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

Pré, M.

Chapter 3

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

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Abstract

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

Introduction

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues. Jasmonates are linolenic acid (18:3)-derived cyclopentanone based compounds of wide distribution in the plant kingdom and play crucial roles in responses to mechanical and insect wounding, pathogen infection and UV irradiation (Turner et al., 2002). The defense response involving JAs is a two-step process. First, perception of the external stress induces JA biosynthesis. Then, JA production results in signal transduction leading to the expression of a large number of defense-related genes, including genes encoding vegetative storage proteins (VSPs), the plant defensins (PDFs) or enzymes involved in the biosynthesis of protective secondary metabolites (Turner et al., 2002; Atallah and Memelink, 2004; Pauw and Memelink, 2005). Arabidopsis mutants that are impaired in JA production, such as the *fatty acid desaturase fad3/fad7/fad8* (*fad*) triple mutant, or JA perception, such as the *coronatine insensitive1* (*coi1*) mutant, exhibit enhanced susceptibility to a variety of pathogens (Vijayan et al., 1998; Thomma et al., 1998; Norman-Setterblad et al., 2000). This indicates that JA is necessary for resistance against certain pathogens. Several molecular players in the jasmonate signaling network have been characterized (Lorenzo and Solano, 2005).

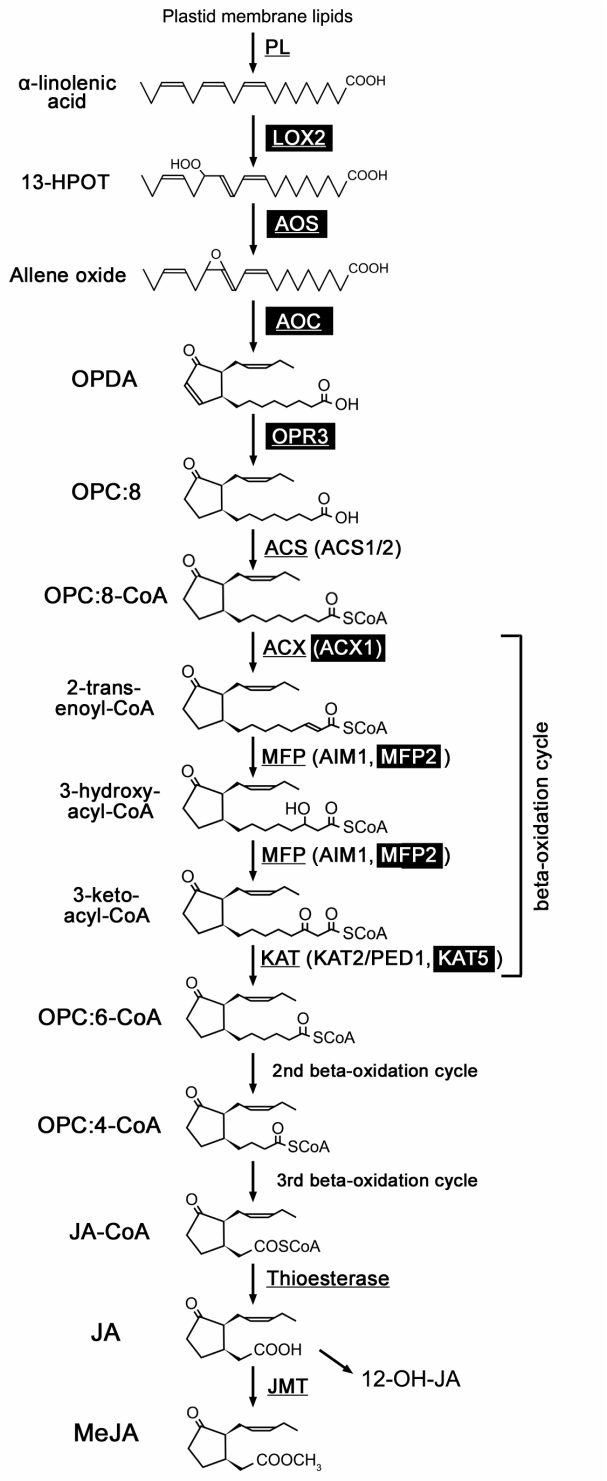

Figure 1. Octadecanoid pathway for JA biosynthesis. Abbreviations for enzyme names are underlined or in brackets. Enzymes whose corresponding genes are upregulated in *ORA47*-overexpressing plants are black-boxed. PL, phospholipase; LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, OPDA reductase; ACS, peroxisomal acyl-coenzyme A (CoA) synthetase; ACX, acyl-CoA oxidase; MFP, multifunctional protein; AIM1, abnormal inflorescence meristem 1; KAT, 3-ketoacyl-CoA thiolase; PED1, peroxisome defective 1; JMT, *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase. 13-HPOT, (9*Z*, 11*E*, 15*Z*, 13*S*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; OPDA, 12-oxo-10,15(*Z*)-octadecatrienoic acid; OPC:8, 3-oxo-2(2'(*Z*)-pentenyl)-cyclopentane-1-octanoic acid; JA, jasmonic acid; 12-OH-JA, 12-hydroxyjasmonic acid; MeJA, methyl jasmonate.

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

Most of the enzymes involved in the so-called octadecanoid pathway leading to JA biosynthesis have now been identified by a combination of biochemical and genetic approaches (Creelman and Mulpuri, 2002; Turner et al., 2002). The enzymes are located in two different subcellular compartments (Figure 1; Vick and Zimmerman, 1984; Schaller, 2001; Wasternack and Hause, 2002). The first part of the pathway directs the conversion of α-linolenic acid to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The second part of the pathway takes place in peroxisomes, where OPDA is reduced by OPDA reductase (OPR3) to give 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), followed by three rounds of beta-oxidation involving three enzymes to yield (+)-7-*iso*-JA which equilibrates to the more stable (-)-JA (Figure 1). Subsequently, JA can be metabolized in the cytoplasm by at least seven different reactions. Among them, methylation to methyl-jasmonate (MeJA) by *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase (JMT; Seo et al., 2001), conjugation to amino acids by JA amino acid synthase (JAR1; Staswick and Tiryaki, 2004) or hydroxylation to 12-hydroxyjasmonic acid (12-OH-JA) are of preferential importance.

