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

Pré, M.

# Chapter 4

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

Martial Pre and Johan Memelink

**Abstract**

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

**Introduction**

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

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

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

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

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

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

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

## Results

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

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

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

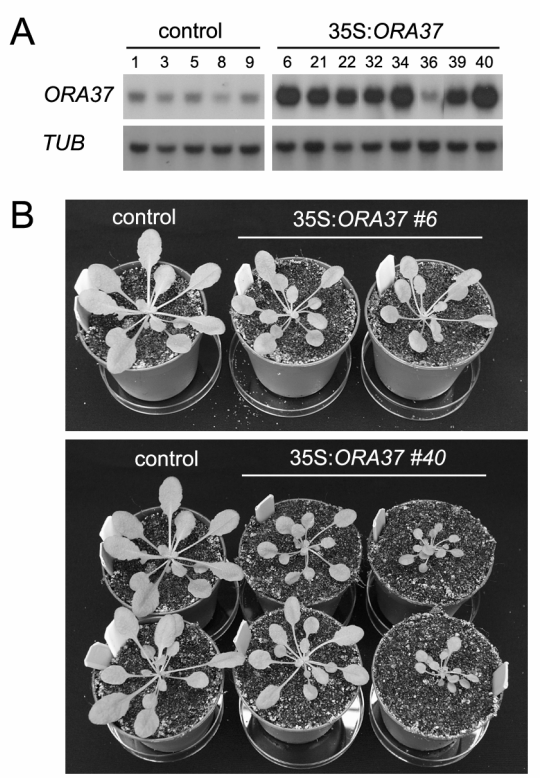


**Figure 1.** Overexpression of the *ORA37* gene in plants results in a mild dwarf phenotype. **(A).** RNA gel blot analyses with two-weeks-old independent transgenic lines constitutively overexpressing the *ORA37* gene (35S:*ORA37*) and control lines. The control lines (1301 lines) carry the *GUS* gene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

