cassette containing *HA-ORA1* was excised with *Hin*dIII and introduced into pCAMBIA1300. pCAMBIA1300-ORA constructs and the *GUS* gene-containing vector pCAMBIA1301 (accession number AF234297) were introduced into *Agrobacterium tumefaciens* by electroporation. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MA medium (Masson and Paswkowski, 1992) containing 20 mg/L hygromycin and 100 mg/L timentin.

Northern blot analysis

T2 seeds from Arabidopsis lines transformed with pCAMBIA1300-ORA or pCAMBIA1301 were surface-sterilized and grown as described in Chapter 2 in liquid MA medium containing 20 mg/L hygromycin for selection. Ten-days old seedlings were collected, frozen in liquid nitrogen and stored at –80 °C. RNA extraction and Northern blot hybridization were performed as described before (Chapter 2).

Protoplast isolation

Protoplasts were isolated from an Arabidopsis cell suspension culture (Axelos *et al*., 1992). A one week-old cell suspension culture was diluted 10-fold in 250 ml Erlenmeyer flasks containing 50 ml medium (3.2 g/L Gamborg's B5 basal medium with minimal organics (Sigma), 3% sucrose, 1 µM 1-naphtalene acetic acid (NAA), pH 5.8) and incubated overnight at 25 °C with shaking. A total of 150 ml of cell culture were then left to sediment. After removal of most of the medium, cells were centrifuged in a 50 ml tube at 600 rpm for 5 min at room temperature and the supernatant was removed. Cell walls were digested by addition of 20 ml of enzyme mix (0.1% Pectolyase (Sigma), 2% cellulase Onozuka R10 (Yakult), 12% sorbitol pH 5.8) for 1 hour at 37 °C. The protoplasts were filtrated through a 40 µM stainless steel sieve and transferred to a 50 ml tube in a total volume of 30 ml of Proto medium (Gamborg's B5 Basal Medium (Sigma), 0.1 M glucose, 0.25 M mannitol, 1 µM 1-NAA, pH 5.8). The protoplasts were centrifuged at 600 rpm for 5 min, washed with 50 ml of Proto medium and re-centrifuged. After addition of 15 ml of Proto medium, the number of protoplasts was determined. Finally, the volume of the protoplast suspension was adjusted to $4x10^6$ cells/ml.

Transient expression assay

In a 60 mm Petri dish 250 µl of protoplast suspension containing 10^6 cells were mixed with a total of 10 µg of DNA consisting of a mixture of the reporter construct 4RV-GusSH-47 (Menke *et al*., 1999) and a pMOG overexpression vector carrying *ORA1*, *ORA2*, or *ORA4* cDNA fused in sense or anti-sense orientation to the CaMV 35S promoter in a ratio of 1:3 and 250 µl of PEG solution (25% PEG 6000, 0.45 M mannitol, 0.1 M $Ca(NO_3)_2.4H_2O$, pH 9). The *ORA1* insert was amplified with the primer set 5'-CGGGATCCATATGACGGCGGATTCTCAATC-3' and 5'-CGGGATCCTTATAAAACCAATAAACGATC-3', cloned as a BamHI fragment in pBluescript SK+, excised with BamHI and cloned into the overexpression vector pMOG181. This *ORA1* derivative used for transient expression assays contained a single point mutation that changed the amino acid residue at position 154 from W to R. This mutation in the AP2 domain did not affect the *in vitro* binding affinity or specificity (data not shown). Co-transformation of the reporter construct with an empty overexpression vector (pMOG463) served as a control. After incubation for

15 min at room temperature 4.5 ml of Proto Medium was added, and the mixture was incubated for 1 hour at room temperature. Plates were incubated at 25 °C in the dark. Twenty hours after transformation, protoplasts were collected in a 15 ml tube and centrifuged at 800 rpm for 10 min. Protoplasts were washed 2 times with 0.5 ml of protoplast washing solution (0.33 M KCl, 18 mM $CaCl_2$, 5 mM MES, pH 5.7). The protoplast pellet was frozen in liquid nitrogen and stored at –80 °C. GUS activity was measured as described by van der Fits and Memelink (1997). The experiment was done in triplicate.

Results

Structures of ORA1, ORA2 and ORA4

The AP2 domains of ORA1, ORA2 and ORA4 are phylogenetically closely related (Chapter 2), and also very similar to the AP2 domains of the ORCA2 and ORCA3 proteins from Catharanthus. Fig. 1 shows an alignment of the amino acid sequences of the three ORA proteins. ORA1 shares 51% overall amino acid sequence identity with ORA2, and 43% with ORA4. ORA2 and ORA4 show 43% of amino acid identity over the entire sequence. The 58 amino acid AP2 domains of ORA1 (amino acids 146 to 203) and ORA2 (amino acids 117 to 174) differ only at a single position, whereas the ORA4 AP2 domain (amino acids 92 to 149) is less similar. Conserved acidic regions, which may function as transcriptional activation domains, are found N-terminal of the AP2 domain in ORA1 (amino acids 64 to 87), ORA2 (amino acids 43 to 66) and ORA4 (amino acids 29 to 52) (Fig. 1). A short stretch of basic amino acids, which may function as a nuclear localization signal (NLS), is found C-terminal of the AP2 domain in ORA1 (amino acids 233 to 236), ORA2 (amino acids 212 to 215) and ORA4 (amino acids 163 to 166) (Fig. 1). Furthermore, a serine-rich region is present in the C-terminus of the ORA2 protein (amino acids 189 to 206) (Fig. 1). A similar serine-rich cluster is found in the ORCA3 protein, where it hase a negative effect on transcriptional activation (van der Fits and Memelink, 2001).

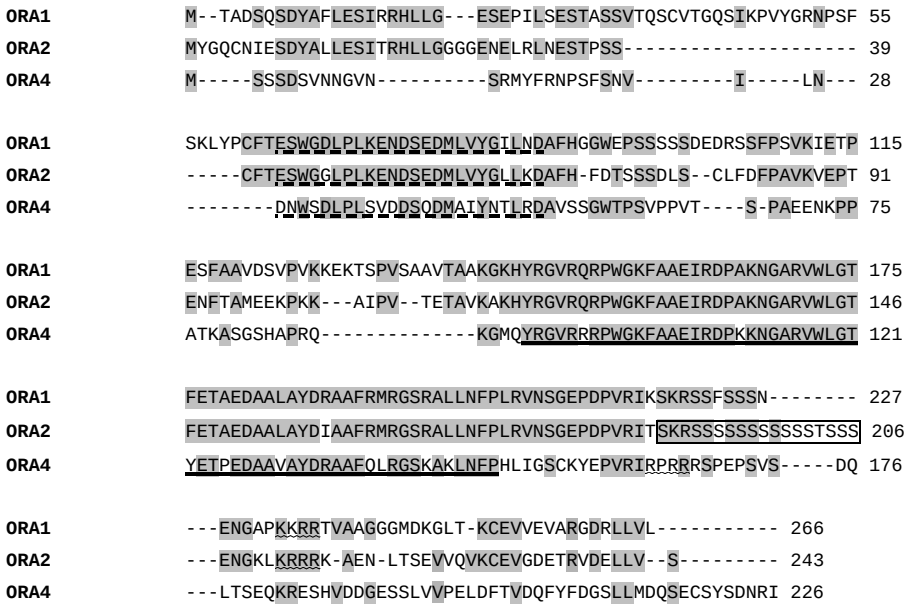


Figure 1. Sequence comparison of the ORA1, 2 and 4 proteins. Identical amino acids are marked with grey boxes. The AP2 domain is underlined**.** The nuclear localization signal is indicated with wavy lines whereas the putative acidic region is marked with a dotted line. The serine-rich region in ORA2 is boxed.

Binding of ORA proteins to the JA-responsive RV element

The fact that the *ORA* genes are induced by JA, suggests that the encoded proteins regulate JA-responsive genes by interacting with JA-responsive promoter elements. The RV fragment is a well-established autonomous JA-responsive element derived from the *STR* promoter, which interacts with the ORCA AP2-domain proteins from Catharanthus (Menke *et al*., 1999; van der Fits and Memelink, 2001).

To establish whether the ORA proteins from Arabidopsis are able to bind to the RV fragment, we performed electrophoretic mobility shift assays (EMSAs). In addition to wildtype RV, a set of mutant RV derivatives was used, in which adjacent blocks of six nucleotides were changed into their complementary nucleotides (Fig. 2A). Binding of the Catharanthus proteins ORCA2 and ORCA3 is abolished by mutations M2, M3, or M4 (Menke *et al*., 1999; van der Fits and Memelink, 2001). The *ORCA1* gene, which is not induced by JA, encodes a protein that does not bind to mutants RVM3 and RVM4 (Menke *et al*., 1999). To test the binding, GST-ORA fusion proteins were incubated with the labeled RV wildtype or

mutant fragments. All three proteins bound to the wildtype RV fragment (Fig. 2B). ORA1 binding was reduced by mutations M3 and M4. ORA2 showed similar binding specificity as ORA1. The binding of ORA4 was strongly reduced by mutations M2, M3 and M4. Therefore, the binding specificity of ORA1 and ORA2 with regard to the RV mutants is similar to that of Catharanthus ORCA1, whereas the specificity of ORA4 is similar to those of Catharanthus ORCA2 and ORCA3.

