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**Author:** Khurshid, Muhammad  
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CHAPTER 2

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

Martial Pré¹, Muhammad Khurshid¹, Arnaud Remay¹, Otto Miersch², Claus Wasternack², Bettina Hause², Johan Memelink¹.³

¹Institute of Biology, Leiden University, Sylviusweg 72, P.O. Box 9505, 2300 RA Leiden, The Netherlands
²Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle (Saale), Germany
³To whom correspondence should be addressed. E-mail: j.memelink@biology.leidenuniv.nl; Phone: +31-71-5274751
ABSTRACT
Jasmonates (JAs) are important signaling molecules in plant defense. The regulation of their biosynthesis is not well understood at the molecular level. The expression of genes encoding JAs biosynthetic enzymes is increased by jasmonic acid (JA), indicating that JAs biosynthesis is controlled by a positive feedback loop. Here, we report that the AP2/ERF-domain transcription factor ORA47 functions in the regulation of the JAs biosynthesis pathway. Overexpression of the ORA47 gene conferred JAs-related phenotypes, such as inhibition of growth and anthocyanin production, and induced the expression of all biosynthesis genes of the JAs pathway tested. JAs measurements in ORA47-overexpressing plants showed an increase in the amounts of the JA precursor 12-oxophytodienoic acid (OPDA), JA, the bioactive form jasmonoyl isoleucine (JA-Ile) and the inactive derivative 12-hydroxy-JA. Probably as a consequence of JAs production several JAs-responsive defense genes including the gene encoding vegetative storage protein1 (VSP1) were upregulated in ORA47-overexpressing plants. Our findings indicate that ORA47 acts as the key regulator in the positive feedback loop by controlling the expression of the JAs biosynthesis genes.

INTRODUCTION
Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of oxylipins that regulate plant responses to environmental and developmental cues. JAs are produced in response to mechanical and insect wounding, pathogen infection and UV irradiation (Turner et al., 2002). The biosynthetic enzymes are located in two different subcellular compartments (Fig. 1; Vick and Zimmerman, 1984; Schaller, 2001; Wasternack, 2007 Schaller and Stintzi, 2009). 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
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; 13-HPOT, (9Z, 11E, 15Z, 13S)-13-hydroperoxy-9,11,15-octadecatrienoic acid; OPDA, 12-oxo-10,15(Z)-octadecatrienoic acid; OPC:8, 3-oxo-2(Z,Z)-pentenyl)cyclopentane-1-octanoic acid; JA, jasmonic acid.
of the pathway takes place in peroxisomes, where OPDA is reduced by OPDA reductase (OPR3) to give 3-oxo-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 (Fig. 1). Subsequently, JA can be metabolized in the cytoplasm by many different reactions (Koo and Howe, 2012), including 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; Miersch et al., 2007) (Fig. 2). The major bioactive JAs is the amino acid conjugate jasmonoyl-isoleucine (JA-Ile; Fonseca et al., 2009). Its concentrations are not only determined by JAR1-mediated synthesis but also by catabolism via an ω-oxidation pathway involving members of the CYP94 family of cytochromes P450 (Kitaoka et al., 2011; Koo et al., 2011; Koo and Howe, 2012; Heitz et al., 2012).

Figure 2. Metabolism of JA. Shown are several metabolic reactions relevant for this chapter. Enzymes whose corresponding genes are upregulated in ORA47-overexpressing plants are black-boxed. JMT, S-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase; MES10, MeJA esterase 10; JAR1, JA RESISTANT 1; JA, jasmonic acid; 12-OH-JA, 12-hydroxyjasmonic acid; MeJA, methyl jasmonate; JA-Ile, jasmonoyl isoleucine.
JA-Ile is perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1) (Thines et al., 2007; Sheard et al., 2010) which is part of an SCF complex with putative E3 ubiquitin ligase activity. This leads to degradation of JAZ repressor proteins (Chini et al., 2007; Thines et al., 2007), which sets in motion defense gene expression programmes leading to the expression of a large number of defense-related proteins, including vegetative storage proteins (VSPs), the plant defensins (PDFs) or enzymes involved in the biosynthesis of protective secondary metabolites (Memelink, 2009).