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

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

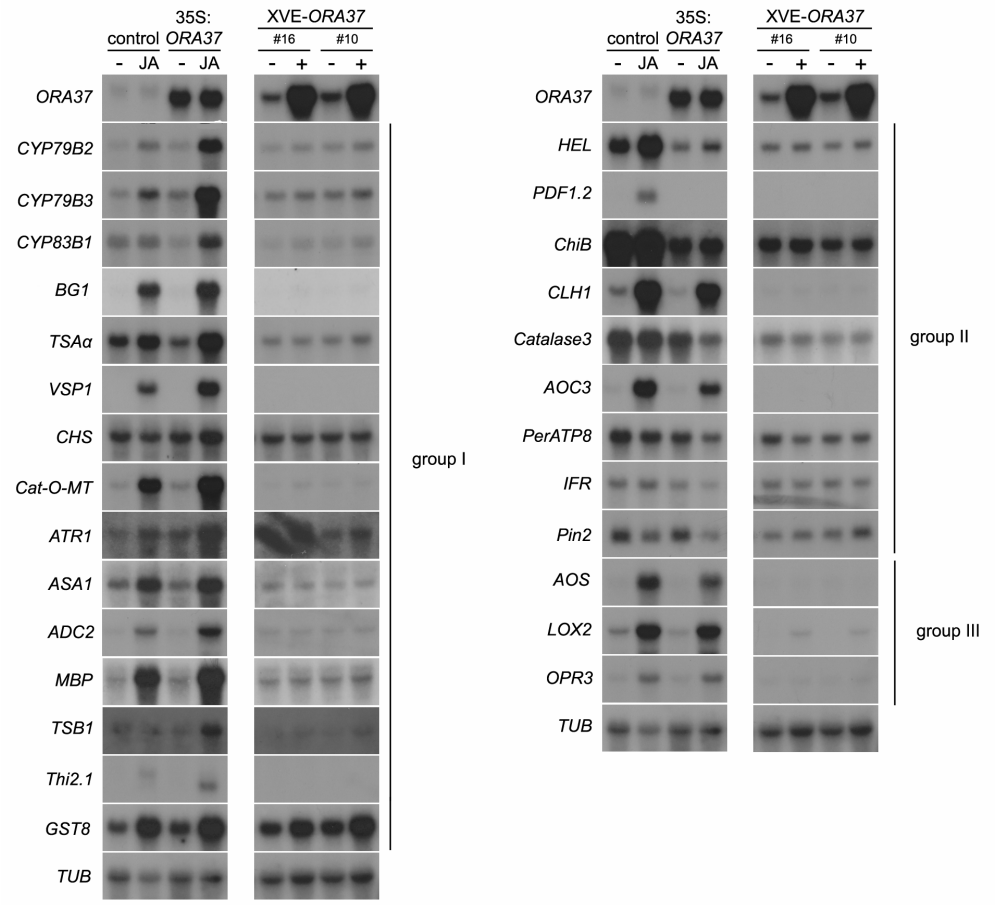


**Figure 2.** Overexpression of *ORA37* positively or negatively affects the JA-induced expression of distinct sets of JA-responsive genes. Two-weeks-old seedlings from transgenic line 35S:*ORA37* (line #40) and the control line (1301-5) were treated for 8 hours with 50 µM JA or with 0.1% of the solvent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

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

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

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

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

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

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

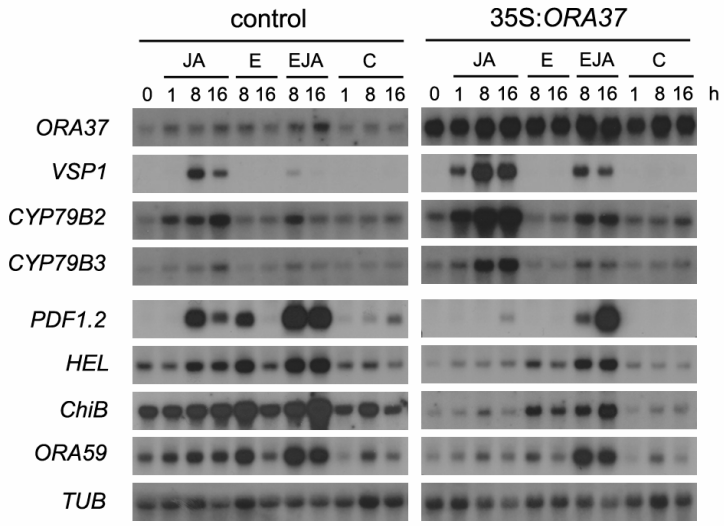


**Figure 3.** Overexpression of *ORA37* positively or negatively affects the JA- and/or ethylene-induced expression of distinct sets of JA-responsive genes. Two-weeks-old seedlings from transgenic line 35S:*ORA37* (line #40) and the control line (1301-5) were treated for the number of hours (h) indicated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

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

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

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

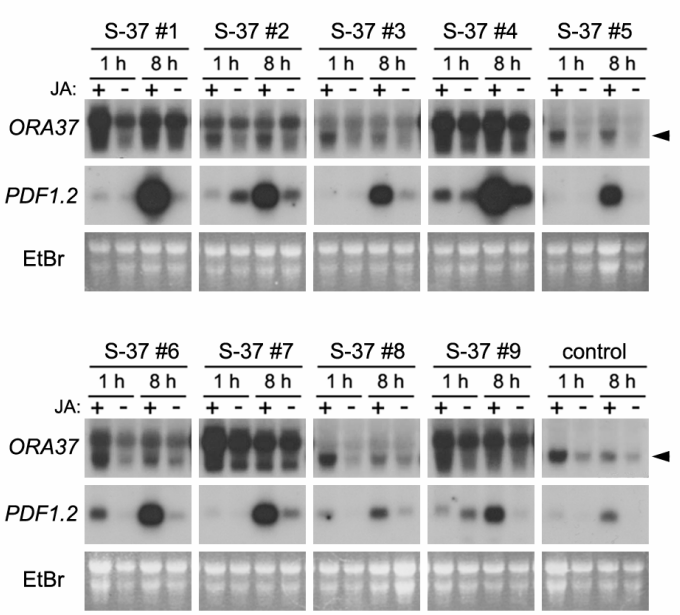


**Figure 4.** Post-transcriptional silencing of *ORA37* causes increased JA-responsive expression of *PDF1.2.* Two-weeks-old seedlings from nine independent transgenic lines carrying the *ORA37*-silencing construct (S-37) and a transgenic control line were treated for 1 or 8 hours with 50 µM JA (+) or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