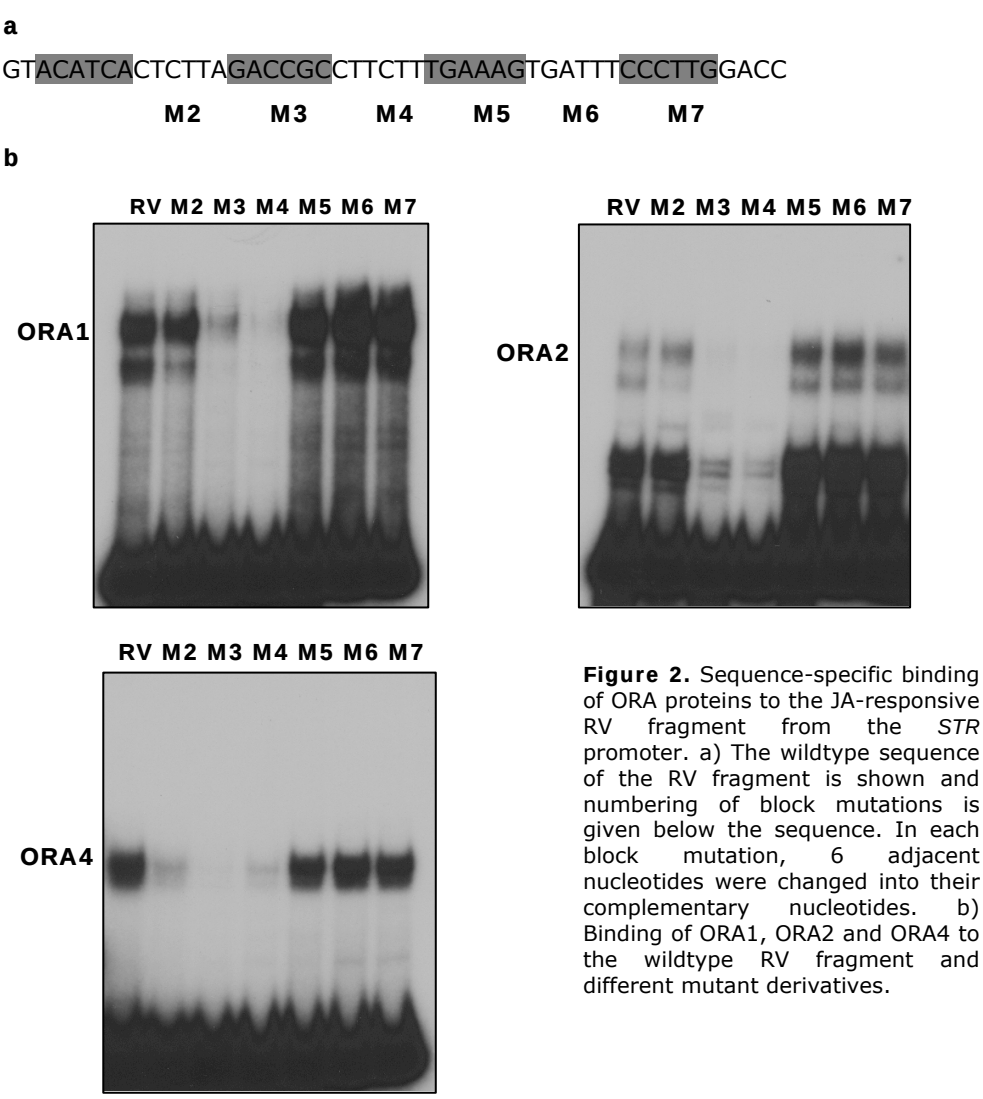


Figure 2. Sequence-specific binding of ORA proteins to the JA-responsive RV fragment from the *STR* promoter. a) The wildtype sequence of the RV fragment is shown and numbering of block mutations is given below the sequence. In each block mutation, 6 adjacent nucleotides were changed into their complementary nucleotides. b) Binding of ORA1, ORA2 and ORA4 to the wildtype RV fragment and different mutant derivatives.

ORA1, 2, and 4 proteins are transcriptional activators

ORA1, ORA2 and ORA4 have a conserved acidic domain (Fig. 1), which may function in transcriptional activation. To establish whether these ORAs are transcriptional activators, their ability to transactivate gene expression via binding to the RV fragment was investigated.

Arabidopsis protoplasts were co-transformed with a reporter vector carrying a tetramer of the RV fragment fused to the minimal CaMV 35S promoter (-47 to +27) and the *GUS* reporter gene, and an effector plasmid carrying *ORA* genes fused in sense or antisense orientation to the CaMV 35S promoter. Reporter gene activity increased between 1.25 and 2.75 fold upon co-transformation with ORA1, ORA2, and ORA4 effector plasmids compared to the empty overexpression plasmid (Fig. 3). No activation of the reporter gene was observed with effector plasmids carrying *ORA* genes fused in the anti-sense orientation to the CaMV 35S promoter. This experiment demonstrates that ORA1, ORA2, and ORA4 are able to function as transcriptional activators of gene expression in Arabidopsis cells.

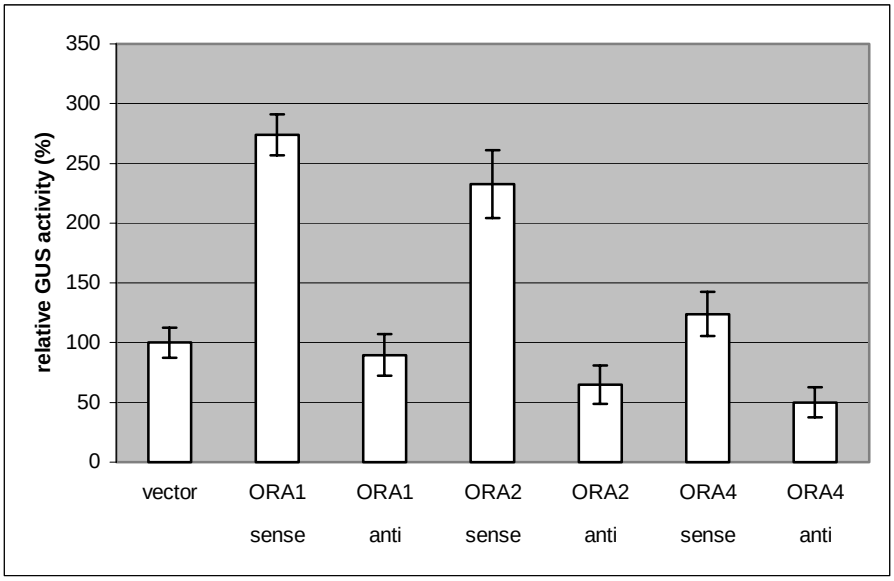


Figure 3. ORA1, ORA2 and ORA4 trans-activate RV-mediated gene expression in Arabidopsis protoplasts. Arabidopsis protoplasts were co-transformed with a reporter plasmid carrying a tetramer of RV fused to the CaMV 35S minimal promoter and *GUS*, and overexpression vectors containing *ORA1*, *ORA2* or *ORA4* cDNA fused in sense or antisense orientation to the CaMV 35S promoter. GUS activities are shown as percentages of the vector control. Bars represent means $\pm$ SE (n=3).

ORA over-expression activates defense-related genes

The *ORA1*, *2* and *4* genes are induced in response to JA (Chapter 2). The encoded proteins can bind *in vitro* to an established JA-responsive element (Fig. 2B), and activate transcription *in vivo* in a transient assay via this element (Fig. 3). Together, these observations strongly suggest that ORA transcription factors are terminal components of the JA signal transduction pathway regulating defense gene expression. In order to test this hypothesis, transgenic Arabidopsis plants over-expressing ORA1, 2, and 4 under the control of the CaMV 35S promoter were generated and RNA was extracted from several independent transgenic lines (Fig. 4).

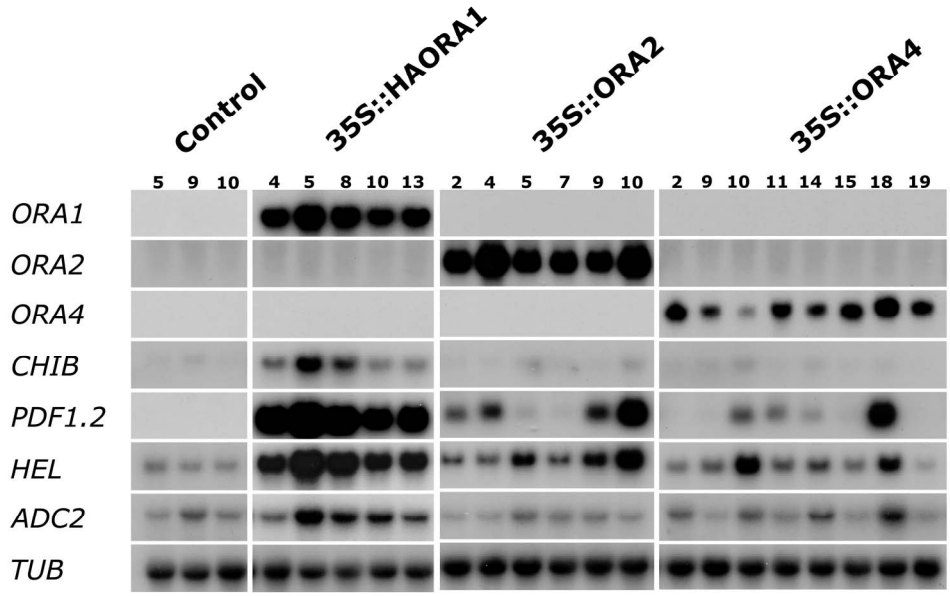


Figure 4. Overexpression of ORA1, ORA2 or ORA4 activates defense-related genes. RNA was extracted from 10 days old transgenic Arabidopsis seedlings grown in liquid MA culture. Ten μg samples of total RNA from independent lines of transgenic control plants overexpressing GUS or of transgenic plants overexpressing HA-ORA1, ORA2 or ORA4, respectively, were hybridised with the genes indicated at the left of the panels.

A number of candidate target genes (Table 1), which are known to be responsive to JA and/or ethylene, were selected and their expression was measured. Northern blot analysis revealed that *PDF1.2*, *HEL*, *CHIB* and *ADC2* (At4g34710, encoding arginine decarboxylase) transcript levels were higher in the 35S::*HAORA1* transgenic plants in comparison with control plants transformed with pCAMBIA1301 (Fig. 4).

Table 1. List of JA-responsive genes tested for expression in ORA-overexpressing lines. The AGI gene codes are indicated. Their expression levels in transgenic lines overexpressing ORA1, ORA2 or ORA4 relative to expression in control lines are indicated. (-) no difference in expression compared with control lines, (+) increased expression level in ORA-overexpressing lines.