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

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

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

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

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

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

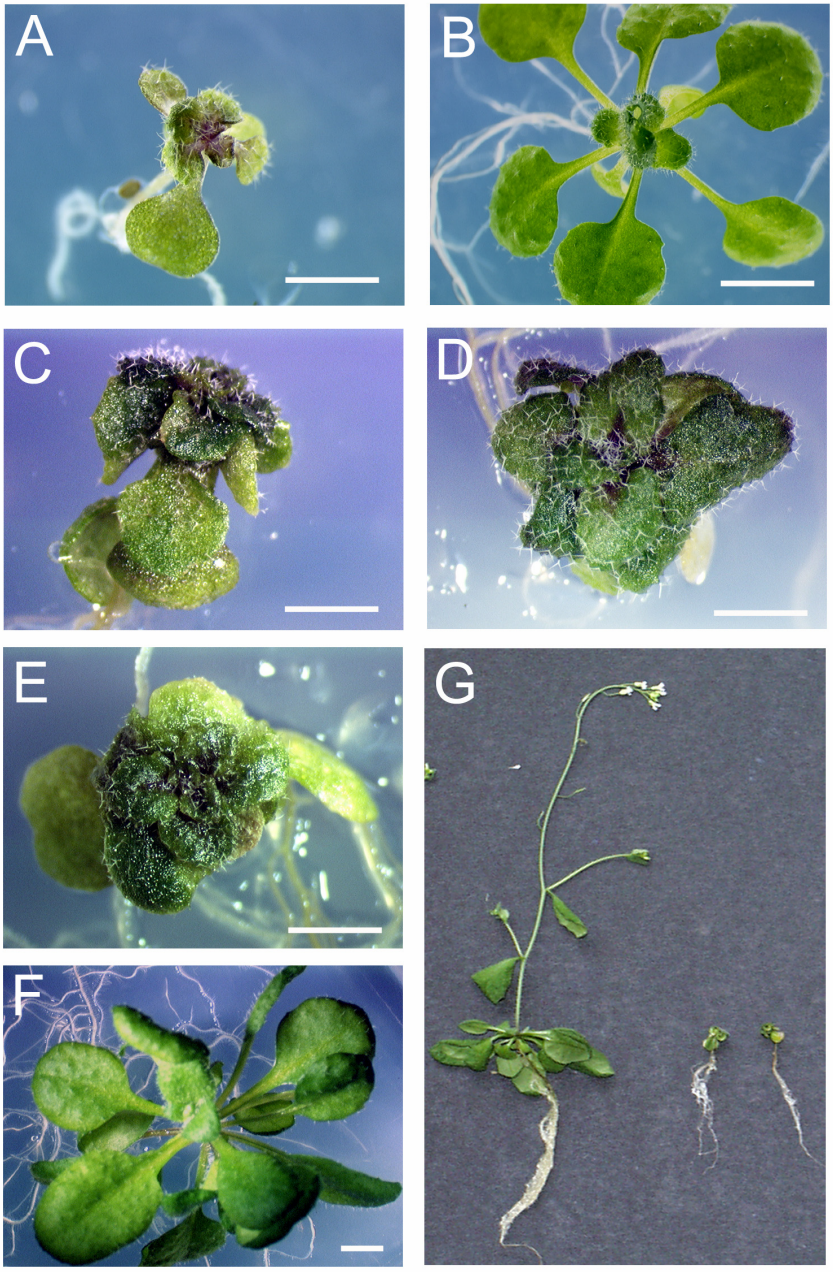

Figure 2. Arabidopsis plants constitutively overexpressing *ORA47* show dwarfism and anthocyanin production. Primary transformants carrying the 35S:*ORA47* construct **(A, C, D, E, G** (right)**)** or empty vector **(B, F, G** (left)**)** were germinated on selective medium. Bars represent 5 millimeters. (**A)** and **(B)**. Three-weeks-old plants. **(C-F).** Five-weeks-old plants. **(G).** Eight-weeks-old mature control plant (left) and two independent primary transformants carrying the 35S:*ORA47* construct (right).

Results

ORA47 overexpression causes severe dwarfism and partially phenocopies exposure to JA

To investigate the role of the transcription factor ORA47 in JA signaling, transgenic Arabidopsis plants constitutively expressing the *ORA47* gene under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter were constructed. As shown in Figure 2, three-weeks-old T1 transformants carrying a 35S:*ORA47* construct (Figure 2A) were smaller than control plants (Figure 2B) and accumulated anthocyanins in the shoot apex. After 5 weeks of growth, 35S:*ORA47* plants (Figure 2C-E) were showing a severe dwarf phenotype with no stem elongation compared to control plants (Figure 2F). Small and disorganized dark rosette leaves were initiated (Figure 2C-E). At a later stage, two-months-old plants remained extremely small compared to control plants (Figure 2G). The sterility caused by the absence of stem and flower development rendered these primary transformants difficult to use for further analyses. Therefore, the function of ORA47 was studied using transgenic plants carrying the *ORA47* gene under the control of the estradiol-inducible XVE system (Zuo *et al*, 2000). T2 seeds from XVE-*ORA47* and XVE-*GUS* transformants were germinated on solid medium with or without 4 µM of the inducer estradiol. Five-days-old XVE-*ORA47* seedlings growing on non-inducing medium showed a normal phenotype similar to the XVE-*GUS* control line (Figure 3A).