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

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

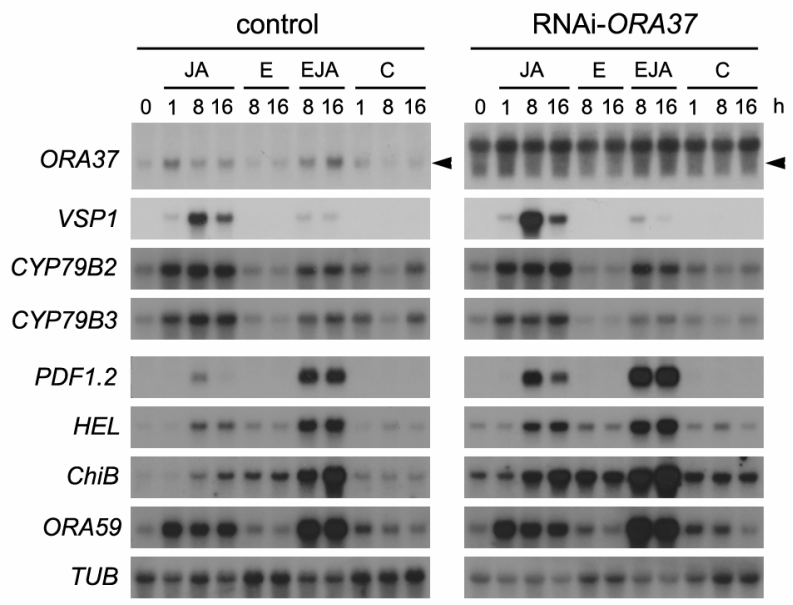


**Figure 5.** Post-transcriptional silencing of the *ORA37* gene enhances the JA- and/or ethylene-responsiveness of several defense genes. Two-weeks-old seedlings from transgenic *ORA37*-silencing line # 4 (RNAi-*ORA37*) and a control line were treated for the number of hours (h) indicated with 50 µM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

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

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

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

**ORA37 acts antagonistically to the transcription factor ORA59**

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

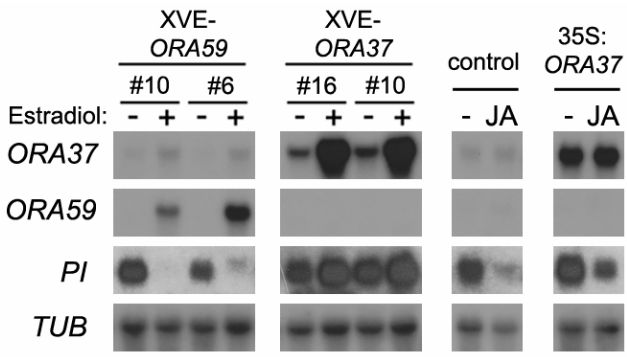


**Figure 6.** ORA37 and ORA59 have opposite effects on the expression of the *PI* gene. Two-weeks-old seedlings from transgenic line 35S:ORA37 (line #40) and the control line (1301-5) were treated for 8 hours with 50 µM JA or with 0.1% of the solvent DMSO (-). Two-weeks-old seedlings from independent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