JA biosynthesis	**AGI**	**35S:: HAORA1**	**35S:: ORA2**	**35S:: ORA4**
Allene oxide synthase (AOS)	At5g42650	-	-	-
tomato Allene oxide cyclase 3 homolog (tAOC3)	At3g25770	-	-	-
Lipoxygenase 2 (LOX2)	At3g45140	-	-	-
S-adenosyl L-methionine: JA methyl carboxyl methyltransferase (JMT)	At1g19640	-	-	-
12 oxo-phytodienoate reductase (OPR3)	At2g06050	-	-	-
Defense				
β-glucosidase homolog (BG1)	At1g52400	-	-	-
Thionin (THI2.1)	At1g72260	-	-	-
putative β-1,3 glucanase (β-1,3 gluc)	At2g01630	-	-	-
Plant defensin (PDF1.2)	At5g44420	+	+	+
Proteinase inhibitor 2 (PIN2)	At2g31980	-	-	-
Hevein-like gene (HEL)	At3g04720	+	+	+
Chitinase B (CHIB)	At3g12500	+	-	-
Vegetative storage protein 1 (VSP1)	At5g24780	-	-	-
Primary metabolism				
Anthranilate synthase 1 (ASA1)	At5g05730	-	-	-
Tryptophan synthase alpha chain (TSAα)	At3g06050	-	-	-
Arginine decarboxylase 2 (ADC2)	At4g34710	+	-	-
3-deoxy-D-arabino-heptulosonate –Phosphate synthase (DHS)	At4g39980	-	-	-
Secondary metabolism				
Putative Catechol O-methyl transferase	At1g76790	-	-	-
Putative flavonol sulfotransferase	At1g74100	-	-	-
Myrosinase binding protein-like	At3g16470	-	-	-
Chalcone synthase	At5g13930	-	-	-
Others				
Glutathione S-transferase (GST8)	At1g78380	-	-	-
Chlorophyllase (CLH)	At5g43860	-	-	-

Transgenic plants overexpressing ORA1 without HA tag, previously generated, expressed the same target genes at higher levels (data not shown). This *ORA1* derivative contained a single point mutation that changed the amino acid residue at position 154 from W to R. Northern blot analysis of 35S::*ORA2* and 35S::*ORA4* (Fig. 4) transgenic lines showed an increase in the mRNA levels of *PDF1.2* and *HEL*. In contrast to the ORA1 lines (Fig. 4), the expression levels of these defense genes were not tightly correlated with *ORA2 or ORA4* mRNA levels. Other tested JA-responsive genes, which were related to JA biosynthesis, defense or primary or secondary metabolism, did not show an increase in their expression levels in the transgenic ORA overexpressing plants (Table 1).

Discussion

We previously identified 14 JA-responsive *ORA* genes encoding AP2-domain transcription factors (Chapter 2). We speculated that these ORA proteins function in regulating defense genes in response to JA. Here, we demonstrated that ORA1, 2 and 4, when over-expressed, increased the expression of JA-responsive defense-related genes. Furthermore, ORA1, 2 and 4 bound in a sequence-specific manner to the JA-responsive RV element from the *STR* promoter, and they acted as transcriptional activators of a reporter gene driven by RV. Phylogenetic analysis showed that ORA1, 2 and 4 clustered together (Chapter 2) suggesting that these genes have most likely arisen from recent genomic duplication events. Therefore these genes can be functionally redundant, which can be studied by generating single, double and triple knockout plants in order to specify the function of each ORA and thus its target genes.

Differences in the binding specificities of the ORA proteins for the RV mutants were observed. ORA2 showed a similar binding specificity as ORA1. Both proteins do not interact with mutants RVM3 and RVM4. The wildtype sequence corresponding to the M3 and M4 mutations contains a GCC-box. These results are consistent with previously reported binding studies with ORA1 and ORA2 (called AtERF1 and AtERF2, respectively, by these authors), which were shown to bind to the GCC-box (Fujimoto *et al.,* 2000). ORA4 binding specificity was identical to those of ORCA2 and ORCA3 (Menke *et al.*, 1999; van der Fits and Memelink, 2001). The AP2 domain of ORA4 is more similar to that of ORCA3 than those of ORA1 and ORA2 are to that of ORCA3.

Transformation of *Arabidopsis* protoplasts with a reporter gene driven by a tetramer of the RV element and the ORAs driven by the constitutive CaMV 35S promoter demonstrates that ORA1, 2 and 4 activate gene expression. The results for ORA1 and ORA2 are consistent with those of Fujimoto *et al.* (2000) showing that ORA1 and ORA2 activate a reporter gene driven by the GCC box. This strongly suggests that the ORAs activate the RV element via direct interaction with the GCC-like box. However, this remains to be rigorously proven via co-expression of ORAs with reporter genes driven by wildtype and mutant versions of the RV region in Arabidopsis protoplasts, to establish whether the sequence-specific transcriptional activities of ORA1, 2 and 4 *in vivo* correlate with their *in vitro* DNA binding specificities.

Previous studies have shown that certain AP2-domain transcription factors are important in regulating plant responses to stress including pathogen attack, drought and cold (Berrocal-Lobo *et al.*, 2002; Haake *et al.*, 2002; Jaglo-Ottosen *et al*., 1998; Kasuga *et al.*, 1999; Brown *et al.*, 2003). However specific functions for ORA1, 2 and 4 in defense were not clear until recently, when it was shown that overexpression of ORA2 upregulates the expression of the defense-related *PDF1.2*, *THI2.1* and *CHIB* genes (Brown *et al.,* 2003). Therefore, we analyzed the transcripts levels of a number of putative target genes for the ORAs, responsive to JA or JA/ethylene (Schenk *et al.*, 2000; Sasaki *et al.*, 2001), in transgenic plants overexpressing *ORA1*, *2* and *4*.

The genes selected were related to JA biosynthesis, defense, and primary or secondary metabolism (Table 1). Our results demonstrated that constitutive over-expression of ORA1, 2 and 4 resulted in expression of the defense-related genes *PDF1.2* and *HEL*. Moreover transgenic ORA1 overexpressing plants showed an increase in *CHIB* and *ADC2* mRNA levels. Our results are partly consistent with the results reported by Brown *et al.* (2003), showing that transgenic plants overexpressing ORA2 have elevated levels of *PDF1.2* gene expression. In contrast to that report, we could not detect an increase in the mRNA level of *CHIB* via Northern blot analysis. This could be due to differences in growth conditions. We used 10 days liquid grown seedlings while they used 3 weeks old plants grown in a greenhouse. The other genes tested (Table 1) did not show any increase in their mRNA levels in the transgenic ORA plants compared to control transformed plants.

These results suggest that JA-responsive expression of *PDF1.2*, *HEL*, *CHIB* and *ADC2* is mediated by ORA1, 2 and 4, whereas expression of the other tested JA-responsive genes appears to be mediated by other transcription factors. Expression of *PDF1.2*, *CHIB* and *HEL* is also activated in transgenic plants overexpressing ERF1 (Lorenzo *et al*., 2003). This raises the question whether all these AP2-domain transcription factors normally regulate the JA-responsive expression of these defense genes, and if not, which ones, if any, do. The normal functions of transcription factors depend on the tissue specificity and/or their specific responses to environmental stimuli. Therefore, to understand the real role that a transcription factor plays during defense responses, overexpression data need to be interpreted in conjunction with other supporting data, such as the expression patterns of the transcription factor and the phenotype of knockout mutants (Zhang, 2003).

The expression of *PDF1.2*, *HEL* and *CHIB* is super-induced by combined treatment with JA and ethylene (Chapter 2). This suggests that these genes are regulated by a transcription factor, which is also transcriptionally super-induced by JA and ethylene. Whereas this is the case for the *ERF1* gene (Lorenzo *et al.*, 2003), *ORA1*, *2* and *4* expression is not synergistically or additively induced by JA and ethylene (Chapter 2). Therefore, ERF1 seems a much better candidate for regulating the JA-responsive expression of these defense genes under natural conditions. However, also for ERF1, evaluation of its role in defense gene expression awaits analysis of knockout mutants.

Acknowledgements

The authors thank Adel Zarei for construction of the pGEX-KG-ORA1 plasmid and for isolation of ORA1, ORA2 and ORA4 GST fusion proteins, and Hugo Bink for assistance with Arabidopsis protoplast isolation. Anthony Champion was supported by a Marie Curie Intra-European fellowship within the 5th European Community Framework Programme (contract QLK5-CT-2002-51650).

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Chapter 4

Salicylic acid and related phenolics induce AP2-domain transcription factor genes from Arabidopsis in an NPR1-independent manner

Mirna Atallah and Johan Memelink

Abstract

Plant defense responses operate through the action of the small signaling molecules jasmonic acid (JA), salicylic acid (SA), or ethylene. These three signaling molecules do not activate defenses independently, but rather initiate signal transduction pathways with complex interactions, which provides the plant with the regulatory potential to fine-tune the defense response. Positive and negative interactions occur between SA and JA signaling. However the primary mode of action appears to be mutually antagonistic. Recently we identified fourteen JA-responsive genes encoding AP2-domain transcription factors from Arabidopsis that we called ORAs (Octadecanoid-Responsive Arabidopsis AP2-domain proteins). The *ORA* gene expression response to JA is rapid and occurs within the first four hours of exposure. Further analysis of the specificity of *ORA* gene expression revealed that SA induced a subset of *ORA* genes, but the level of mRNA accumulation was low relative to the level in response to JA. SA repressed the basal expression level of two other *ORA* genes. Surprisingly, SA induced the expression of the SA-responsive *ORA* gene subset even more efficiently in transgenic NahG and mutant *npr1-1* plants impaired in their SA-dependent defense response. In addition, SA induced in these mutants several *ORA* genes that did not respond in the wildtype. Catechol, the NahG-mediated product of SA, and other small phenolics such as benzoic acid and *trans*-cinnamic acid also induced the expression of SA-responsive *ORA* genes. These results show that SA and related small phenolic compounds induce *ORA* gene expression independent of NPR1. The results also show that the NahG enzyme has other effects on gene expression than those caused by simply eliminating SA.

Introduction

Plants are capable of activating distinct defense responses that are effective specifically against the invader encountered (Feys and Parker, 2000; Pieterse and van Loon, 1999). Defense responses are regulated by a network of interconnected signal transduction pathways in which salicylic acid (SA) and jasmonic acid (JA) function as key signaling molecules (Reymond and Farmer, 1998; Glazebrook, 2001; Thomma *et al.*, 2001). Both SA and JA are accumulated by plants after pathogen attack and trigger the activation of a number of defense genes (Reymond and Farmer, 1998; Dong, 1998).

In plants, SA has been proposed to be synthesized by the phenylpropanoid pathway via *trans*-cinnamic acid and benzoic acid, or via the isochorismate pathway from chorismic acid (Shah, 2003; Wildermuth *et al*., 2001) (Fig. 1). SA participates in local defense responses, including the hypersensitive cell death response (HR), and in systemic defense reactions and in systemic acquired resistance (SAR) in response to bacterial, fungal and viral infection (Klessig *et al.*, 2000; Feys and Parker, 2000). SA-dependent defense responses are associated with the local and systemic expression of defense genes, encoding pathogenesis-related (PR) proteins (Ward *et al.*, 1991). The onset of SA-dependent defense is also characterized by an increase in SA levels both locally at the infection site and systemically (Malamy *et al.*, 1990). SA is a necessary signal for *PR* gene expression because transgenic NahG plants that express the bacterial enzyme salicylate hydroxylase, which converts SA into catechol, do not express *PR* genes in response to infection by pathogens (Gaffney *et al.*, 1993). Moreover, *PR* gene expression requires the SA-dependent nuclear translocation of the NPR1 protein (Kinkema *et al.*, 2000), also known as SAI1 and NIM1 (Shah *et al.*, 1997; Ryals *et al.*, 1997). Although NPR1 plays a key role in SA signaling pathway leading to defense responses, several studies (Shah, 2003) showed the presence of NPR1-independent SA-dependent defense mechanisms.