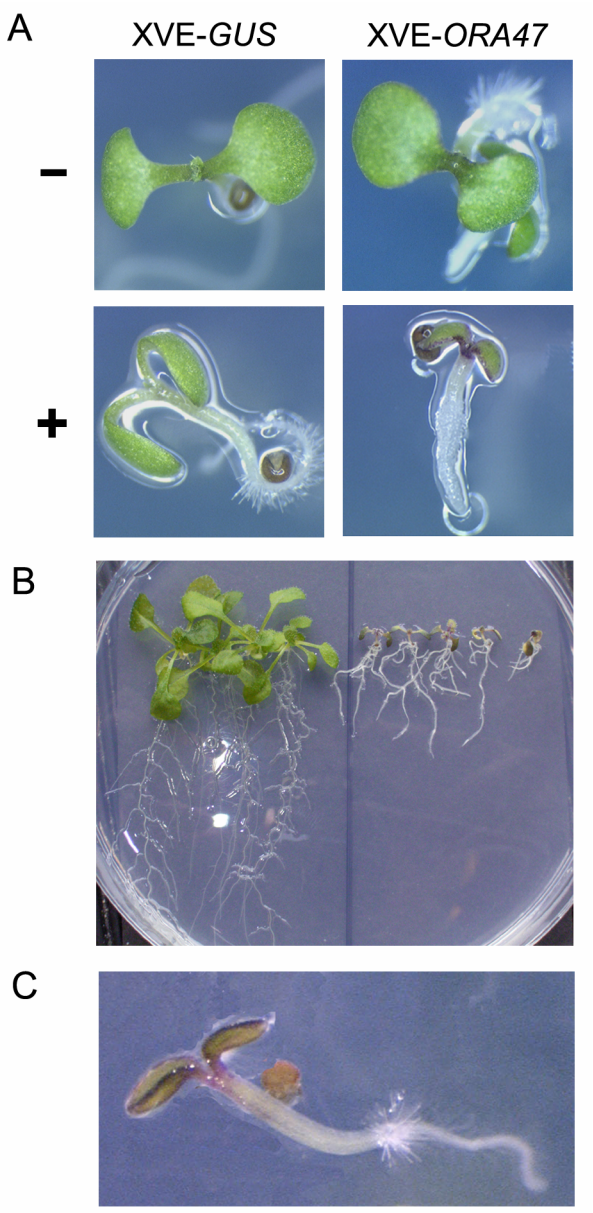

Figure 3. Growth inhibition and anthocyanin production in estradiol-induced *ORA47* overexpressing plants. **(A).** Transgenic seeds carrying an overexpression module with the *ORA47* or *GUS* gene under the control of an inducible promoter (XVE-*ORA47* and XVE-*GUS*, respectively) were germinated for five days on medium with 4 µM of the inducer estradiol (+) or with the solvent DMSO (-). **(B).** Five-days-old seedlings grown on non-inducing medium were transferred to inducing medium containing 4 µM estradiol and grown for ten additional days; left: XVE- *GUS* seedlings; right: XVE-*ORA47* seedlings. **(C).** Four-days-old wild-type Arabidopsis seedling grown on medium containing 50 µM jasmonic acid.

Growth of XVE-*GUS* control seedlings was similar on induction medium and normal medium, indicating that the presence of estradiol in the medium had no effect on plant growth (Figure 3A). Estradiol-treated XVE-*ORA47* seedlings accumulated anthocyanins in the shoot apex and were smaller, with shorter and thicker roots compared to XVE-*GUS* control plants grown under the same conditions. Anthocyanin production and dwarfism were also observed when 5-days-old non-induced XVE-*ORA47* seedlings were transferred to estradiol-containing medium and grown for an additional week (Figure 3B), indicating that these phenotypes were directly correlated to *ORA47* overexpression. These morphological traits were similar to those observed in 35S:*ORA47* seedlings constitutively overexpressing *ORA47*. In wild-type Arabidopsis seedlings, JA or methyl jasmonate (MeJA) treatment inhibits root growth (Staswick et al., 1992) and induces purple coloration in the cotyledon margins and shoot apex, due to the accumulation of anthocyanins (Figure 3C; Feys et al., 1994). Similarly, the *cev1* mutant plants carrying constitutively elevated levels of JA exhibit stunted growth and anthocyanin accumulation (Ellis and Turner, 2001; Ellis et al., 2002). In tobacco, exogenous JA causes a reduction in the number of flower buds (Barendse et al., 1985). Therefore, the phenotypes observed in *ORA47*-overexpressing plants resemble those of JA-treated wild-type plants, which prompted us to investigate whether ORA47 might be involved in the regulation of jasmonate production.

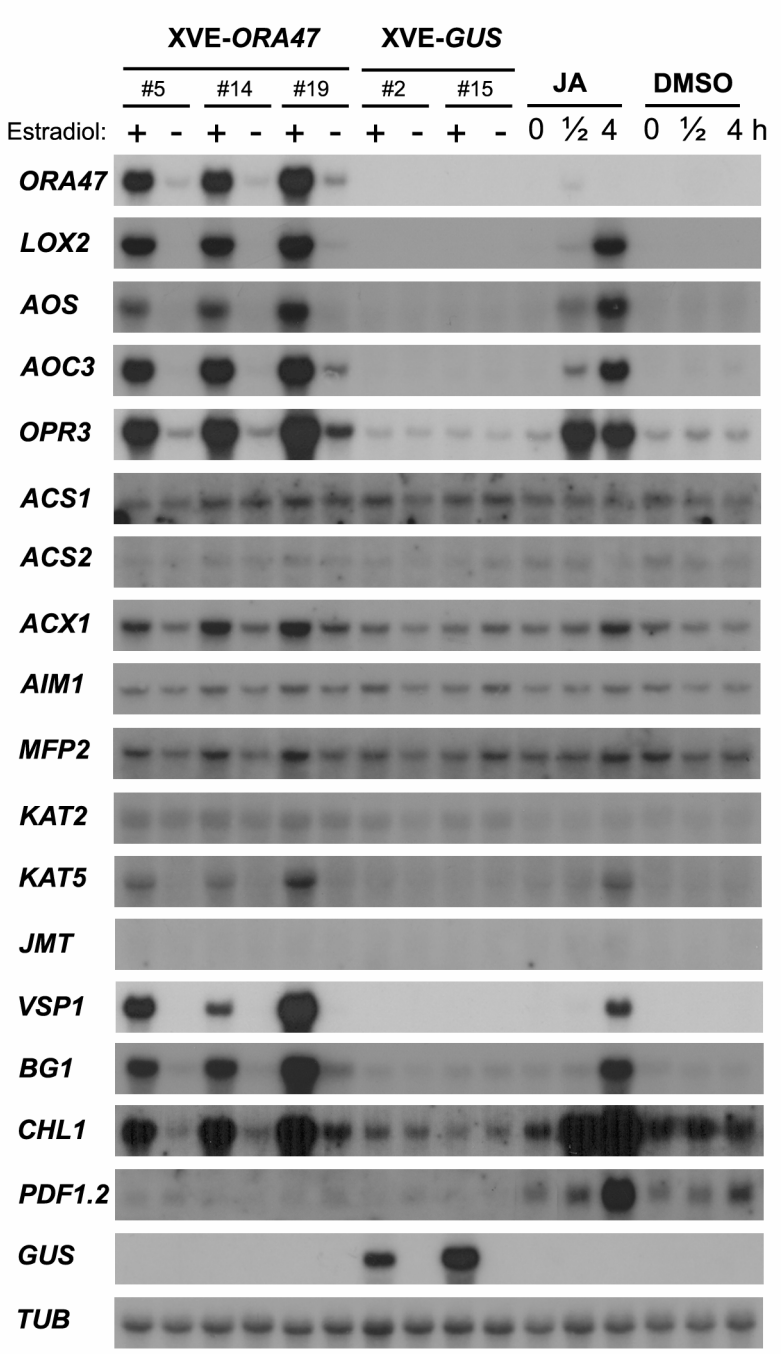

Figure 4. *ORA47* overexpression increases the expression of JA biosynthesis genes. RNA gel blot analyses with two-weeks-old independent XVE-*ORA47* and XVE-*GUS* lines treated for 24 hours with 2 µM estradiol (+) or with the solvent DMSO (-). To study JA-responsive gene expression, two-weeks-old wild-type plants were treated with 50 µM jasmonic acid (JA) or the solvent DMSO for the number of hours (h) indicated. The *TUB* probe was used to verify RNA loading.