The expression of JAs biosynthesis genes, including LOX2, AOS, AOC and OPR3, is induced by wounding or treatment with exogenous JA or MeJA (Turner et al., 2002; Sasaki et al., 2001; Pauwels et al., 2008). The observation that wounding induces the expression of JAs biosynthesis genes suggests that, at least partly, the wound-induced production of JAs is a result of the increased transcription of genes encoding the pathway enzymes. In addition, transcriptional activation of the JAs biosynthesis genes by (Me)JA indicates that JAs signaling is amplified by a positive feedback loop initiated by JAs (Turner et al., 2002).

The developmental regulation of JAs biosynthesis was reported to be regulated positively and negatively by different classes of TCP (TEOSINTE BRANCHED/ CYCLOIDEA/PCF) transcription factors via the regulation of LOX genes (Schommer et al., 2008; Danisman et al., 2012). A LOX2 promoter with mutated TCP binding sites has a lower basal activity but still responds to MeJA treatment (Schommer et al., 2008), indicating that TCPs control JAs biosynthesis linked to development but not in response to JAs signaling. Candidate transcription factors for regulating the positive feedback loop in JAs biosynthesis were reported by Pauwels et al. (2008). In a transient assay to screen for activators the basic helix-loop-helix transcription factor MYC2 and the AP2/ERF-domain transcription factor ORA47 were able to activate the LOX3 promoter in tobacco protoplasts. Whereas MYC2 has been extensively studied and is one of the major JAs-responsive transcription factors (Lorenzo et al., 2004, Boter et al., 2004; Dombrecht et al., 2007; Memelink, 2009; Pauwels and Goossens, 2011), little is known about the function of ORA47. The ORA47 gene
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responds to (Me)JA treatment (Wang et al., 2008; Pauwels et al., 2008) in a COI1-dependent manner (Wang et al., 2008) and to wounding (Walley et al., 2007). This has led to the hypothesis that ORA47 together with MYC2 may control the positive feedback loop in JAs biosynthesis (Pauwels et al., 2008). However, overexpression of ORA47 using the constitutive CaMV 35S promoter has been reported to result in elevated expression of the defense gene VSP2 whereas a negative effect was found on the expression of the LOX3 gene (Wang et al., 2008). These authors also did not report JAs-related phenotypes in the transgenic plants, such as growth inhibition or anthocyanin production.

In this study we investigated the possible function of the transcription factor ORA47 in JAs signaling. We found that plants constitutively overexpressing the ORA47 gene showed a strong dwarf phenotype and produced anthocyanins. Inducible ORA47 overexpression led to the activation of a large number of genes involved in JAs biosynthesis. Oxylipin measurements in plants overexpressing ORA47 revealed increased JAs levels. In addition, many JAs-responsive defense genes, such as VSP1, were highly expressed in ORA47-overexpressing plants. Our results indicate that ORA47 is responsible for the regulation of the auto-stimulatory loop in JAs biosynthesis.

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 3, three-weeks-old T1 transformants carrying a 35S:ORA47 construct (Fig. 3A) were smaller than control plants (Fig. 3B) and accumulated anthocyanins in the shoot apex. After 5 weeks of growth, 35S:ORA47 plants (Fig. 3C-E) were showing a severe dwarf phenotype with no stem elongation compared to control plants (Fig. 3F). Small and disorganized dark rosette leaves were initiated (Fig. 3C-E). At a later stage, two-months-old
Figure 3. 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).

Plants remained extremely small compared to control plants (Fig. 3G). The sterility caused by the absence of stem and flower development rendered these primary transformants difficult to use for further analyses. Therefore, the
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 (Fig. 4A).

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 (Fig. 4A). 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 (Fig. 4B), 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 (Fig. 4C; Feys et al., 1994). Similarly, cev1 mutant plants having 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 JAs production.

