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

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Chapter 5

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

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

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

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

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

Results

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

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

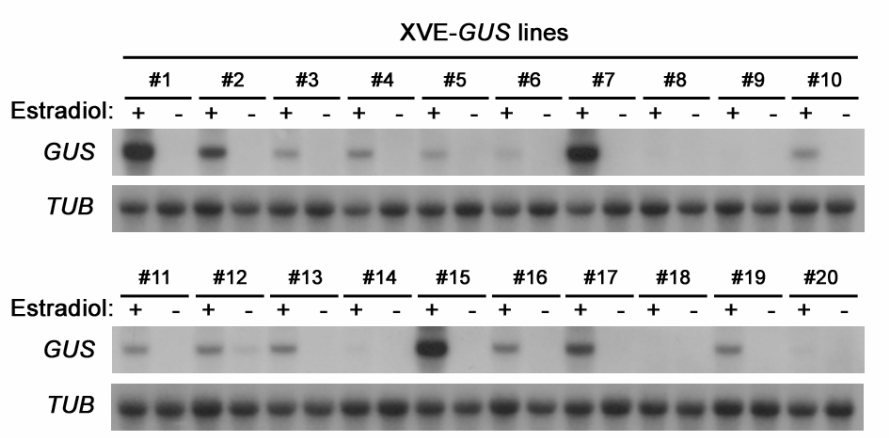

Figure 1. The *GUS* gene is induced by estradiol in the majority of independent transgenic XVE-*GUS* lines. Ten-days-old seedlings from 20 independent transgenic lines containing the *GUS* gene under the control of the XVE module were treated for 24 hours with 2 µM estradiol (+) or with the solvent DMSO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

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

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

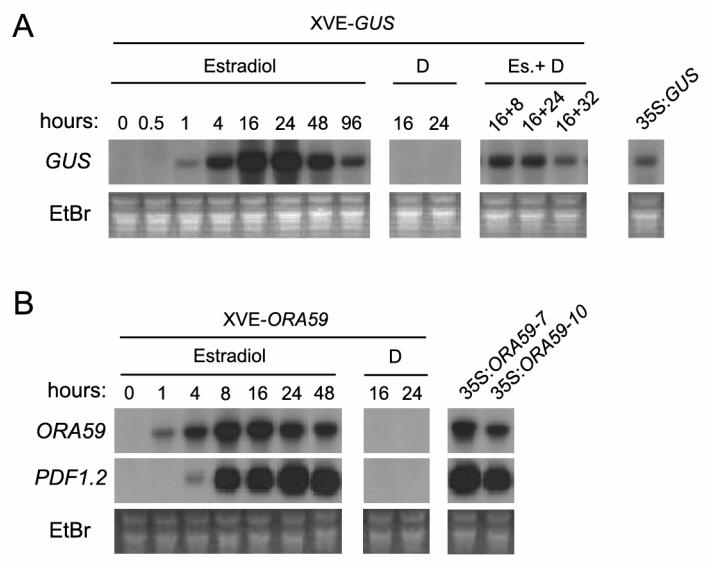

Figure 2. Time course analysis of XVE-controlled transgene expression in response to estradiol.
(A). Two-weeks-old XVE-*GUS* (line 15) seedlings were treated for the number of hours indicated with 2 µM estradiol (Es) or the solvent DMSO (D). **(B).** Two-weeks-old XVE-*ORA59* (line 6) seedlings were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