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

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

Although the first enzymatic steps leading to OPC:8 synthesis are well characterized (Figure 1), less is known about the identity and regulation of the β-oxidation genes involved in the biosynthesis of jasmonates. Due to their *in vitro* activity, two peroxisomal acyl-coenzyme A (CoA) synthetases, ACS1 (At4g05160) and ACS2 (At5g63380) identified within the group of 25 4-coumarate:CoA-like ligases were suggested as candidates for the coupling of a CoA group to the fatty acid chain of the OPC:8 precursor (Schneider et al., 2005). *At4g05160* gene expression was shown to be induced by MeJA treatment (Schneider et al., 2005). This prompted us to test whether the expression of these genes is controlled by ORA47. The results in Figure 4 show that, under our experimental conditions, the expression of the *At4g05160* and *At5g63380* genes did not increase in XVE-*ORA47* plants following estradiol treatment, or in wild-type plants in response to JA.

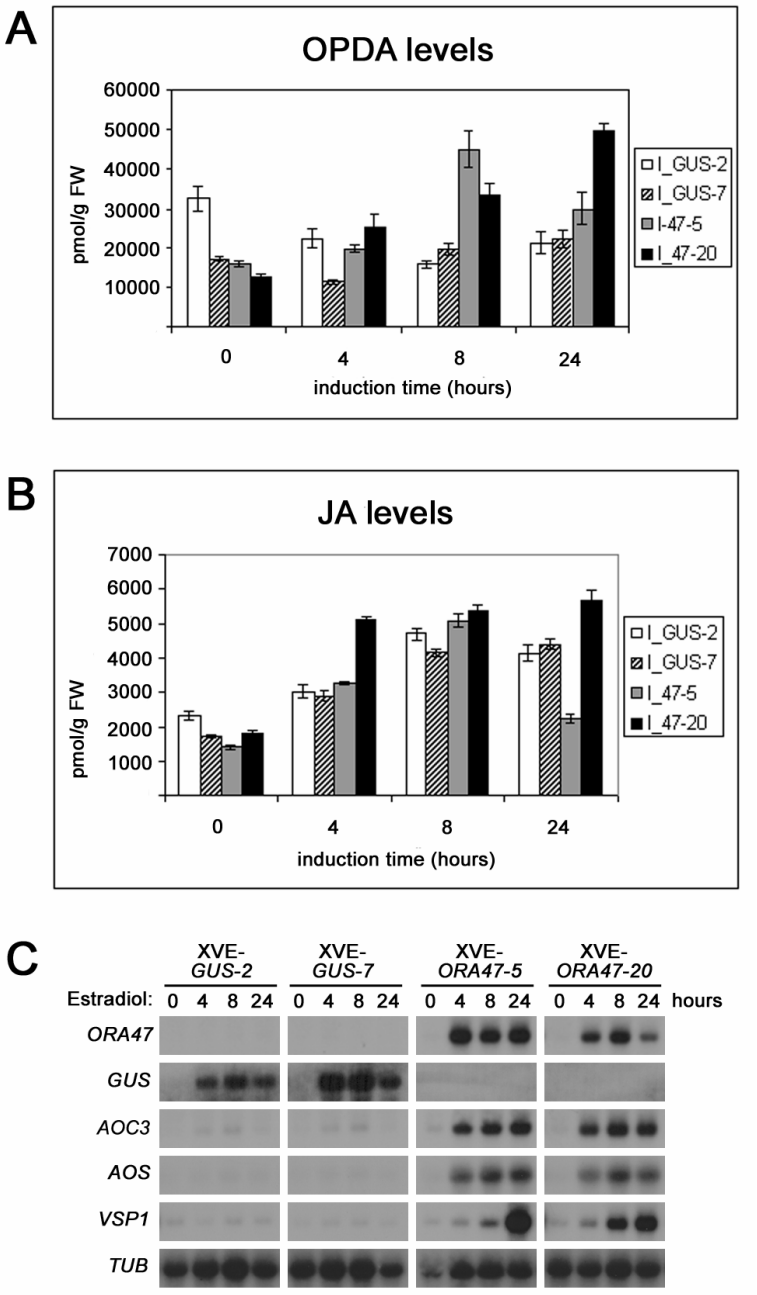

Figure 5. *ORA47* overexpression increases endogenous OPDA levels. Sixteen-days-old seedlings from two XVE-*ORA47* and two XVE-*GUS* lines were treated with 5 µM estradiol and tissue samples were collected after 4, 8 and 24 hours. OPDA **(A)** and JA **(B)** contents in XVE-*ORA47*-5 (grey bars), XVE-*ORA47*-20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

Following attachment of a CoA group, three consecutive cycles of β-oxidation are necessary to yield JA. Each round of β-oxidation requires the concerted action of acyl-CoA oxidases (ACX), multifunctional proteins (MFP) with enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase activities, and 3-ketoacyl-CoA thiolases (KAT; Figure 1). Wounding induces the local and systemic expression of *ACX1* and *KAT2/PED1,* whereas *ACX1* and *KAT5* transcripts accumulate in response to JA (Cruz Castillo et al., 2004). The *ped1* mutant shows a reduced JA level after wounding, indicating that KAT2/PED1 is needed for JA biosynthesis in wounded leaves (Afitlhile et al., 2005). As shown in Figure 4, expression of the *ACX1* and *KAT5* genes, as well as the *MFP2* gene, was slightly induced in XVE-*ORA47* plants treated with estradiol as well as in JA-treated wild-type plants. In contrast, *AIM1,* encoding a multifunctional protein, and *KAT2* transcripts remained constant in all treatments. The *JMT* gene, encoding an enzyme responsible for the methylation of JA to form MeJA (Figure 1), is induced by JA in leaves of mature plants. In contrast, *JMT* expression is undetectable in young seedlings even after JA treatment (Seo et al., 2001). Our results shown in Figure 4 confirmed the absence of *JMT* expression in JA-treated wild-type seedlings, and show that ORA47 induction failed to induce *JMT* gene expression at this developmental stage.

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

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

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

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

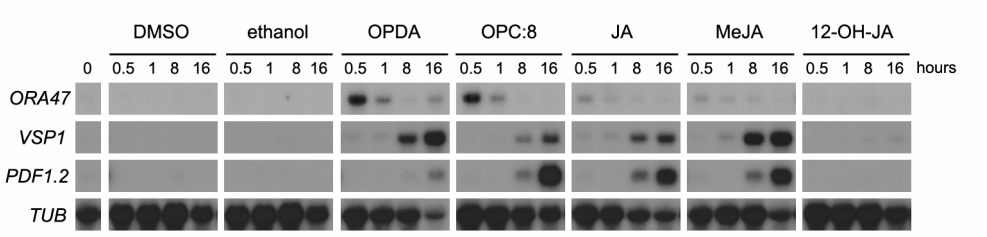

Figure 6. OPDA induces *VSP1* gene expression but has limited effect on the *PDF1.2* gene expression. Two-weeks-old Arabidopsis seedlings were treated for the number of hours indicated with 50 µM OPDA, OPC:8, JA, MeJA or 12-OH-JA, or with the solvents DMSO and ethanol. The RNA gel blot was hybridized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