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

**Discussion**

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

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

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

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

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

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

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

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

The ethylene signaling pathway was shown to repress the wound-induced expression of several wound-responsive genes, including the *VSP1* and *CYP79B2* genes (Rojo et al., 1999; Mikkelsen et al., 2000). Similarly, the JA-induced expression of the *VSP1* and *CYP79B2* genes was reduced in control plants treated with a combination of ethephon and JA compared to control plants treated with JA alone (Figure 3). This indicates that ethylene has a negative effect on a branch of the JA signaling pathway. Therefore, the role of ORA37 might be to repress the inhibitory effect of ethylene on the expression of a subset of JA- and/or wound-inducible genes. Overexpression of the ethylene-responsive AP2/ERF gene *ERF1* has been shown to inhibit the expression of the *VSP2* gene in response to JA (Lorenzo et al., 2004). It was suggested that the negative regulation of the *VSP2* gene by ethylene is executed through ERF1. It is possible that ORA37 antagonizes the ERF1-mediated negative effect of ethylene, presumably by competing for the same AP2/ERF-domain binding site. Identification of the direct target genes of ORA37 is an important prerequisite to unravel the precise function of ORA37 in the JA and/or ethylene signaling pathways. In the absence of ethylene treatment, JA treatment in *ORA37*-overexpressing plants resulted in higher expression of the *VSP1* and *CYP79B2* genes compared to JA-treated control plants. The increased expression of these genes in *ORA37*-overexpressing plants in response to JA alone may be due to ORA37-mediated repression of the negative effect of the basal level of ethylene.

**ORA37 versus ORA59**

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

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

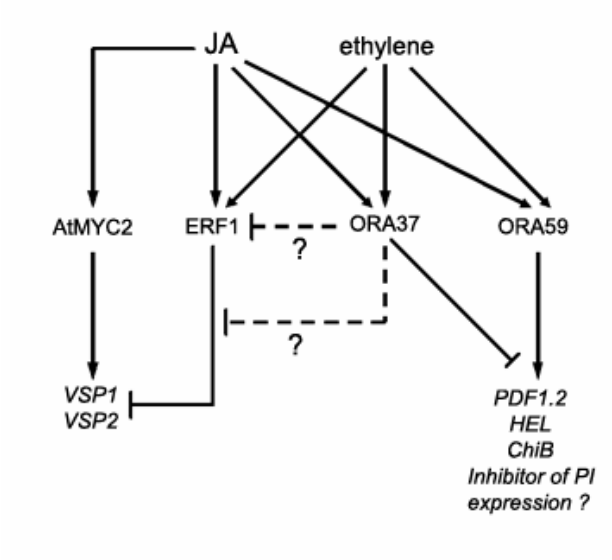


**Figure 7.** Model showing the role of several transcription factors in JA and ethylene signaling. Perception of JA and/or ethylene leads to the transcriptional activation of the transcription factors *ORA37*, *ORA59*, *ERF1* and *AtMYC2*. ORA59 integrates both JA and ethylene signals to control the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

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

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

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

## Materials and Methods

### *Biological materials, growth conditions and treatments*

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

Surface-sterilized seeds were grown on plates containing MA medium (Masson and Paszkowski, 1992) supplemented with 0.6% agar. Transgenic plants from the T2 generation were selected on solid MA

medium containing 100 mg/L timentin and either 25 mg/L kanamycin for *ORA37*-silenced plants and related control plants or 20 mg/L hygromycin for *ORA37*-overexpressing plants and related control plants. Following stratification for 3 days at 4ºC, seeds were incubated at 21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) for 10 days, after which 15 to 20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml MA medium and incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated for different time periods with 50 μM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.1% final concentration), 1 mM of the ethylene-releasing compound ethephon (Sigma) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) or a combination of JA and ethephon. As controls, seedlings were treated with 0.1% DMSO, 0.5 mM sodium phosphate or a combination of both. Transgenic plants carrying an XVE expression module containing the *ORA37* or *GUS* gene were treated with 2 μM estradiol (Sigma) dissolved in DMSO (0.2% final concentration). As a control, seedlings were treated with 0.2% DMSO.