JA, its volatile methylester (MeJA) and biologically active precursors and derivatives, collectively called jasmonates (JAs), are fatty acid derivatives, which are synthesized via the octadecanoid pathway (Mueller, 1997). JAs trigger SA-independent defense mechanisms, and induce the expression of genes encoding toxic proteins such as defensin (PDF1.2) and thionin (THI2.1) (Penninckx *et al.*, 1998; Epple *et al.*, 1995). In addition, the induced systemic resistance (ISR) response triggered by non-pathogenic rhizobacteria is also mediated by JA (Pieterse *et al.*, 1998).

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). In Arabidopsis, SA inhibits JA synthesis and signaling. Arabidopsis mutants impaired in SA accumulation such as *eds4* and *pad4* exhibit enhanced expression of *PDF1.2* in response to JA (Gupta *et al.*, 2000). Moreover, the *cpr6* mutant constitutively expresses both *PR1* and *PDF1.2*

(Clarke *et al.*, 1998) and an *eds5cpr6* double mutant has a reduced SA level, and increased *PDF1.2* gene expression (Clarke *et al.*, 2000). SA has been shown to be a potent suppressor of JA-inducible gene expression (Gupta *et al*., 2000) in an NPR1-dependent manner (Spoel *et al*., 2003). JA also inhibits SA signaling. The Arabidopsis mutant *cev1* constitutively produces JA, and SA-dependent defense gene expression is suppressed (Ellis *et al.*, 2002). The *mpk4* mutation blocks JA-responsive *PDF1.2* gene expression and causes constitutive expression of the SA-responsive *PR1* gene (Petersen *et al.*, 2000).

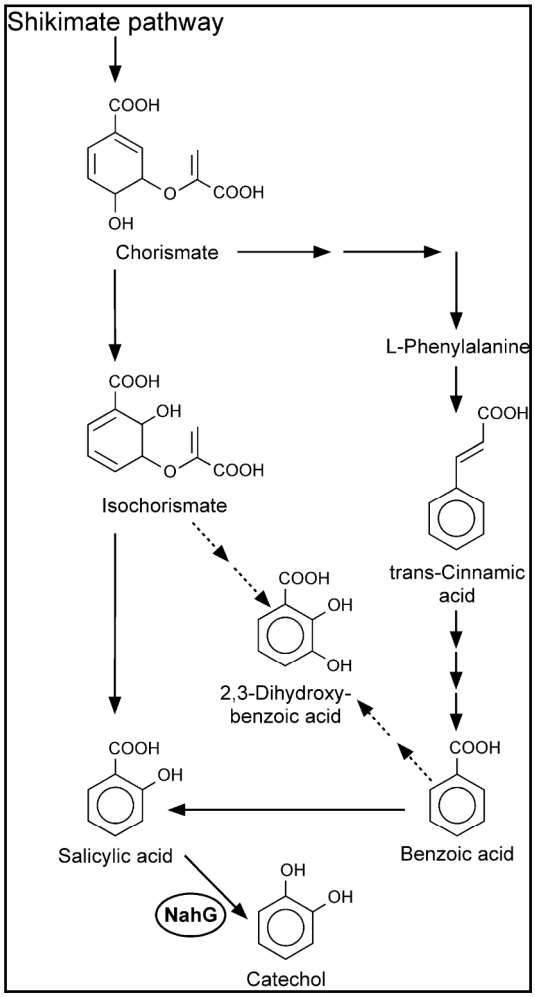

Figure 1: SA biosynthesis via the isochorismate or the phenylpropanoid pathways.

However, SA and JA can act synergistically. In a micro-array analysis of 2375 Arabidopsis genes more than 50 defense-related genes were induced both by SA and JA (Schenk *et al.*, 2000).

In Arabidopsis, we previously identified 14 JA-responsive genes encoding AP2-domain transcription factors called ORAs (Octadecanoid-Responsive Arabidopsis AP2-domain proteins). Their response to JA was dependent on the central JA signal transduction protein COI1. In this study, we analyzed the effect of SA on *ORA* gene expression. SA was found to induce or repress subsets of *ORA* genes. SA induced the SA-responsive *ORA* subset in an *npr1* mutant background, and even in NahG transgenic plants. Consistent with the latter finding, catechol and a number of other small phenolics induced the expression of these *ORA* genes. These results indicate that SA and related small phenolics induce *ORA* gene expression via an NPR1-independent signaling pathway. Our results also indicate that in transgenic NahG plants SA can still have effects on gene expression by conversion into the related small phenolic catechol, which can also induce *ORA* gene expression.

Materials and methods

Plant material, growth conditions, and treatments

The *Arabidopsis thaliana* wildtype (WT), mutant (*npr1-1*, *coi1-1*) and transgenic (NahG) plants used were Columbia (Col-0) ecotype. Seeds were surface-sterilized by incubation for 1 minute in 70 % ethanol, for 15 minutes in 50% bleach, followed by 5 rinses with sterile water. Per treatment 3 mg corresponding to approximately 150 seeds were added to 50 ml of MA medium (Masson and Paszkowski, 1992) in a 250 ml widemouth Erlenmeyer flask capped with aluminium foil and stratified for 3 days at 4 °C. Following 10 days of incubation in a growth chamber (16 h light/8 h dark, 4000 lux) at 21 °C on a shaker at 120 rpm, seedlings were treated for different time periods with 50 μM JA (Sigma) dissolved in dimethylsulfoxide (DMSO; 0.1 % final concentration in the culture medium), 1 mM SA (Sigma) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration), or a combination of SA and JA. Control seedlings were treated with final concentrations of 0.1 % DMSO, 0.5 mM sodium phosphate or a combination of both. Seedlings were harvested in liquid nitrogen, and stored at –80 °C until RNA isolation.

Treatments with the different phenolic compounds (Sigma) at 1 mM final concentrations were performed similarly. Stocks were dissolved in 50 mM sodium phosphate pH 7 and the final solvent concentration was 0.5 mM.

RNA extraction and Northern blotting

RNA extraction and Northern blotting were performed as described before (Chapter 2). Ten μg of total RNA were loaded onto the gels. Specific non-conserved parts of the coding regions of the ORAs outside of the conserved AP2 domain were generated via PCR amplification and used as probes.
DNA fragments corresponding to the open reading frame of the gene encoding β-tubulin (*TUB*; *At5g44340*), pathogenesis related-1 protein (*PR-1*, *At2g19990*) and a 300 bp fragment at the 3' end of the lipoxygenase 2 gene (*LOX2*; *At3g45140*) were amplified by PCR from Arabidopsis genomic DNA. The PCR primer sets used were (*TUB*) 5'- CGGAATTCATGAGAGAGATCCTTCATATC-3' and 5'-CCCTCGAGTTAAGTCTCGTACTCCTCTTC-3'; (*LOX2*) 5'-CGGGATCCGTGCGGAACA TAGGCCACGG-3' and 5'-CGGGATCCGGAACACCCATTCCGGTAAC-3'; (*PR-1*) 5'-GTA GGTGCTCTTGTTCTTCC-3' and 5'-TTCACATAATTCCCACGAGG-3'.

Results

Several *ORA* genes are induced by SA in an NPR1-independent manner

We previously identified 14 JA-responsive *ORA* genes encoding AP2-domain transcription factors from Arabidopsis. The gene expression response to JA is rapid and occurs well within four hours after JA addition. Several of the *ORA* genes are synergistically induced by a combination of JA and ethylene (Chapter 2). Here we analysed their gene expression in response to 1 mM SA in 10 days old seedlings. Three different types of responses to SA were observed. A first group consisting of *ORA1* (*At4g17500*), *ORA2* (*At5g47220*), *ORA44* (*At1g43160*) and *ORA68* (*At5gg13330*) was induced by SA after 4 to 8 hours of treatment. In contrast, the steady state mRNA levels of *ORA37* (*At3g15210*) and *ORA59* (At1g06160) were reduced after SA treatment. The third group containing *ORA 4* (*At2g44840*), *ORA19* (*At2g22200*), *ORA31* (*At5g47230*), *ORA33* (*At4g34410*), *ORA47* (*At1g74930*), *ORA63* (*At5g61890*), *ORA71* (*At5g07310*) and *ORA91*

(*At1g12630*) did not show changes in their gene expression level in response to SA after 4 or 8 hours (Fig. 2).

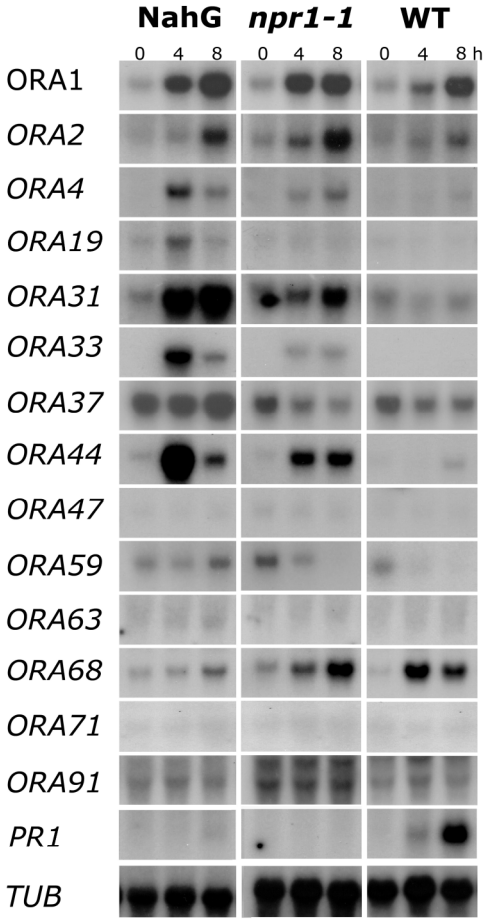

Figure 2: Expression of *ORA* transcription factor genes in wild type, *npr1-1* and NahG seedlings after SA treatment. Ten days old seedlings grown in liquid culture were treated with 1 mM SA for the time periods indicated in hours. Specific *ORA* probes were used to avoid cross-hybridization. *Tubulin* probe was used to verify integrity and equal loading of RNA. *PR1* probe was used to verify efficiency of SA treatment and identity of mutant and transgenic seedlings.