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

The defense-related genes *VSP1* and *PDF1.2* are two well-characterized markers of the JA response (Benedetti et al., 1995; Penninckx et al., 1996). Overexpression of *ORA47* led to increased expression of the *VSP1* gene, but not the *PDF1.2* gene. Furthermore, plants overexpressing the *ORA47* gene accumulated a high level of OPDA, but not of JA. These results led us to hypothesize that a JA-related signaling molecule synthesized in *ORA47*-overexpressing plants (e.g. OPDA) would activate *VSP1* gene expression, whereas *PDF1.2* gene expression would require another JA-related signaling molecule (e.g. JA) that is not produced in *ORA47*-overexpressing plants. To test whether exogenous application of different JA-related compounds in wild-type plants results in differential expression of the *VSP1* and *PDF1.2* genes, two-weeks-old wild-type seedlings were treated for various time periods with JA or the JA-precursors OPDA and OPC:8 and the JA-derivatives MeJA and 12-hydroxyjasmonic acid (12-OH-JA; Figure 1). Several lines of evidence suggest that a number of JA-related oxylipins, such as OPDA or 12-OH-JA, might have a biological activity distinct from JA or MeJA (Schaller et al., 2005). All treatments were performed using an identical concentration of all compounds for direct comparison of gene induction. As shown in Figure

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

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

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

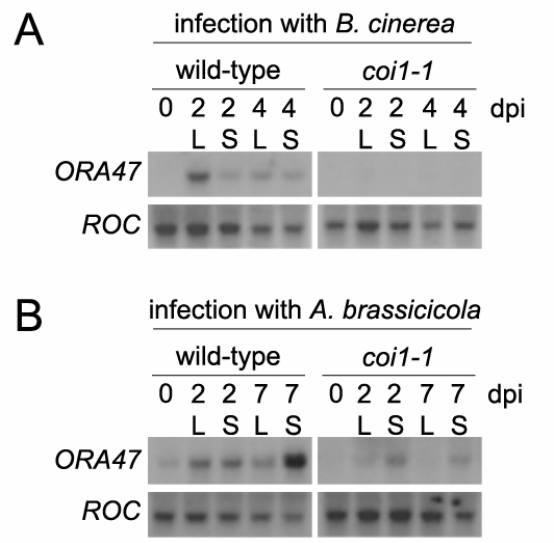

Figure 7. Infection with necrotrophic fungi activates *ORA47* expression in a COI1-dependent manner. Four-weeks-old wild-type and *coi1-1* mutant plants were infected with *Botrytis cinerea* (**A**) or *Alternaria brassicicola* (**B**) and RNA was extracted from infected local (L) and non-infected systemic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

Activation of JA biosynthetic gene expression by ORA47 requires COI1

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

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

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

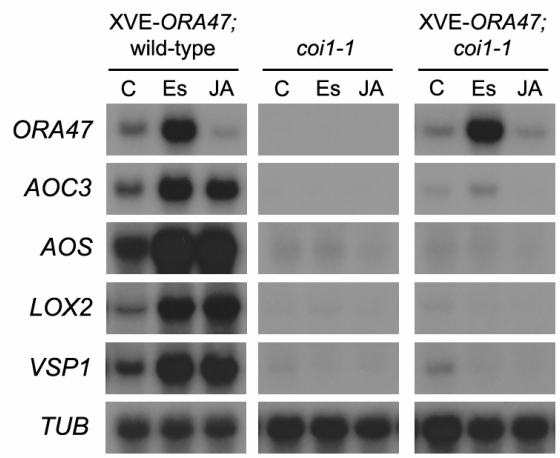

Figure 8. Activation of JA biosynthetic gene expression by ORA47 requires COI1.
RNA gel blot analyses with two-weeks-old *coi1-1* mutant plants and transgenic plants carrying the XVE-*ORA47* expression module in the *coi1-1* (XVE-*ORA47*; *coi1-1*) or wild-type (XVE-*ORA47*; wild-type) backgrounds treated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

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

To further investigate the role of ORA47 in JA signaling leading to gene expression, we analyzed the expression of the JA biosynthetic genes in response to JA in *ora47* mutant plants. The *ora47-1* mutant line SALK_109440 contains a T-DNA inserted in the promoter region of the *ORA47* gene (155 base pairs upstream of the transcriptional start site; data not shown). As shown in Figure 9A, JA failed to induce *ORA47* gene expression in the mutant plants compared to wild-type plants. Instead, *ora47* plants exhibited a low but detectable level of *ORA47* transcript. This indicates that the promoter of the *ORA47* gene has lost the ability

in the *ora47-1* mutant to respond to JA but has conserved the potential to express a basal level of the full-length *ORA47* mRNA. Expression of the JA biosynthesis genes, such as *AOC3* and *LOX2*, was similar in the *ora47-1* mutant compared to wild-type plants in response to JA (Figure 9A).

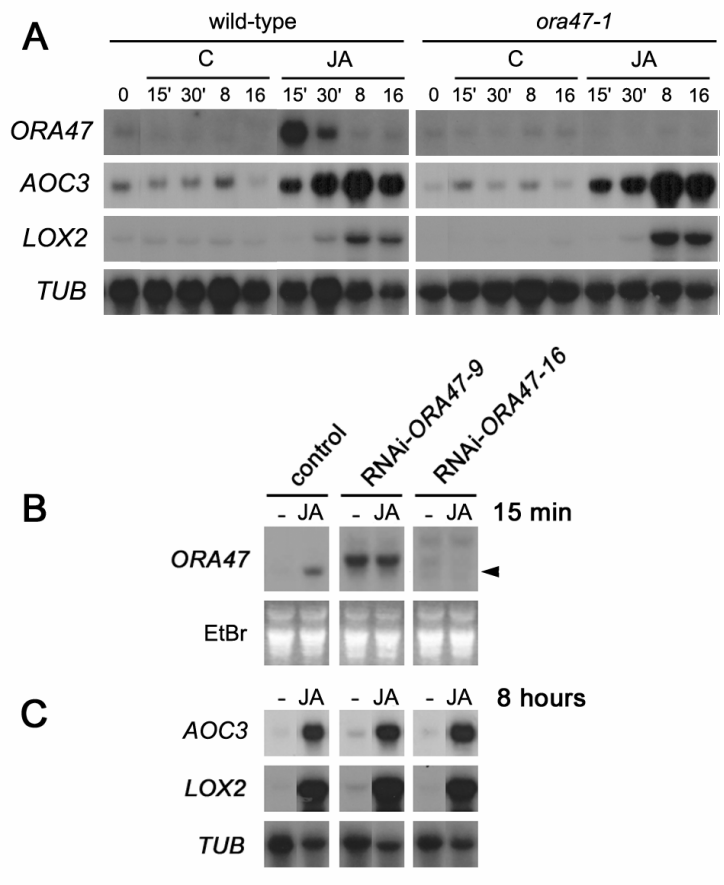

Figure 9. Expression of JA biosynthetic genes in response to JA in the *ora47* mutant and transgenic *ORA47*-silenced plants. **(A)**. RNA gel blot analyses with two-weeks-old *ora47-1* mutant and wild-type plants treated for 15 and 30 minutes or for the number of hours indicated with 50 µM jasmonic acid (JA) or 0.1 % of the solvent DMSO (C). **(B)**. and **(C)**. RNA gel blot analyses with two-weeks-old transgenic *ORA47*-silenced lines 9 and 16 and a control line (S-GUS-6) treated for 15 minutes (**B**) or 8 hours (**C**) with 50 µM jasmonic acid (JA) or 0.1 % of the solvent DMSO (-). The black arrowhead indicates the position of the *ORA47* mRNA on the RNA gel. The RNA gel blots were hybridized with the indicated probes. Equal loading was verified by ethidium bromide (EtBr) staining of the RNA gel prior to blotting (**B**). The *TUB* probe was used to verify RNA loading (**C**).