**ORA47 overexpression increases the expression of JAs biosynthesis genes**
To test whether ORA47 might transcriptionally control JAs biosynthesis, we examined the expression of the JAs biosynthesis genes in XVE-ORA47 plants in response to estradiol treatment. RNA gel blots revealed that expression of the
Figure 4. 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.
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LOX2, AOS, AOC2, OPR3 and JAR1 genes, encoding enzymes involved in the synthesis of the bioactive JA-Ile, were strongly induced in XVE-ORA47 plants treated with estradiol (Fig. 5). Similar results were obtained in the three independent XVE-ORA47 transgenic lines. No expression of the biosynthetic genes was observed in the XVE-ORA47 lines without inducer, except for line 19 where AOC2 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 JAs 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, AOC2, OPR3 and JAR1 genes is due to the specific activation of ORA47 transgene expression by estradiol. Whereas LOX2, AOS, AOC2 and OPR3 were induced by JA treatment in 10-days-old wild-type Arabidopsis seedlings (Fig. 5) consistent with other reports (Sasaki et al., 2001), JAR1 was not upregulated at the tested time points.

Although the first enzymatic steps leading to OPC:8 synthesis are well characterized (Fig. 1), less is known about the identity and regulation of the β-oxidation genes involved in the biosynthesis of JAs. 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 OPC8 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 5 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 the expression of JAs biosynthesis genes. RNA gel blot analyses of 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. For gene names see legends of Figs. 1 and 2. VSP1, Vegetative Storage Protein 1; BG1, Beta-Glucosidase 1; PDF1.2, Plant Defensin 1.2.

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; Fig. 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 JAs biosynthesis in wounded leaves (Afitlhile et al., 2005). As shown in Figure 5, 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.

We were unable to detect the expression of the JMT gene, encoding an enzyme responsible for the methylation of JA to form MeJA, suggesting that it did not respond to ORA47 overexpression. It has been reported that JMT is expressed in mature plants but not in seedlings (Seo et al., 2001). However the MES10 gene, encoding an enzyme that can demethylate MeJA to form JA, was strongly induced by estradiol treatment of XVE-ORA47 plants. Similar to the JAR1 gene the MES10 gene was not induced by JA treatment of wildtype seedlings at the tested time points.

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

**ORA47 overexpression increases endogenous JAs levels**

The increase in transcript abundance of JAs biosynthesis genes in ORA47-overexpressing plants prompted us to determine the amounts of JA, its
precursor OPDA, the bioactive JA-Ile and the inactive 12-OH-JA in these plants.
Two-weeks-old seedlings from six XVE-ORA47 and four XVE-GUS lines were treated with 5 µM estradiol and tissue samples were collected after 0, 4, 8 and 24 hours. Endogenous JAs contents were determined by LC-MS analysis.
Estradiol-induced expression of ORA47 led to gradual increases over time in the target JAs levels with on average a 4-fold increase in the OPDA level, a 10-fold increase in the JA level, a 1.8-fold increase in the JA-Ile level and a 9-fold increase in the 12-OH-JA level after 24 hrs (Fig. 6A). RNA was isolated from estradiol-treated seedlings from 2 lines for each construct to monitor gene expression over time. As shown by RNA gel blot analyses, estradiol treatment effectively induced the expression of the ORA47 and GUS genes in the XVE-ORA47 and XVE-GUS lines, respectively, even at the shortest time point (Fig. 6B). Consistent with the results from Figure 5, the AOS, AOC2 and VSP1 genes were expressed exclusively in the XVE-ORA47 lines. The JAs biosynthesis genes were switched on somewhat earlier than the defense gene VSP1.

12-OH-JA is inactive in defense gene induction
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) and are commonly used as marker genes for the two branches of JAs-responsive signaling (Memelink, 2009). It has been reported that 12-OH-JA is inactive in inducing defense genes in potato (Miersch et al. 2008). To test the activity in Arabidopsis we compared responses to OPDA, JA and 12-OH-JA added at identical concentrations. As shown in Figure 7, OPDA and JA effectively induced the expression of ORA47, VSP1 and PDF1.2, whereas 12-OH-JA had no effect on gene expression. Control treatments with the solvents DMSO and ethanol did not affect gene expression (data not shown).