To establish whether the addition of SA induced the expression of *ORA1*, *2*, *44* and *68* via the well-established NPR1-dependent SA signal transduction pathway, the response to SA was studied in transgenic NahG and in *npr1-1* mutant seedlings. Northern blot analysis showed that SA induced *ORA1*, *2*, *44*

and *68* in both NahG and *npr1-1* seedlings. Surprisingly, *ORA37* and *ORA59*, which are repressed by SA in the wildtype, were both induced by SA in NahG seedlings. In *npr1-1* seedlings *ORA37* and *ORA58* were still repressed suggesting that the repression is NPR1-independent. The *ORA* genes of the third group, which did not respond to SA in the wildtype, showed two types of SA responses in the SA pathway mutants. One subgroup consisting of *ORA4*, *19*, *31* and *33* were induced by SA in NahG and *npr1-1* seedlings. The second subgroup containing *ORA47*, *63*, *71* and *91* were not responsive to SA in NahG and *npr1-1* seedlings (Fig. 2). Analysis of *Tubulin* mRNA levels showed equal loading of RNA (Fig. 2). Analysis of *PR1* mRNA levels showed that the SA treatment was effective, and that the *npr1-1* mutant and the NahG transgenic seedlings did not induce *PR1* gene expression in response to SA.

ORA gene induction by SA is independent of the JA signaling pathway

One possible explanation for the observation that SA induces the expression of a subset of *ORA* genes independent of the canonical SA signal transduction pathway, is that SA causes a stress response resulting in the biosynthesis of JA, which is a known inducer of the *ORA* genes (Chapter 2). Therefore, we analysed the expression levels of the SA-responsive *ORA* genes after SA treatment of the JA-insensitive *coi1-1* mutant. *ORA1*, *2*, *44* and *68* were induced to similar levels by SA both in the wildtype and in the *coi1-1* mutant (Fig. 3). Repression of *ORA37* and *ORA59* gene expression by SA was still observed in *coi1-1* seedlings (Fig. 3). It can be concluded that the observed effects of SA on gene expression are caused directly by SA rather than indirectly via an increase in the JA level.

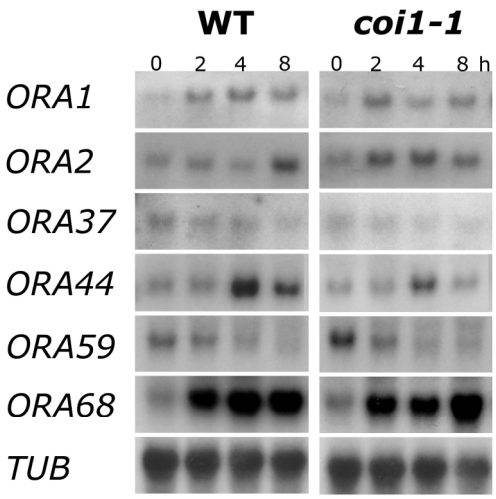

Figure 3: SA-responsive *ORA* gene expression is COI1-independent. *ORA* mRNA levels were analysed after addition of 1 mM SA to wildtype and *coi1-1* seedlings for the number of hours indicated. Specific probes for the *ORA* genes indicated were used. *Tubulin* probe was used to verify integrity and equal loading of RNA.

Several *ORA* genes are induced by small phenolics

We found that the effects of SA on expression of certain *ORA* genes do not require the canonical SA pathway component NPR1, and that SA does not act indirectly via the JA signaling pathway. The observation that SA induces *ORA* gene expression in the transgenic NahG seedlings suggests that SA acts as a small phenolic, and that catechol, the NahG-mediated product of SA, can also act as an inducing agent. To test this hypothesis wildtype seedlings were treated with 1 mM catechol. Northern blots analysis revealed that *ORA1*, *2*, *44* and *68* were induced in wildtype seedlings after catechol treatment. The SA-responsive marker gene, *PR-1*, was not induced by catechol, while an increase in its mRNA level was observed after treatment with SA (Fig. 4). *ORA37* and *ORA59*, which were repressed by SA in the wildtype, were induced by catechol. *ORA4*, *19* and *31*, which showed no response to SA in the wildtype, were induced by catechol. This demonstrates that the induction of these genes by SA in NahG seedlings (Fig. 2) is due to its conversion into catechol.

The finding that the small phenolic catechol induces the expression of several *ORA* genes raises the question whether other common plant phenolics would have similar effects. Analysis of the *ORA* gene expression response to the

phenolic compounds benzoic acid (BA), trans-cinnamic acid (CA), and 2,3-dihydroxybenzoic acid (DHBA) showed that some of these compounds are able to induce certain *ORA* genes. The treatment with BA induced the expression of *ORA1*, *2*, *4*, *33* and *68* genes whereas the treatment with DHBA had little effect on *ORA* gene expression with only small increases in the mRNA levels of *ORA1*, *2* and *13*. CA induced *ORA1*, *2*, *33*, *44* and *68* gene expression. *ORA37* and *ORA59*, which were repressed by SA, were also repressed by CA. *ORA59* expression was also repressed after treatment with BA and DHBA. The SA-responsive control gene *PR1* was induced by SA, BA and DHBA. The JA-responsive *LOX2* gene was induced only after catechol treatment. None of the other treatments induced *LOX2* gene expression. Analysis of *TUB* mRNA levels showed equal loading of RNA (Fig. 4).

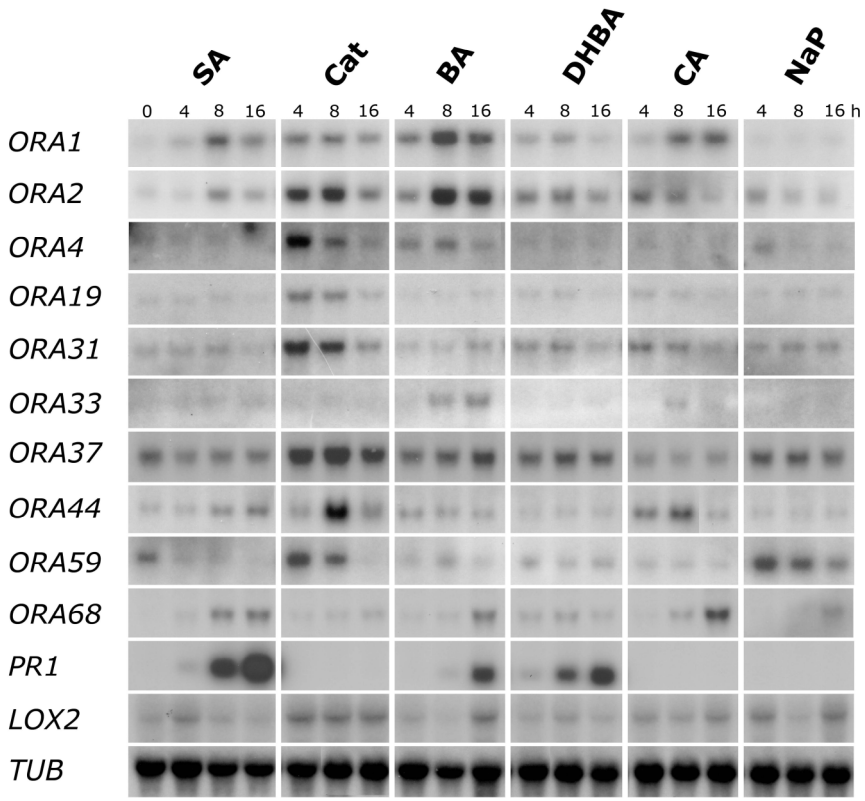

Figure 4: Expression of *ORA* transcription factor genes in wildtype seedlings after treatment with different phenolics. Ten days old seedlings grown in liquid culture were treated with 1 mM SA, catechol (Cat), benzoic acid (BA), 2,3-dihydroxybenzoic acid (DHBA), trans-cinnamic acid (CA) or sodium phosphate (NaP) for the time periods indicated in hours. Specific probes for *PR-1*, *LOX2* and *ORA* genes were used for RNA hybridization. *Tubulin* probe was used to verify integrity and equal loading of RNA.

Effects of combined treatment of JA and SA on the expression of SA-repressible *ORA* genes

Previously, the JA-responsive expression of the genes *LOX2*, *VSP2* and *PDF1.2* was shown to be repressed by SA in an NPR1-dependent manner (Spoel *et al.*, 2003). This raises the questions whether the JA-responsive expression of *ORA37* and *ORA59* genes is also repressed by SA, and whether this possible inhibitory effect of SA requires a functional NPR1 protein. Fig. 5 shows that the *ORA37* and *ORA59* mRNA levels were increased both in wildtype and *npr1-1* seedlings after 30 min of JA treatment, whereas SA caused a similar reduction after 4 and 8 hours of treatment. *ORA37* and *ORA59* gene expression was induced in the combined treatment after 30 min of treatment. However there was a difference between the *ORA37* and *ORA59* expression patterns. The *ORA37* expression level was reduced at all time points compared to the JA treatment both in wildtype and *npr1-1* plants, whereas the *ORA59* expression level was only slightly reduced in the *npr1-1* mutant plants (Fig. 5). Therefore, it can be concluded that SA has a weak inhibitory effect on JA-responsive *ORA37* expression, which is NPR1-independent. Analysis of *TUB* mRNA levels showed equal loading of RNA (Fig. 5).

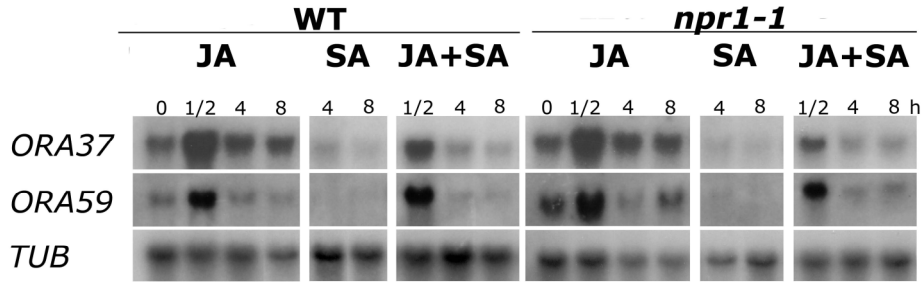

Figure 5: SA-mediated repression of *ORA* gene expression is independent of NPR1. Northern blot analysis of *ORA37* and *ORA59* gene expression after treatment with 50 μM JA, 1 mM SA, and a combination of JA and SA for the time periods indicated in wildtype and *npr1-1* plants. Specific probes for *ORA37* and *ORA59* were used for RNA hybridization. *Tubulin* probe was used to verify integrity and equal loading of RNA.