This indicates that a JA-induced increase in the *ORA47* transcript level is not required for the JA-responsive expression of the JA biosynthesis genes *AOC3* and *LOX2*. Expression of full-

length *ORA47* mRNA in the *ora47-1* mutant line suggested that this line is not a null-allele mutant line. It is likely that the functionality of the ORA47 protein is not compromised in this mutant line. Therefore, transgenic plants showing post-transcriptional silencing of the *ORA47* gene (RNAi-*ORA47*) were constructed. Expression analyses of RNAi-*ORA47* lines treated with JA showed that 27 out of 29 independent lines induced the *ORA47* gene to a similar level than in a JA-treated control line (data not shown), indicating that these transgenic lines did not effectively silence the *ORA47* gene. On the other hand, the lines RNAi-*ORA47-9* and RNAi-*ORA47-16* showed undetectable levels of *ORA47* mRNA after 15 minutes of treatment with JA (Figure 9B). Instead, hybridization with a specific probe for *ORA47* detected a prominent band corresponding to a large RNA species in the RNAi-*ORA47-9* line, independently of the treatment (Figure 9B). This RNA species is most likely the complete unspliced hairpin RNA encoded by the silencing transgene. In the RNAi-*ORA47-16* line, a smeary signal was observed with a probe specific for *ORA47*. As with the *ora47-1* mutant line, expression of the *AOC3* and *LOX2* genes in response to 8 hours treatment with JA was similar in the *ORA47*-silenced lines compared to the control line (Figure 9C), indicating that JA can induce *AOC3* and *LOX2* gene expression in the absence of *ORA47* gene expression. This suggests that the ORA47 transcription factor is not strictly required for the expression of the JA biosynthesis *AOC3* and *LOX2* genes in response to JA.

Discussion

Jasmonic acid is a signaling molecule that regulates certain aspects of development as well as diverse responses to stress. Little is known about the regulatory mechanisms controlling JA biosynthesis. In this report, we demonstrate that ORA47, a member of the Arabidopsis AP2/ERF-domain class of transcription factors, plays a major role in the regulation of jasmonate biosynthesis. We show that overexpression of the *ORA47* gene resulted in the activation of JA biosynthesis genes, and led to elevated amounts of endogenous OPDA, a bioactive signaling molecule as well as a precursor of JA. This is the first identification of a plant transcription factor involved in the regulation of jasmonate biosynthesis.

ORA47 positively regulates the JA biosynthesis genes

Overexpression of *ORA47* gene in Arabidopsis activated the expression of all established genes encoding enzymes of the JA biosynthetic pathway, such as *LOX2, AOS, AOC3* and *OPR3*. Additionally, several genes encoding enzymes of the β-oxidation steps, including *ACX1, MFP2* and *KAT5,* were also expressed at higher levels in *ORA47*-overexpressing

plants. These findings suggest that the enzymes encoded by these genes are likely to be involved in the last steps of JA production. These genes were also induced in response to JA treatment, which is also consistent with a putative role in JA biosynthesis. In contrast, expression of two peroxisomal acyl-CoA synthetases *ACS1* and *ACS2* genes was not induced either by *ORA47* overexpression or by JA treatment. Schneider et al. (2005) showed that the A*CS1* (*At4g05160)* gene was expressed in response to MeJA treatment and that recombinant ACS1 and ACS2 were able to use the JA precursor OPC:8 as a substrate *in vitro*. These contradictory results do not allow us to clarify the role of these two enzymes in JA biosynthesis. It is possible that ACS1 and ACS2 are involved in the JA pathway with no requirement for *de novo* protein synthesis.

Our results indicate that ORA47 controls the expression of the *KAT5* gene, encoding a 3-ketoacyl-CoA thiolase, but not the homologous *KAT2* gene. Under our experimental conditions, expression of the *KAT2* (also referred to as *PED1*) gene was not induced either by *ORA47*-overexpression or by JA treatment. *KAT2* transcripts accumulate in wounded leaves and a *ped1* mutation results in lower accumulation of JA in wounded tissues (Cruz Castillo et al., 2004; Afitlhile et al., 2005), suggesting a role in wound-induced JA production. Moreover, He et al. (2002) suggested a role for KAT2/PED1 in senescence-induced JA synthesis. Therefore it is likely that the *KAT2/PED1* gene is expressed in response to wounding or senescence without the requirement for ORA47, whereas a different signal initiating the auto-stimulatory loop would recruit ORA47 to activate the *KAT5* gene.

ORA47 controls OPDA production

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

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

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

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

Several successful and unsuccessful attempts to modulate JA levels in plants have been described using transgenic approaches. Overexpression of the *AOS* gene in transgenic Arabidopsis and tobacco and of the *AOC* gene in tomato did not alter the basal level of jasmonic acid, but when wounded, transgenic plants produced a higher level of JA than did wounded control plants (Laudert et al., 2000; Stenzel et al., 2003), suggesting that the production of jasmonates is limited by the availability of substrates (free α-linolenic acid or 13-hydroperoxyoctadecatrienoic acid), the levels of which are enhanced after wounding. In contrast, overexpression of *JMT* led to elevated levels of MeJA, while the JA content remained unchanged. Plants overexpressing *JMT* exhibited constitutive expression of JA-responsive genes and increased resistance against *B. cinerea* (Seo et al., 2001). The *cas1* and *cet1* mutant plants contain constitutively high levels of jasmonate (Kubigsteltig and Weiler, 2003; Hilpert et al., 2001). These mutants exhibit a severe growth inhibition phenotype. This phenotype is likely to be due to the high jasmonate contents present in these mutants, as it is also observed in wild-type plants treated with exogenous JA. We speculate that the dwarf phenotype observed in plants constitutively overexpressing the *ORA47* gene is a consequence of high OPDA levels and downstream gene activation. The biosynthesis of OPDA is likely to induce the constitutive expression of a large number of jasmonate-responsive genes, thereby generating a stress condition that compromises plant development and overall fitness. Indeed, overexpression of *ORA47* induced the expression of several JA-responsive genes, including *VSP1, BG1* and *CHL1*.