JA activates the JAs biosynthesis genes in an ora47 knock-down mutant and in ORA47-silenced plants
To further investigate the role of ORA47 in JAs signaling leading to gene expression, we analyzed the expression of the JAs biosynthesis genes in
Figure 6. ORA47 overexpression increases endogenous JAs levels. Two-weeks-old seedlings from six XVE-ORA47 and four XVE-GUS lines were treated in triplicate with 5 µM estradiol and tissue samples were collected at time zero and after 4, 8 and 24 hours. (A) Levels of the indicated JAs in pmol/g fresh weight (FW). Values represent means of all lines and replicates ± SE. Significant differences between ORA47 and GUS values are indicated with an asterisk (Wilcoxon test, p < 0.05) (B) Gene expression patterns in 2 lines for each construct. The RNA gel blot was hybridized with the indicated probes. The TUB probe was used to verify RNA loading. Panels with the same probe were on the same blot and exposed for the same time.
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 8A, 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 JAs biosynthesis genes, such as *LOX2* and *AOC2*, was similar in the *ora47*-1 mutant compared to wild-type plants in response to JA (Fig. 8A).

![Figure 7.](image)

**Figure 7.** 12-OH-JA is inactive in defense gene induction. Two-weeks-old Arabidopsis seedlings were treated for the number of hours indicated with 50 µM OPDA, JA, or 12-OH-JA. The RNA gel blot was hybridized with the indicated probes. The TUB probe was used to verify RNA loading. Panels with the same probe were on the same blot and exposed for the same time.

This indicates that a JA-induced increase in the *ORA47* transcript level is not required for the JA-responsive expression of the JAs biosynthesis genes *LOX2* and *AOC2*. 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 8B). 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 8B). 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 LOX2 and AOC2 genes in response to 8 hours treatment with JA was similar in the ORA47-silenced lines compared to the control line (Figure 8C).

As a third approach to silence ORA47 expression we used the artificial microRNA (amiRNA) method (Ossowski et al., 2008; Schwab et al., 2006). The ORA47-specific amiRNA construct was introduced in Arabidopsis plants both under control of the constitutive CaMV 35S promoter as well as under the control of the estradiol-responsive XVE system (Zuo et al., 2000). Two-week-old seedlings of 14 independent transgenic T2 lines containing the constitutively expressed construct were treated for 15 min with 50 µM JA whereas 20 independent lines containing the inducible construct were first treated for 24 hrs with 5 µM estradiol followed by a 15 min treatment with JA. Northern blot hybridization analysis of the extracted RNAs revealed that none of the lines had an ORA47 mRNA level that differed from wildtype seedlings or 2 XVE-GUS lines respectively (results not shown). Therefore these amiRNA lines were not further analysed.

DISCUSSION

JAs are signaling molecules that regulate certain aspects of development as well as diverse responses to stress. Little is known about the regulatory mechanisms
Figure 8. Expression of JAs biosynthetic genes in response to JA in the *ora47* mutant and in transgenic *ORA47*-silenced plants. (A) RNA gel blot analyses with two-weeks-old *ora47-1* mutant and wild-type seedlings treated for 15 and 30 min 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 (SGUS-6) treated for 15 minutes (B) or 8 hours (C) with 50 µM 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).
controlling JAs 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 JAs biosynthesis. We show that overexpression of the ORA47 gene resulted in the activation of JAs biosynthesis genes, and led to elevated levels of endogenous JAs.

**ORA47 positively regulates the JAs biosynthesis genes**

Overexpression of ORA47 gene in Arabidopsis activated the expression of all established genes encoding enzymes of the JAs biosynthetic pathway, such as LOX2, AOS, AOC2, OPR3 and JAR1. Additionally, several genes encoding enzymes of the β-oxidation steps, including ACX1, MFP2 and KAT5, were also expressed at moderately increased 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) reported that the ACS1 (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; Afithile et al., 2005), suggesting a role in wound-induced JAs production. Moreover, He et al. (2002) suggested a role for KAT2/PED1 in senescence-induced JAs synthesis. Therefore it is likely that the KAT2/PED1 gene is
expressed in response to signals related to wounding or senescence without the requirement for ORA47, whereas JAs signaling initiating the auto-stimulatory loop would recruit ORA47 to activate the KAT5 gene.

Our finding that the LOX2 gene is upregulated in response to ORA47 overexpression fits well with the report that ORA47 can transactivate the LOX3 promoter in tobacco protoplasts (Pauwels et al., 2008). It suggests that LOX genes are direct target genes of ORA47. Our finding is in disagreement with the report that the LOX3 gene is downregulated in transgenic plants constitutively overexpressing the ORA47 gene (Wang et al., 2008). Although we were able to obtain T1 plants after flower dip transformation, these plants were extreme dwarfs and they never flowered. Wang et al. (2008) do not mention aberrant phenotypes displayed by the transgenic plants. We hypothesize that these authors were working with plants with very low levels of ORA47 overexpression which were insufficient for induction of JAs biosynthesis genes and JAs production.