Discussion

Plant defense responses are regulated by the signaling molecules JA, SA and ethylene. However, these defense signals do not act independently. Positive and negative crosstalk has been observed between the signaling pathways leading to

a fine-tuned defense response (Feys and Parker 2000; Reymond and Farmer 1998). In Chapter 2 we showed that *Arabidopsis* possesses 14 JA-responsive *ORA* genes encoding AP2-domain transcription factors. Several of the *ORA* genes are synergistically induced by a combination of JA and ethylene (Chapter 2). Here we analysed *ORA* gene expression in response to SA. SA had different effects on individual *ORA* genes, indicating that ORA transcription factors have multiple and complex functions. We found that SA acted positively on the expression of some *ORA* genes, negatively on the expression of others and had no effect on a third *ORA* gene subset. The effects of SA on *ORA* gene expression occurred at later time points than JA-mediated responses. The *ORA* gene expression response to SA was not abolished in plants impaired in the canonical SA signal transduction pathway. However, SA did not act indirectly by inducing JA biosynthesis, since the *coi1-1* mutant, affected in the JA signaling pathway, showed a wildtype *ORA* gene expression response to SA.

SA induced in NahG seedlings all the SA-responsive *ORA* genes, the SA-repressible *ORA37* and *ORA59* genes, and a subset of the *ORA* genes that were non-responsive to SA in the wildtype. This suggested that catechol might be the inducing agent. This was confirmed by treating wildtype plants with catechol. Catechol induced the expression of all *ORA* genes that were induced by SA in NahG seedlings, except *ORA33*. This indicates that for those *ORA* genes SA-responsive expression in NahG seedlings is due to catechol.

Moreover, we observed an induction of the SA-responsive *ORA* genes in *npr1-1* mutant plants after SA treatment. SA therefore regulates *ORA* gene expression in an NPR1-independent manner. In this respect, the SA-responsive *ORA* genes are similar to the early SA-responsive genes glutathione S-transferase (*GST6*) and glucosyltransferase (*IEGT*), which are also regulated by SA via an NPR1-independent pathway (Uquillas *et al*., 2004). The existence of an NPR1-independent SA signaling pathway is supported by studies of various Arabidopsis constitutive-defense mutants. For example, the Arabidopsis *suppressor of SA-insensitivity2* (*ssi2*) mutant confers resistance to *Pseudomonas syringae* and *Peronospora parasitica*, which is compromised by *eds5* and NahG but is retained in the *npr1* mutant background (Shah *et al.*, 2001; Shah, 2003). Also, *ORA37* and *ORA59* were repressed by SA in an NPR1-independent manner. Surprisingly, *ORA* gene induction by SA was much stronger in the *npr1-1* mutant, and some *ORA*

genes such as *ORA31* and *ORA33*, which are unresponsive to SA in the wildtype, were induced in the *npr1-1* mutant.

Since both SA and catechol, phenolic compounds with a simple structure, are able to induce *ORA* gene expression, and since the SA response is NPR1-independent, this suggests that SA is acting as a phenolic inducing compound. To establish whether other phenolic compounds with a similar structure might induce *ORA* gene expression, we studied the effects of the common plant phenolic compounds BA, DHBA and CA on *ORA* gene expression. All of the SA-responsive *ORA* genes were induced by one or more of these different phenolic compounds, albeit to different levels.

The interaction between the SA and JA signaling pathways is known to be complex with both antagonistic and synergistic interactions reported (Glazebrook, 2001; Rojo *et al.*, 2003). However the primary mode of action appears to be mutually antagonistic. For example, the SA-responsive *PR-1* gene is repressed by JA (Rao *et al.*, 2000). Conversely, the JA-responsive *PDF1.2*, *VSP2* and *LOX2* genes are repressed by SA (Spoel *et al.*, 2003). Similarly, our results showed that the *ORA37* and *ORA59* genes were induced by JA and repressed by SA. However, in contrast to Spoel *et al.* (2003), the SA-mediated suppression of the *ORA37* and *ORA59* genes was not abolished in mutant *npr1-1* seedlings, indicating that NPR1 is not essential for the inhibition of *ORA* gene expression by SA. A negative effect of SA on JA-responsive expression was only observed with *ORA37.* This suggests that the transcription factor ORA37 might play a role in the negative cross-talk between JA and SA, where SA acts via an NPR1-independent pathway.

Transgenic NahG plants have been used in many studies to prove that certain defense responses are mediated by SA (Spoel *et al.*, 2003; Delaney *et al.*, 1994; Gaffney *et al.*, 1993) Our data show that the effect of the bacterial *NahG* gene on plant gene expression is different from what would be expected if it merely inactivates SA. The salicylate hydroxylase enzyme creates the new phenolic molecule catechol, which can be a more powerful inducer of gene expression than SA itself. Several studies have reported aberrant defense responses in NahG plants that are not explained by depletion of SA (van Wees and Glazebrook, 2003; Heck *et al*., 2003). It was also shown that catechol compromised the normal defense response to *Pseudomonas syringae* pv. *phaseolicola* via an unknown mechanism, which appeared to involve the

production of reactive oxygen species (van Wees and Glazebrook, 2003). Our results give some insight in the effects of catechol on gene expression, which may in part account for the observed effect on defense. Together with previous studies, it can be concluded that the use of NahG plants should be treated with caution and previous results should be re-evaluated. For example, Spoel *et al*. (2003) concluded from the observation that *LOX2* gene expression was more strongly induced by infection with *Pseudomonas syringae* pv. tomato DC3000 in NahG plants than in wildtype, that the elevated SA level in wildtype suppressed JA signaling. Our results show that increased *LOX2* induction in NahG plants can also be caused by catechol production.

Our data suggest that SA has two distinct signaling activities, one based on its specific SA structure, and one based on its small phenolic character. Other common plant phenolics may also have signaling activities. Our data further suggest that Arabidopsis possesses a phenolic receptor (family) distinct from a SA-specific receptor (family), which activates an NPR1-independent signal transduction pathway that leads to activation of *ORA* gene expression. The observation that SA has a stronger inducing effect on *ORA* gene expression in the *npr1-1* mutant indicates that NPR1 has a negative effect on the phenolics signaling pathway (Fig. 6).

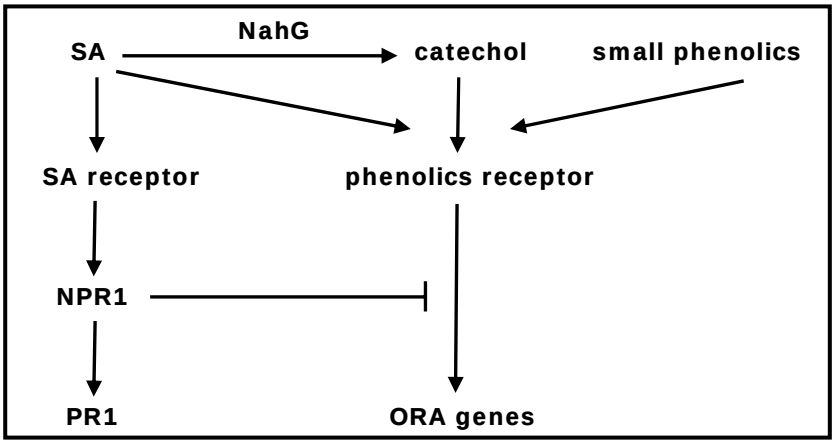

Figure 6: Model for the transcriptional regulation of *ORA* expression by SA. SA induces *PR-1* and *ORA* gene expression differently. SA induces *ORA* gene expression by acting as a small phenolic compound. Other small phenolics may also act as signals. NPR1 has a negative effect on the phenolic signaling pathway leading to induction of *ORA* gene expression.

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Chapter 5

Summary

Perception of stress or pathogens by a plant triggers rapid defense responses via a number of signal transduction pathways (Yang *et al.*, 1997). An important aspect of plant stress signal transduction is the biosynthesis of one or a combination of the hormone-like compounds jasmonic acid (JA), salicylic acid (SA) and ethylene (Reymond and Farmer, 1998). These hormones interact cooperatively or antagonistically in a variety of responses, leading to fine-tuning of the complex defense response and to the expression of defense genes (Glazebrook, 2001; Rojo *et al.*, 2003).

Jasmonates are a family of cyclopentanone derivatives synthesized from linolenic acid via the octadecanoid pathway (Liechti and Farmer, 2002; Turner *et al.*, 2002; Atallah and Memelink, 2004). They regulate several aspects of plant growth and development, and are involved in responses to several environmental stress factors (Turner *et al.*, 2002; Atallah and Memelink, 2004).

At present, it is largely unknown how stress signals affect JA synthesis. The mechanisms whereby JA signaling triggers gene expression are just starting to be elucidated. In *Catharanthus roseus*, a JA- and elicitor-responsive element (JERE) in the promoter of the JA-responsive terpenoid indole alkaloid biosynthetic gene *Strictosidine synthase* (*STR*) has been identified (Menke *et al.*, 1999). The JERE interacts with two transcription factors called Octadecanoid-Responsive Catharanthus AP2/ERF-domain proteins (ORCAs) (Menke *et al.*, 1999; van der Fits and Memelink, 2001). Both belong to the AP2-domain family of plant transcription factors, which are not found in animals or yeast and are characterized by the AP2 DNA-binding domain. The expression of the *ORCA* genes themselves is JA-responsive (Menke *et al.*, 1999, van der Fits and Memelink, 2001). Moreover, the expression of several alkaloid biosynthesis genes is regulated by ORCA3 (van der Fits and Memelink, 2000). Based on these observations, it was postulated that JA-responsive gene expression in Arabidopsis is also mediated by members of the AP2-domain transcription factor family, and that the corresponding genes are also expressed in a JA-responsive manner.

The studies described in this thesis are focused on the molecular mode of action of the important plant stress hormone jasmonic acid (JA) in gene expression in Arabidopsis. The aim was to find JA-responsive members of the large AP2-domain transcription factor gene family in Arabidopsis, at clarifying their role in the JA signal transduction network, and at establishing whether the

proteins encoded by these members are involved in JA-responsive gene expression.