ORA47 is involved in the JA auto-stimulatory loop

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

still unclear how JA production initially occurs in response to stress and what is the integrator of such stress that leads to JA biosynthesis.

Another hypothesis preferred by us is that an early step following perception of the stress signal involves covalent modifications of pre-existing ORA47 protein without *de novo* protein synthesis. Activation of ORA47 and resulting expression of the JA biosynthesis genes would lead to production of a small amount of jasmonate that would activate transcription of the *ORA47* gene and subsequent amplification of the signal by the feedback loop. Overexpression of the *ORA47* gene in a *coi1* mutant background did not lead to activation of the JA biosynthesis genes, suggesting that these genes are not primary targets of ORA47. However, it is possible that the stress-induced activation of ORA47 requires COI1-dependent modifications (or COI1-dependent co-factors) to bind to the promoters of the JA biosynthesis genes. Within that scenario, some JA should be produced to initiate these modifications. The low but significant induction of *AOC3* observed in the *coi1* mutant background in response to *ORA47* overexpression might lead to the production of that small quantity of JA.

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

Materials and Methods

Biological Materials, Growth Conditions and Treatments

Arabidopsis thaliana ecotype Columbia (Col-0) is the genetic background for all wild-type, transgenic and *coi1-1* and *ora47-1* mutant plants. Seeds were surface-sterilized by incubation for 1 minute in 70 % ethanol, 15 minutes in 50% bleach, and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for three hours (http://plantpath.wisc.edu/~afb/vapster.html).

Surface-sterilized seeds were grown on plates containing MA medium (Masson and Paszkowski, 1992) supplemented with 0.6% agar. Transgenic plants from T1 and T2 generations were selected on MA medium containing either 25 mg/L kanamycin for *ORA47*-silenced plants or 20 mg/L hygromycin for *ORA47*-overexpressing plants. Following stratification for 3 days at 4ºC, seeds were first germinated at

21ºC in a growth chamber (16 h light/8 h dark, 2500 lux) on solid MA medium supplemented with the above mentioned appropriate antibiotics for 10 days, after which 15 to 20 seedlings were transferred to 50 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 ml liquid MA medium without antibiotic and incubated on a shaker at 120 rpm for 4 additional days before treatment.

Treatments with JA were performed by adding 50 μM (+/-)-JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO; 0.1% final concentration) to the liquid medium. As controls, seedlings were treated with 0.1% DMSO. Transgene expression in plants transformed with pER8 derivatives containing the *ORA47* or *GUS* gene was induced by adding 2-5 μM estradiol (Sigma) dissolved in DMSO (0.1% final concentration) to the liquid medium. As control, seedlings were treated with 0.1% DMSO. Alternatively, XVE-*ORA7* and XVE-*GUS* plants were germinated on solid MA medium containing 4 μM estradiol or 0.1 % DMSO as control (Figure 3A).

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

Treatments with the different oxylipins were performed at a final concentration of 50 μM. The compounds JA, MeJA, OPC:8 and 12-OH-JA were dissolved in DMSO whereas OPDA was dissolved in ethanol. Plants were treated with DMSO and ethanol (0.1 % final concentration) as controls.

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

Binary constructs and plant transformation

The *ORA47* (*At1g74930*) open reading frame (ORF) was PCR-amplified from Arabidopsis genomic DNA using the primer set 5'-GAA GAT CTC ATA TGG TGA AGC AAG CGA TGA AG-3' and 5'-GAA GAT CTT CAA AAA TCC CAA AGA ATC AAA G-3' and, following digestion with BglII, was cloned in pIC-20R (Marsh *et al*., 1984).

For the construction of transgenic lines constitutively overexpressing *ORA47,* the *ORA47* insert was excised with BglII and inserted into pMOG183 (Mogen International, Leiden, The Netherlands) digested with BamHI. The pMOG183 vector is a pUC18 derivative carrying a double-enhanced Cauliflower Mosaic Virus (CaMV) 35S promoter and the *nos* terminator separated by a BamHI site. The CaMV 35S cassette containing the *ORA47* gene in sense orientation was excised with SacI/HindIII and cloned into the binary vector pCAMBIA1300 (accession number AF234296).

For the construction of transgenic lines showing post-transcriptional gene silencing of the *ORA47* gene, the *ORA47* ORF was cloned into pIC-20H (Marsh *et al*., 1984) digested with BglII and into pBluescript SK+ (Stratagene, La Jolla, CA) digested with BamHI, such that the 5'-end of the *ORA47* ORF flanked the EcoRI restriction sites of the respective plasmids. The *ORA47* insert was excised from pIC-20H with EcoRI/XhoI and cloned into the pHANNIBAL vector (accession number AJ311872) to generate pHAN-ORA47as. To create an inverted repeat, the *ORA47* ORF was excised from pBluescript SK^+-ORA47 with XbaI/HindIII and cloned into pHAN-ORA47as to generate pHAN-ORA47sas. For the construction of control lines, the *GUS* ORF was excised from GusSH (Pasquali *et al*., 1994) with SalI/HindIII and cloned into pHANNIBAL digested with XhoI/HindIII. The pHANNIBAL expression cassettes were cloned into the binary vector pART27 (Gleave, 1992) using NotI.

For the construction of plants with estradiol-responsive transgene expression, the *GUS* ORF was excised from pGUSN358→S (Clontech, Palo Alto, CA) with SalI/EcoRI and cloned into pBluescript SK+ to

generate pSK-GUS. The *ORA47* ORF and the *GUS* ORF were excised from the pBluescript vector, with ApaI/SpeI and XhoI/XbaI, respectively, and cloned into the binary vector pER8 (Zuo *et al*, 2000) digested with ApaI/SpeI and XhoI/SpeI, respectively.

The binary vector pCAMBIA1300-ORA47 was introduced into *Agrobacterium tumefaciens* strain LBA1115 (containing the Vir plasmid pSDM3010). The binary vectors pART27-ORA47 and pART27-GUS were introduced into *A. tumefaciens* strain LBA4404 while pER8-ORA47 and pER8-GUS were introduced into *A. tumefaciens* strain EHA105. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MA medium containing 100 mg/L timentin and 20 mg/L hygromycin, except for pART27 transformants which were selected on 25 mg/L kanamycin.