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

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

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

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

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

Identification of ORA-regulated genes

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

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

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

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

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

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

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

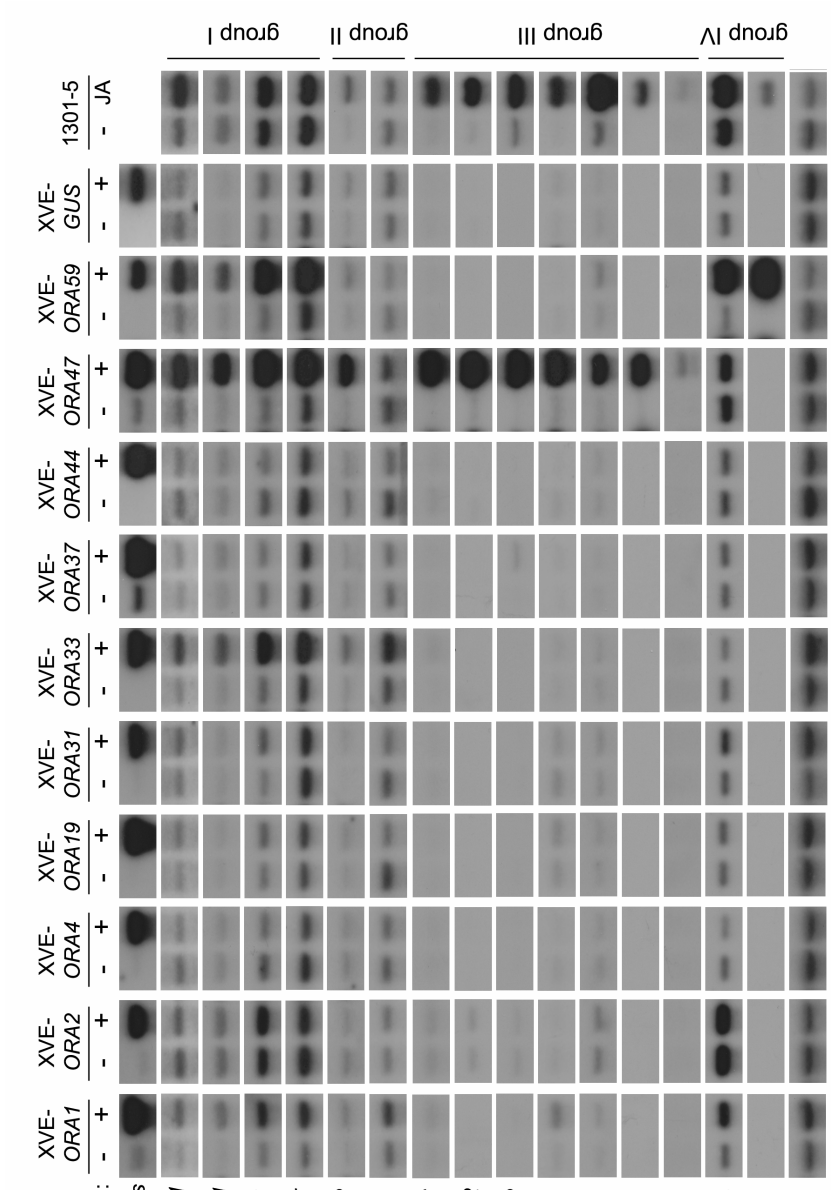

Figure 3. Expression of selected JA-responsive genes in the different *ORA*-overexpressing lines. Two-weeks-old Arabidopsis seedlings from transgenic lines carrying the *ORA1, ORA2, ORA4, ORA19, ORA31, ORA33, ORA37, ORA44, ORA47, ORA59* or *GUS* gene in the XVE module, were treated for 24 hours with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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Biological materials, growth conditions and treatments

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

Surface-sterilized seeds were grown on plates containing MA medium (Masson and Paszkowski, 1992) supplemented with 0.6% agar. Transgenic plants from the T2 generation were selected on solid MA medium containing 100 mg/L timentin and 20 mg/L hygromycin. Following stratification for 3 days at 4ºC, seeds were incubated at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days, after which 15 to 20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml MA medium and were incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated for different time periods with 2 µM estradiol (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.2% final concentration). As control, seedlings were treated with 0.2% DMSO. Treatments with JA were performed by adding 50 µM JA (Sigma) dissolved in DMSO (0.1% final concentration) to the liquid medium. As controls, seedlings were treated with 0.1% DMSO.

Binary constructs and plant transformation

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

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

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

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

Northern blot analyses

Total RNA was extracted from frozen tissue by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70 % ethanol, and resuspended in water. As described by Memelink et al. (1994), 10 µg RNA samples were subjected to electrophoresis on 1.5% agarose/1% formaldehyde gels, and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). All probes were ^{32}P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994), with minor modifications (Chapter 3). Blots were exposed on X-ray films (Fuji, Tokyo, Japan).

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

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