Chapter 2 describes the identification of JA-responsive AP2-domain transcription factor genes in Arabidopsis: so called *ORA* (Octadecanoid-Responsive Arabidopsis AP2 domain) genes. The approach used was to amplify all 126 genes encoding transcription factors with a single DNA-binding domain of the AP2 type from Arabidopsis, found after a database search, by PCR, and to use these genes as probes in Northern blot hybridizations to study their expression in 10 days old Arabidopsis seedlings grown in liquid culture and exposed to JA for various time periods up to 4 hours. This strategy resulted in the identification of 14 *ORA* genes, which showed increased expression in response to JA within 4 hours. The *ORA* genes showed four different types of expression kinetics. A first group consisting of *ORA1*, *ORA2* and *ORA47*, showed a rapid transient expression by JA with a peak at 15 min of treatment, and returned to basal levels within 4 hours of exposure to JA. A second group with *ORA4*, *ORA31*, *ORA33*, *ORA37* and *ORA59*, showed intermediately transient kinetics of expression. JA induced these genes within a short interval of time, starting after 15-30 min and peaking after 30 min of treatment. A third group with *ORA63* and *ORA71* showed an extremely transient expression, which was only detectable after 1 hour of treatment, but not after 30 min or 2 hours. And a fourth group with *ORA19*, *ORA44*, *ORA68* and *ORA91* was characterized by a more prolonged expression in response to JA up to the longest time point of 4 hours. Analysis of gene expression in response to SA and ethephon (an ethylene releaser) showed that a number of *ORA* genes were induced by SA or ethephon. However this response was not as fast as for JA.
Six of the *ORA* genes were previously functionally characterized. These encode ORA1, 2, 4, 31, 37 and 44, which were previously called AtERF1, AtERF2, AtERF13, AtERF5, AtERF4 and RAP2.6 respectively (Fujimoto *et al.*, 2000; Onate-Sanchez and Singh, 2002; Chen *et al.*, 2002). In these studies, these *ORA* genes were shown to be induced by ethylene, wounding, pathogens or virus attack.

The related expression patterns of groups of *ORA* genes were not strictly correlated to the similarity between the corresponding encoded proteins. Protein similarities were only observed between ORA1, 2 and 4 and between ORA63, 68 and 71.

To further determine how JA controls *ORA* gene expression, mutants affected in JA, SA or ethylene responses were used. Induction of JA-responsive *ORA* gene expression was not affected in the *npr1-1* mutant or in transgenic NahG plants impaired in the SA pathway, whereas the JA-responsive expression of all *ORA* genes depended on COI1, an F-box protein required for all known JA responses. Analysis of the ethylene-insensitive mutants *etr1-1* and *ein2-1* revealed a subset of five *ORA* genes (*ORA31*, *37*, *44*, *59* and *68*) of which the JA-responsive expression depended on ETR1 and EIN2 as well as on COI1. Interestingly, the expression of this subset of *ORA* genes was also found to be synergistically induced by a combination of JA and ethephon, suggesting that the encoded ORA proteins integrate JA and ethylene signal inputs to coordinate the appropriate gene expression response in a manner similar to the previously identified ERF1 transcription factor (Lorenzo *et al.*, 2003). Although this chapter provides an in depth analysis of the regulation of *ORA* gene expression, no specific function of any of the ORA proteins was determined.

In **Chapter 3** the attention is focused on ORA1, ORA2 and ORA4. These three proteins are more related to each other than to the other ORA proteins, and they are also most related to the ORCA proteins. *In vitro* binding experiments demonstrated that ORA1, ORA2 and ORA4 interact with the JERE present in the *STR* promoter. Using a set of mutant RV derivatives, in which adjacent blocks of six nucleotides were changed into their complementary nucleotides, it turned out that ORA1 and ORA2 binding was reduced by mutations M3 and M4. The binding of ORA4 was strongly reduced by mutations M2, M3 and M4. Therefore, the binding specificity of ORA1/ORA2 and ORA4 with regard to the RV mutants is similar to that of Catharanthus ORCA1 and ORCA2/ORCA3 respectively. In Arabidopsis, a number of AP2-domain transcription factors have been implicated in stress responses (Shinozaki and Yamaguchi-Shinozaki, 2000). Certain AP2-domain transcription factors are important in regulating plant responses to stress including pathogen attack, drought and cold (Berrocal-Lobo *et al.*, 2002; Haake *et al.*, 2002; Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Brown *et al.*, 2003). Specific functions for ORA1, 2 and 4 in defense were not clear until recently, when it was shown that overexpression of ORA2 upregulates the expression of the defense-related genes *PDF1.2*, *THI2.1* and *CHIB* (Brown *et al.*, 2003). Results described in Chapter 3 indicate the involvement of ORA1, ORA2 and ORA4 in

transcriptional regulation of defense genes. Transgenic plants overexpressing ORA1, ORA2 and ORA4 had increased expression levels of the defense-related genes *PDF1.2*, *HEL*, *CHIB*, and *ADC2*. *PDF1.2*, *CHIB* and *HEL* expression is also increased in transgenic plants overexpressing ERF1 (Lorenzo *et al*., 2003). This raises the question whether all these AP2-domain transcription factors normally regulate the JA-responsive expression of these defense genes, and if not, which ones, if any, do.

Although it seems likely that ERF1 is a much better candidate for regulating the JA-responsive expression of these defense genes under natural conditions, since the *ERF1* gene (Lorenzo *et al.*, 2003) as well its putative target genes (Chapter 2) are super-induced by combined treatment with JA and ethylene, an evaluation of the roles of individual AP2-domain transcription factors including ERF1 in defense gene expression awaits analysis of knockout mutants.

Chapter 4 describes how SA and related phenolics modulate the expression of *ORA* genes in an NPR1-independent manner. Plants are capable of activating distinct defense responses through the action of the small signaling molecules JA, SA, or ethylene (Feys and Parker, 2000; Pieterse and van Loon, 1999), which do not activate defense independently, but rather initiate signal transduction pathways with complex interactions. This provides the plant with the regulatory potential to fine-tune the defense response. 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). SA induced *ORA1*, *ORA2*, *ORA44* and *ORA68* expression. In contrast *ORA37* and *ORA59* expression was reduced in response to SA. The other *ORA* genes did not show changes in their expression level in response to SA in wildtype plants. Interestingly the *ORA* gene expression response to SA was not abolished in mutant *npr1-1* and transgenic NahG plants impaired in the canonical SA signal transduction pathway. In contrast a larger number of *ORA* genes were induced by SA in *npr1-1* and NahG plants. This demonstrates that SA induces *ORA* gene expression via an NPR1-independent manner. In the NahG plants catechol, the NahG-mediated conversion product of SA, may be the inducer. Indeed, catechol as well as several other plant small phenolics, such as benzoic acid, trans-

cinnamic acid, and 2,3-dihydroxybenzoic acid, induced *ORA* genes in wildtype plants.

Since the interaction between the SA and JA signaling pathways is known to be complex with both positive and negative interactions reported (Glazebrook, 2001; Rojo *et al.*, 2003), it was studied whether SA acted indirectly by inducing JA biosynthesis. This was not the case since the *coi1-1* mutant affected in the JA signaling pathway showed wildtype *ORA* gene expression responses to SA. Moreover, the effect of a combined treatment with JA and SA on the expression of the SA-repressible *ORA37* and *ORA59* genes was studied. A negative effect of SA on JA-responsive expression was only observed with the *ORA37* gene. This suggests that the transcription factor ORA37 might play a role in the negative cross-talk between JA and SA, where SA acts via an NPR1-independent pathway. The data presented in Chapter 4 suggest that in the induction of *ORA* gene expression SA is acting as a small phenolic compound, which is perceived by a receptor distinct from the SA receptor initiating an NPR1-independent signal transduction pathway.

The studies described in this thesis have led to the identification of 14 JA-responsive AP2-domain transcription factor genes (*ORA*), putatively involved in the JA signaling and defense. Several ORA transcription factors seem to be acting as integrators of the JA, ethylene and SA signaling pathway inputs. This work contributes to a better understanding of the molecular mode of action of JA in gene expression in Arabidopsis. However, future challenging work remains in unravelling the specific roles of ORAs in defense gene expression and their roles in crosstalk between different signaling pathways. Analysis of knockout mutants and transgenic plants overexpressing ORAs will help to unravel the roles of ORA proteins in JA signaling and defense.

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Samenvatting

Planten maken gebruik van verschillende signaalsystemen om te reageren op stress, verwonding, vraat of de aanwezigheid van ziekteverwekkers. Belangrijk daarbij is de aanmaak van endogeen geproduceerde hormoonachtige signaalstoffen, met name jasmonzuur (JA), salicylzuur (SA) en ethyleen. Welke hormonen worden gemaakt hangt af van het type ziekteverwekker, van de soort stress en van het soort herbivoor. Deze hormonen veroorzaken hun eigen specifieke effecten, maar versterken of verzwakken bovendien elkaars effect, wat leidt tot een nauwkeurige afregeling van verschillende afweerreacties en van de expressie van specifieke sets van afweergenen. In dit proefschrift ligt de nadruk op de rol van JA. JA en verwante jasmonaten vormen een familie van cyclopentanoonverbindingen, die door middel van de zogenaamde octadecanoidsyntheseweg worden gemaakt van linoleenzuur. Ze reguleren verschillende aspecten van plantengroei en ontwikkeling, en zijn betrokken bij reacties op allerlei stressomstandigheden.

Momenteel is het vrijwel onbekend hoe stress-signalen leiden tot de aanmaak van JA. Ook zijn de mechanismen waardoor JA signalering leidt tot expressie van bepaalde genen pas sinds kort onderwerp van studie. Voor het onderzoek naar deze stress-signalering worden vaak gezuiverde verbindingen van ziekteverwekkers gebruikt, die afweer induceren, zogenaamde elicitors. *Catharanthus roseus* reageert op een elicitor met de aanmaak van beschermende terpenoïde indool alkaloïden (TIA). In deze plant is in de promoter van het JA-responsieve gen *Strictosidine synthese* (*STR*), dat betrokken is bij de synthese van TIA, een JA- en elicitor-responsief element (JERE) ontdekt. Het JERE kan twee transcriptiefactoren binden, de zogenaamde Octadecanoïde-responsieve Catharanthus AP2/ERF-domein eiwitten (ORCAs). Beide behoren tot een speciale familie van transcriptiefactoren, die niet voorkomt bij gist of bij dieren. Kenmerkend is de aanwezigheid van het DNA-bindende zogenaamde AP2 eiwitdomein. De expressie van *ORCA* genen wordt geïnduceerd door JA. Van ORCA3 is inmiddels bekend dat het de JA-responsieve expressie van verscheidene genen reguleert die betrokken zijn bij de aanmaak van TIA. Deze waarnemingen hebben geleid tot een model, waarin een elicitor de JA synthese stimuleert, JA de ORCA eiwitten in stelling brengt en de ORCA eiwitten op hun beurt de syntheseweg van beschermende stoffen (TIA) aanzwengelen.