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

RNA extraction and Northern blot analyses

Total RNA was isolated from frozen tissue ground in liquid nitrogen by extraction with two volumes of hot phenol buffer (1:1 mixture of phenol and 100 mM LiCl, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 100 mM Tris) and one volume of chloroform. After centrifugation, the aqueous phase was re-extracted with one volume of chloroform. RNA was precipitated overnight with LiCl at a final concentration of 2 M, washed twice with 70% ethanol, and resuspended in water. Northern blot analyses were performed as described (Memelink et al., 1994) with the following modifications. Ten µg RNA samples were subjected to electrophoresis on 1.5% agarose/1% formaldehyde gels, and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). Blots were prehybridized for several hours in 1 M NaCl, 10% dextran sulfate (sodium salt, Sigma), 1% SDS, and 50 µg/ml denatured salmon sperm DNA at 65ºC before addition of denatured ^{32}P-labeled DNA probes. After overnight hybridization, blots were washed twice at 42ºC for 30 min with 0.1 × SSPE (saline/sodium phosphate/EDTA) and 0.5% SDS. Finally, the blots were washed briefly with 0.1 × SSPE at room temperature. Blots were exposed on X-ray films (Fuji, Tokyo, Japan).

For probe preparation, DNA fragments corresponding to the complete open reading frame of the following genes were PCR amplified using the following primer sets: 5'- CGG GAT CCG TGC GGA ACA TAG GCC ACG G- 3' and 5'- CGG GAT CCG GAA CAC CCA TTC CGG TAA C- 3' for *Lipoxygenase2* (*LOX2, At3g45140*); 5'- ATG GCT TCT ATT TCA ACC CC- 3' and 5'- CTA AAA GCT AGC TTT CCT TAA CG- 3' for *Allene oxide synthase* (*AOS* , *At5g42650*); 5'- ATG GCT CT TCA GCA GTG TC- 3' and 5'- TTA GTT GGT ATA GTT ACT TAT AAC- 3' for *Allene oxide cyclase3* (*AOC3, At3g25770*); 5'- ATG ACG GCG GCA CAA GGG AAC- 3' and 5'- TCA GAG GCG GGA AGA AGG AG- 3' for *OPDA reductase3* (*OPR3, At2g06050*); 5'- ATG GAG AAA TCC GGC TAC GG- 3' and 5'- GCC ATG ATA CAC TCC CAT AAG- 3' for *peroxisomal acyl-CoA synthetase1* (*ACS1, At4g05160*); 5'- CAG CCG TTT GAT TGA CCG GAG- 3' and 5'- CGG TCG AGG CGA TTA GGT TAC- 3' for *peroxisomal acyl-CoA*

synthetase2 (*ACS2, At5g63380*); 5'- AGC AAT CGA GCT CGG TTG AG- 3' and 5'- CAG CTG CTT TGG AAC ATC CG- 3' for *acyl-CoA oxidase1* (*ACX1, At4g16760*); 5'- TGT CAT GCC AGA GTT GCT GC- 3' and 5'- CTT GAG AAC CCC ACT GTA TC- 3' for *abnormal inflorescence meristem1* (*AIM1, At4g29010*); 5'- CAT CTA AGC CAG TTA AAG CTG- 3' and 5'- GCT CCA ACA ATT CGA TCC TG- 3' for *multifunctional protein2* (*MFP2, At3g06860*); 5'- ATG GAG AAA GCG ATC GAG AG- 3' and 5'- TGA GAC ACC AAA GCG TTG TG- 3' for *3-ketoacyl-CoA thiolase2* (*KAT2/PED1, At2g33150*); 5'- ATG GCT GCT TTT GGA GAT GAC- 3' and 5'- TGC TTT AGT CTC AGG GTC CAC- 3' for *3-ketoacyl-CoA thiolase5* (*KAT5, At5g48880*); 5'- ATG GAG GTA ATG CGA GTT CTT C- 3' and 5'- TCA ACC GGT TCT AAC GAG CG- 3' for *S-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase* (*JMT*, *At1g19640*); 5'- CGG GAT CCA TGA AAA TCC TCT CAC TTT- 3' and 5'- CCC TCG AGT TAA GAA GGT ACG TAG TAG AG- 3' for *Vegetative Storage Protein 1* (*VSP1, At5g24780*); 5'- ATG GTG AGG TTC GAG AAG G- 3' and 5'- CTA GAG TTC TTC CCT CAG C- 3' for *β-Glucosidase1* (*BG1, At1g52400*); 5'- ATG GCG GCG ATA GAG GAC AG- 3' and 5'- CTA GAC GAA GAT ACC AGA AG- 3' for *Chlorophyllase1* (*CHL1, At5g43860*); 5'- CGG AAT TCA TGA GAG AGA TCC TTC ATA TC- 3' and 5'- CCC TCG AGT TAA GTC TCG TAC TCC TCT TC- 3' for *β-tubulin* (*TUB, At5g44340*); 5'-CGG GAA GGA TCG TGA TGG A-3' and 5'-CCA ACC TTC TCG ATG GCC T-3' for *ROC* (*At4g38740*). For *ORA47* (*At1g74930*), a specific DNA fragment that shows few homology with other AP2/ERF genes was PCR amplified from Arabidopsis genomic DNA using the following primer set 5'- GGG GTA CCG GAT CCT CTC CTT CTA CAT CTG CAT CTG TTG-3' and 5'- GCT CTA GAC TCG AGT CCC AAA GAA TCA AAG ATTC-3'.

Measurements of JA and OPDA levels

For JA and OPDA measurements, two independent transgenic lines containing the XVE-*ORA47* expression module and two independent transgenic lines containing the XVE-*GUS* expression module were used. Per treatment and per line, 10 mg of surface-sterilized seeds were germinated in 250 ml Erlenmeyer flasks containing 50 ml MA medium and grown for 16 days. Expression of the transgene was induced by adding 5 µM estradiol dissolved in DMSO (0.05% final concentration) to the growth medium. Seedlings were collected at time zero and after 4, 8 and 24 hours, frozen and ground in liquid nitrogen. One aliquot was kept for RNA extraction while the rest of the powdered tissue was used to measure OPDA and JA levels. Plant material from 20 seedlings was pooled to minimize biological differences and this was done in triplicate. The powdered tissue (500 mg) was homogenized with 10 ml 80% (v/v) methanol after adding 100 ng ($^2H_6$)JA and 100 ng ($^2H_5$)OPDA, respectively, as internal standards. The homogenate was filtered, and the eluate passed through a column filled with 3 ml DEAE-Sephadex A25 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) (Ac^--form, methanol). The column was washed with 3 ml methanol and subsequently with 3 ml 0.1 M acetic acid in methanol. Eluents with 3 ml of 1 M acetic acid in methanol and 3 ml of 1.5 M acetic acid in methanol were collected, evaporated and separated on preparative HPLC using an Eurospher 100-C18 column (5 µm, 250 x 4 mm) (Knauer, Berlin, Germany). A gradient of methanol and 0.2 % acetic acid in H_2O (40% to 100%) was used within 25 min. Fractions at R_t 13 to 14.50 min (corresponding to JA) and at R_t 21.75 to 22.50 min (corresponding to OPDA) were combined separately and evaporated. The evaporated samples were

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

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