**ORA47 controls JAs production**

The increased expression of JAs biosynthesis genes in ORA47-overexpressing plants suggested that these plants might contain enhanced levels of endogenous JAs. Indeed elevated levels of OPDA, JA, JA-Ile and 12-OH-JA were found.

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 JA, 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 JAs 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 JAs-responsive genes and increased resistance against *B. cinerea* (Seo et al., 2001). The cas1 and cet1 mutant plants contain
constitutively high levels of JA (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 JAs 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 JAs levels and downstream gene activation. The biosynthesis of JAs is likely to induce the constitutive expression of a large number of JAs-responsive genes, thereby generating a stress condition that compromises plant development and overall fitness. Indeed, overexpression of ORA47 induced the expression of several JAs-responsive genes, including VSP1 and BG1.

**ORA47 is involved in the JAs auto-stimulatory loop**

Analyses of the *ora47* knock-down mutant and the *ORA47*-silenced plants showed that expression of the JAs biosynthesis genes in response to JA was not altered in these plants. The knock-down line contains a T-DNA insertion in the promoter and still expresses the *ORA47* transcript at a basal level, indicating that JAs-responsive *ORA47* gene expression is not required for activation of its target genes. The RNAi strategy yielded only two putative knock-down lines out of 29 transgenic lines. This frequency is much lower than with 3 other AP2/ERF-domain transcription factors for which we applied the RNAi strategy (Pré, 2006), including ORA59 (Pré et al., 2008). In those cases the large majority of the lines showed successful silencing. In addition lines with successful silencing always showed a prominent smear in Northern blot expression analyses using the corresponding gene as a probe. With the two putative *ORA47* knockdown lines we did not observe this smear. Therefore we hypothesize that these lines still express the *ORA47* gene at a low level sufficient for effective JA-responsive expression of its target genes. Also the amiRNA approach was not successful in generating plants with a reduced *ORA47* mRNA level. Especially in the case of the inducible amiRNA construct this was surprising, since possible lethal effects are unlikely to occur within the relatively short timeframe of induction.
Here we showed that overexpression of ORA47, encoding an AP2/ERF-domain transcription factor, induces the expression of all established JAs biosynthesis genes tested and results in elevated JAs levels. These results tend to place ORA47 upstream of JAs 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 JAs production. However, several lines of evidence indicate that ORA47 is a downstream component in JAs signaling where ORA47 regulates the positive feedback amplification loop. First, induction of the ORA47 gene by JA (Pauwels et al., 2008; Wang et al., 2008) requires the JA-Ile receptor COI1 (Wang et al., 2008), indicating that perception of JAs is required for ORA47 expression in response to stress. Therefore, ORA47 is likely to act as a terminal component in JAs signal transduction, rather than as an integrator of stress signals leading to JAs production. We speculate that the function of ORA47 is to regulate the amplification loop that leads to the expression of the JAs biosynthesis genes in response to JAs. Up to now, it is still unclear how JAs production initially occurs in response to stress and what is the integrator of stress signals that leads to JAs 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. In this context it is worth mentioning that ORA47 was detected as a phosphorylation target of MAP kinases (Popescu et al., 2009). Activation of ORA47 protein and the resulting expression of the JAs biosynthesis genes would lead to production of a small amount of JAs that would activate transcription of the ORA47 gene and subsequent amplification of the signal by the feedback loop.

MATERIAL AND METHODS

Plant material, growth conditions and treatments

Arabidopsis thaliana ecotype Columbia (Col-0) is the genetic background for all wild-type, transgenic 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 4°C, seeds were first germinated at 21°C 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 4A). Treatments with the different JAs were performed at a final concentration of 50 µM. The compounds JA 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.