Omdat JA een algemeen plantenstresshormoon is, en er voorshands geen redenen zijn waarom dit model alleen zou opgaan voor *Catharanthus*, is de werkhypothese opgesteld dat ook in de modelplant Arabidopsis transcriptiefactoren van de AP2-domein familie een rol spelen bij JA-responsieve genexpressie en dat JA de expressie van de overeenkomstige genen induceert. Daarom is eerst gezocht naar transcriptiefactoren van de AP2-domein familie in Arabidopsis, die geactiveerd worden in respons op JA (nu ORA transcriptiefactoren genoemd, voor Octadecanoïde-responsieve Arabidopsis AP2/ERF-domein eiwitten). Vervolgens is hun rol in het JA-signaal transductie netwerk en in JA-responsieve genexpressie onderzocht.

In **Hoofdstuk 2** wordt de ontdekking van Arabidopsis *ORA* genen beschreven. Alle bekende 126 AP2-domeineiwitcoderende genen van Arabidopsis met een enkel DNA-bindend domein werden getest op verhoogde expressie na het toedienen van JA. Dit resulteerde in de vondst van 14 *ORA* genen. Op grond van de snelheid en duur van expressie konden vier typen *ORA* genen worden onderscheiden. Groep 1 bestaat uit *ORA1*, *2* en *47*, die 15 min na toediening van JA al maximaal tot expressie komen, en na 4 uur alweer op het basisniveau van expressie zijn teruggevallen. Groep 2, *ORA4*, *31*, *33*, *37* en *59*, laat een expressiepiek na 30 min zien. Geïnduceerde expressie van een derde groep, *ORA63* en *71*, is zichtbaar na 60 min maar niet na 30 min of na 2 uur. Groep 4 tenslotte, *ORA19*, *44*, *68* en *91*, vertoont een relatief langdurig verhoogd expressieniveau na toedienen van JA, over een periode van tenminste 4 uur.

Als test op JA-specificiteit, is de expressie onder invloed van twee andere plantenstresshormonen getest, namelijk SA en ethyleen. Expressie van een aantal *ORA* genen bleek inderdaad door deze hormonen geïnduceerd te worden, zij het minder snel dan door JA. Zes *ORA* genen bleken al eerder onder een andere naam beschreven te zijn: *ORA1* = *AtERF1*, *ORA2* = *AtERF2*, *ORA4* = *AtERF13*, *ORA31* = *AtERF5*, *ORA37* = *AtERF4*, en *ORA44* = *Rap2.6.* Expressie van deze *ORA* genen is in de literatuur beschreven als induceerbaar door ethyleen, verwonding of infectie door virussen of andere pathogenen.

De expressie van *ORA* genen onder invloed van JA werd vervolgens getest in Arabidopsis mutanten met een verstoorde respons op JA, SA of ethyleen. Als eerste werd een JA mutant getest, en wel de *coi1-1* mutant. COI1 codeert voor een F-box eiwit dat een noodzakelijke rol speelt bij alle tot dusver bekende JA-

responsen in planten. Inderdaad bleek JA geen enkel *ORA* gen tot expressie te kunnen brengen in de *coi1-1* mutant van Arabidopsis. Vervolgens werden twee SA-mutanten getest. Enerzijds Arabidopsis planten die het bacteriële *NahG* gen ingebouwd hebben gekregen en SA afbreken af tot het inactief geachte catechol. Anderzijds planten met een mutatie in het *NPR1* gen, wat Arabidopsis gedeeltelijk ongevoelig maakt voor SA. In beide typen mutanten bleek de expressie van *ORA* genen onder invloed van JA onveranderd. Dit geeft aan dat SA niet betrokken is bij JA-responsieve expressie van *ORA* genen. Tenslotte werden de ethyleen-ongevoelige mutanten *etr1-1* en *ein2-1* van Arabidopsis getest. Daarbij werd een groep van *ORA* genen gevonden (*ORA31*, *37*, *44*, *59* en *68*) die voor hun JA-geïnduceerde expressie tevens afhankelijk bleken van ETR1 en EIN2. Overeenkomstig met deze waarneming bleek deze groep van genen extra hoog tot expressie te komen bij gelijktijdige toediening van JA en ethyleen. Dit suggereert dat de meeste *ORA* genen JA-specifiek zijn, maar dat een bepaalde groep ORA transcriptiefactoren een rol speelt bij de integratie van JA- en ethyleensignalering, zoals ook door anderen gevonden is voor de transcriptiefactor ERF1. Van JA en ethyleen is bekend dat ze samenwerken bij de respons op bepaalde pathogenen, en de waarnemingen beschreven in Hoofdstuk 2 bevestigen dat beeld en geven aan welke transcriptiefactoren hier waarschijnlijk bij betrokken zijn.

Omdat zij de meeste homologie vertonen met de *ORCA* genen, werd speciale aandacht besteed aan *ORA1*, *2* en *4*, zoals beschreven in **Hoofdstuk 3**. Net als de ORCA eiwitten bleken ORA1, 2 en 4 *in vitro* te binden aan het JERE in de *STR* promoter van *C. roseus*. Studie van mutaties van het betreffende promoterfragment maakte duidelijk dat de bindingsspecificiteit van ORA1/2 en ORA4 overeenkomt met die van, respectievelijk, ORCA1 en ORCA2/3. Om een idee te krijgen van de genen waarvan de expressie mogelijk door deze ORAs wordt gereguleerd, werden transgene Arabidopsis planten gemaakt waarin *ORA1*, *2* en *4* apart tot overexpressie werden gebracht. Overexpressie van elk van deze genen bleek te resulteren in een verhoogde expressie van de afweergenen *PDF1.2*, *HEL*, *CHIB* en *ADC2*. Dit veronderstelt een directe rol van deze ORAs bij de expressie van deze genen. Werk van andere groepen liet zien dat overexpressie van *ERF1* verhoogde *PDF1.2*, *HEL*, en *CHIB*-expressie geeft, en dat *ERF2* (= *ORA2*) betrokken is bij expressie van *PDF1.2*, *CHIB* en *THI1.2*. Dit past

in het door ons gevonden patroon. De precieze rol van ORA1, 2 of 4 is nog onduidelijk. Gelet op het feit dat de expressie van *ERF1* en de ERF1 doelwitgenen extra hoog geïnduceerd worden na een behandeling met zowel JA als ethyleen, verdient ook de eventuele rol van ethyleen bij de interpretatie van de functies van deze ORAs extra aandacht.

De relatie tussen JA- en SA-signalering krijgt aandacht in **Hoofdstuk 4.** Hoewel eerder verondersteld werd dat SA niet betrokken is bij JA-geïnduceerde expressie van *ORA* genen (Hoofdstuk 2), bleken SA en verwante fenolische verbindingen (zoals benzoëzuur en kaneelzuur) de expressie van *ORA* genen wel te beïnvloeden. SA induceert de expressie van *ORA1*, *2*, *44* en *68*. De expressie van *ORA37* en *59* wordt daarentegen door SA geremd. De overige *ORA* genen zijn niet SA-responsief. Het eiwit NPR1, betrokken bij de meeste vormen van SA-signalering, speelt bij deze effecten opmerkelijk genoeg geen rol. Ook de aanwezigheid van het NahG eiwit, dat SA omzet in catechol, heeft geen remmende werking. Dit geeft aan dat in plaats van een specifieke SA-signalering, er sprake is van een respons op kleine fenolische verbindingen, inclusief SA en catechol, waarbij een andere signaaltransductieroute actief is dan de "klassieke" SA-weg. De respons op fenolen bleek onafhankelijk van JA- en ethyleen-signalering. Toediening van JA bleek wel de remmende werking van SA op de expressie van *ORA59* op te heffen, maar niet die van *ORA37*. Deze gegevens hebben een nieuwe factor (kleine fenolische verbindingen) toegevoegd aan het netwerk van stressreacties rond JA-geïnduceerde genexpressie. Deze waarneming is relevant omdat JA een belangrijke rol speelt bij wondreacties in planten, en verwonding vaak gepaard gaat met aanwezigheid van kleine fenolische verbindingen. Verder heeft onderzoek door Leidse collega's uitgewezen dat toediening van elicitor in Catharanthus cellen leidt tot productie van dihydroxybenzoëzuur.

Dit proefschrift beschrijft de identificatie van 14 JA- geïnduceerde genen coderend voor AP2-domein transcriptiefactoren, de ORAs, in Arabidopsis. De beschreven resultaten rechtvaardigen de hypothese dat ORA eiwitten een centrale rol spelen in stress-gerelateerde JA-signalering en in expressie van bepaalde afweergenen. Bovendien speelt een aantal ORAs waarschijnlijk een rol bij de integratie van verschillende stress-signaaltransductie wegen, en wel die van SA, verwante fenolische verbindingen, en ethyleen. De precieze rol van elke ORA

moet nu bepaald worden met "gain-of-function" (overexpressie) en "loss-of-function" (knock-out mutanten) strategieën.

Curriculum Vitae

Mirna Atallah was born on the 22nd of February 1975 in Byblos, Lebanon. She attended high school at "Notre Dame de la Paix" in Beirut. In 1992, she entered the Faculty of Agricultural Sciences, at the Lebanese University. In 1996 she received an award from the International Center for Advanced Mediterranean Agronomic studies to finish her Bachelor degree at the Mediterranean Agronomic Institute of Chania, Greece. She joined the department of Horticultural Sciences and Technology in October 1996 and received her DSPU (Diploma of Specialized Post-graduate Studies) in June 1997. From September 1997 until December 1998 she stayed in Greece and received her Master degree from the department of Horticultural Sciences and Technology. She was involved in the project: "Cloning and characterization of novel plant antioxidant genes". In 1999, she worked from April until December at the lab of Dr. Jonathan Chernoff at the Fox Chase Cancer Center in Philadelphia, USA. In December 1999, she started the present work under the supervision of Dr. Johan Memelink at the Institute of Biology at Leiden University, Leiden, The Netherlands.