### *Binary constructs and plant transformation*

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

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

For the construction of transgenic lines showing post-transcriptional gene silencing of the *ORA37* gene, the *ORA37* ORF digested with BamHI was cloned into pIC-19R (Marsh et al., 1984) digested with BamHI such that the 5'-end of the *ORA37* ORF flanked the EcoRI restriction site. The *ORA37* insert was excised from pIC-19R with EcoRI/SalI and cloned into the pHANNIBAL vector (accession number AJ311872) to generate pHAN-ORA37as. To create an inverted repeat, the *ORA37* ORF was cloned as a BamHI fragment into pIC-20H (Marsh et al., 1984) digested with BglII. The *ORA37* ORF was excised from pIC-20H with HindIII and cloned into pHAN-ORA37as to generate a pHANNIBAL construct containing the *ORA37* ORF as an inverted repeat. For the construction of the RNAi control line (S-GUS #6), the *GUS* ORF was excised from GusSH (Pasquali et al., 1994) with SalI/HindIII and cloned into pHANNIBAL digested with XhoI/HindIII. The pHANNIBAL expression cassettes were cloned into the binary vector pART27 (Gleave, 1992) using NotI.

For the construction of plants with estradiol-responsive transgene expression (XVE), the *ORA37* open reading frame was excised from pBluescript SK+ with ApaI/SpeI and cloned into the binary vector pER8 (Zuo et al, 2000) digested with ApaI/SpeI.

The binary vectors pCAMBIA1300-ORA37 and pCAMBIA1301 were introduced into *Agrobacterium tumefaciens* strain LBA1115 (containing the Vir plasmid pSDM3010). The binary vectors pART27-ORA37 and pART27-GUS were introduced into *A. tumefaciens* strain LBA4404 while pER8-ORA37 was introduced into *A. tumefaciens* strain EHA105. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MA medium containing 100 mg/L timentin and 20 mg/L hygromycin, except for pART27 transformants which were selected on 25 mg/L kanamycin. Details for the construction of the XVE-*ORA59* plants are reported in Chapter 2.

### *Northern blot analyses*

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

DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5'- GGG GTA CCG GAT CCC AAC TTT TCT CGA GCT GAG TGA C- 3' and 5'- GCT CTA GAG AAT TCT AGA CAG GAC GCG ACA TCG G- 3' for *ORA37* (*At3g15210*); 5'- GGG GTA CCG GAT CCT CTT AAG TGG AGA GTT TTC C- 3' and 5'- GCT CTA GAC TCG AGG CCA CGG TGG CTT CTT TTC C- 3' for *ORA59* (*At1g06160*); 5'- CGG GAT CCA TGA AAA TCC TCT CAC TTT- 3' and 5'- CCC TCG AGT TAA GAA GGT ACG TAG TAG AG- 3' for *Vegetative Storage Protein 1* (*VSP1, At5g24780*); 5'- TCA AAC TCT TCG GAT CTC AC- 3' and 5'- CCC AAG CGG TTA AAT GAT CG- 3' for *CYP79B2* (*At4g39950*); 5'- CTC CTT CTT CCT TGC AAA TGG- 3' and 5'- GTC CGT TTA GAT CCA ATC CC- 3' for *CYP79B3* (*At2g22330*); 5'- ATG GTG AGG TTC GAG AAG G- 3' and 5'- CTA GAG TTC TTC CCT CAG C- 3' for *β-Glucosidase1* (*BG1, At1g52400*); 5'- ATG GCG ATT GCT TTC AAA TCC- 3' and 5'- TCA AAG AAG AGC AGA TTT AAG- 3' for *Tryptophan synthase α chain* (*TSAα, At3g06050*); 5'- AAT GAG CTC TCA TGG CTA AGT TTG CTT CC- 3' and 5'- AAT CCA TGG AAT ACA CAC GAT TTA GCA CC- 3' for *Plant defensin 1.2* (*PDF1.2, At5g44420*); 5'- GCT TCA GAC TAC TGT GAA CC- 3' and 5'- TCC ACC GTT AAT GAT GTT CG- 3' for *Chitinase B* (*ChiB, At3g12500*); 5'- CGG GAT CCA TAT GAA GAT CAG ACT TAG CAT AAC- 3' and 5'- CGG GAT CCT CAA ACG CGA TCA ATG GCC GAA AC- 3' for *Hevein-like* gene (*HEL, At3g04720*); 5'- ATG GCT TAT TCT AAG GTT GC- 3' and 5'- CTA GGC ACA TTT GAA ACC AG- 3' for *Protease Inhibitor* (*PI*, *At5g4890*); 5'- CGG AAT TCA TGA GAG AGA TCC TTC ATA TC- 3' and 5'- CCC TCG AGT TAA GTC TCG TAC TCC TCT TC- 3' for *β-tubulin* (*TUB, At5g44340*); 5'- TAT AGC CGG TTT AGT AGC GGC- 3' and 5'- TGT GCC GTA CTC ATT GTA CC- 3' for *CYP83B1* (*At4g31500*); 5'- ATG GTG ATG GCT GGT GCT TC- 3' and 5'- TTA GAG AGG AAC GCT GTG CAA G- 3' for *Chalcone synthase* (*CHS*, *At5g13930*); 5'- ATG GGA CAC CTA ATT CCT C- 3' and 5'- CTA AGC CT TTT CGT TAA CTC- 3' for *Chatechol-O-methyl transferase* (*Cat-O-MT*, *At1g76790*); 5'- CAA TTC AAC CGG TCA AAC CG- 3' and 5'- CAT ATT GTC ATC TTC GTT CC- 3' for *ATR1* (*At5g60890*); 5'- ATG CCT GCT TTA GCT TGC G- 3' and 5'- TCA CGC AGA GAT GTA ATC G- 3' for *Arginine decarboxylase2* (*ADC2*, *At4g34710*); 5'- ATG GCG AAA AAG TTG GAA GC- 3' and 5'- TCA

TTT GGC TAT GGG CGC AAC- 3' for *Myrosinase-binding protein* (*MBP*, *At3g16470*); 5'- CAG GCA CCT CTG CTA CTT TC- 3' and 5'- ATA GCT CCA TGG AGG ACA CC- 3' for *Tryptophan synthase β subunit* (*TSB1*, *At5g54810*); 5'- ATG AAA GGA AGA ATT TTG ATT TTG- 3' and 5'- TTA CAA CAG TTT AGG CGG CC- 3' for *Thionin2.1* (*Thi2.1*, *At1g72260*); 5'- ATG GCG AAC GAG GTG ATT C- 3' and 5'- TTA CTC AGG TAC AAA TTT CTT CC- 3' for *Glutathions S-transferase8* (*GST8*, *At1g78380*); 5'- ATG GCG GCG ATA GAG GAC AG- 3' and 5'- CTA GAC GAA GAT ACC AGA AG- 3' for *Chlorophyllase1* (*CLH1*, *At5g43860*); 5'- ATG GAT CCT TAC AAG TAT CG- 3' and 5'- CTA GAT GCT TGG CCT CAC GTT C- 3' for *Catalase3* (*At1g20620*); 5'- ATG GCT CT TCA GCA GTG TC- 3' and 5'- TTA GTT GGT ATA GTT ACT TAT AAC- 3' for *Allene oxide cyclase3* (*AOC3*, *At3g25770*); 5'- ATG GAG AAG AAT ACT TCT CAA AC- 3' and 5'- CTA ATT GAC ACG TGA ACA ATC- 3' for *Peroxidase ATP8* (*PerATP8*, *At4g30170*); 5'- ATG AAA GAG ACT AAT TTT GG- 3' and 5'- TTA TAC GAA AAT TTT CAA ATA TTC- 3' for *Isoflavone reductase* (*IFR*, *At4g13660*); 5'- ATG GCT TCT ATT TCA ACC CC- 3' and 5'- CTA AAA GCT AGC TTT CCT TAA CG- 3' for *Allene oxide synthase* (*AOS*, *At5g42650*); 5'- ATG ACG GCG GCA CAA GGG AAC- 3' and 5'- TCA GAG GCG GGA AGA AGG AG- 3' for *OPDA reductase3* (*OPR3*, *At2g06050*); 5'- CGG GAT CCG TGC GGA ACA TAG GCC ACG G- 3' and 5'- CGG GAT CCG GAA CAC CCA TTC CGG TAA C- 3' for *Lipoxygenase2* (*LOX2*, *At3g45140*).

.

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