**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 AGG-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 BgIII 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 BgIII 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-ORA47as. 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. Primers for artificial micro RNA (amiRNA) targeting ORA47 mRNA were designed using the WMD (Web MicroRNA Designer) program (http://wmd.weigelworld.org). amiRNA was amplified with the 1-4 primers 5’-GAT TGA AAT TAG CAT TGC CGC GGT CTC TCT GTA TTC C-3’, 5’-GAC CGC GGC AAT GCT AAT TTC AAT CAA AGA GAA TCA ATG A-3’, 5’-GAC CAC GGC AAT GCT TAT TTC ATT
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CAC AGG TCG TGA TAT G-3’, 5’-GAA TGA AAT AAG CAT TGC CGT GGT
CTA CAT ATA TAT TCC T-3’ and the a-b primers 5’-CTG CAA GG GAT TAA
GTT GGG TAA C-3’ and 5’-GCG GAT AAC AAT TTC ACA CAG GAA ACA
G-3’. The amiRNA containing precursor was engineered into a 404 bp fragment
containing the MIR319a stem-loop as described (Schwab et al., 2006; Ossowski
et al., 2008). The amiRNA containing precursor cassette was cloned in
pCAMBIA1300 with SphI for constitutive expression and for inducible
expression the stem-loop construct was cloned in pER8 with Xhol/SpeI. The
binary vector pCAMBIA1300-ORA47 was introduced into Agrobacterium
tumefaciens strain LBA1115 (containing the Vir plasmid pSDM3010). The
pART27 binary vectors were introduced into A. tumefaciens strain LBA4404
while pER8 binary vectors and pCAMBIA1300-amiRNA 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.

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 65°C before addition of denatured 32P-labeled DNA probes. After
overnight hybridization, blots were washed twice at 42°C 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 ACC 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 GGT ATA GTT ACT TAT AAC- 3' for Allene oxide cyclase2 (AOC2, 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 CTT 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 TTT TG- 3' for 3-ketoacyl-CoA thiolase2 (KAT2/PED1, At2g33150); 5'- ATG GCT GCT TTT GGA GAT GAC- 3' and 5'- TGA GAT GAG AAA GCG TTT GGA GAT GAC- 3' for 3-ketoacyl-CoA thiolase5 (KAT5, At5g48880); 5'- ATG GCT GTA GAG GGT TTT GGA GAT GAC- 3' and 5'- TCA ACC GTT TCT AAC GAG CG- 3' for S-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase (JMT, At1g19640); 5'- ATG TTT GAG GGT GAA AC- 3' and 5'- TCA AAA GCG TGT GCT GAA G- 3' for JAR1 (At2g46370); 5'- ACA CCA CAT GCA GCA ACA AC- 3' and 5'- TGA GCC TTA GTG AGC ATA GC- 3' for MES10 (At3g50440); 5'- CGG GAT CCA TGA AAA TCC TCT CAC TT- 3' and 5'- CCC TCG AGT TAA GAA GTT ACG TAG TGG AG- 3' for Vegetative Storage Protein 1 (VSP1, At5g24780); 5'- ATG GTG
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AGG TTC GAG AAG G- 3' and 5'- CTA GAG TTC CCT CAG C- 3' for \( \beta - \text{Glucosidase1} \) (BG1, At1g52400); 5'- ATG GCG GCG ATA GAG GAC AG- 3' and 5'- CTA GAC GAA GAT ACC AGA AG- 3' for \( \text{Chlorophyllase1} \) (CHL1, At5g43860); 5'- CTA GAC GAA GAT ACC AGA AG- 3' and 5'- CCC TCG AGT TAA GTC TCG TAC TCC TCT TC- 3' for \( \beta - \text{tubulin} \) (TUB, At5g44340); 5'- CGG AAT TCA TGA GAG AGA TCC TTC ATA TCp 3' and 5'-CCA ACC T TC TCG ATG GCC Tp3'.

Measurements of JAs levels
For JAs measurements, six transgenic lines containing the XVE-ORA47 expression module and four transgenic lines containing the XVE-GUS expression module were used. Per treatment and per line 20 two-weeks-old seedlings were incubated in 50 mL polypropylene tubes containing 10 ml MA medium. 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. Plant material from 20 seedlings was pooled to minimize biological differences and this was done in triplicate. Quantification of OPDA, JA, JA-Ile and 12-OH-JA was done according to Balcke et al. (in preparation). Briefly, homogenized plant material (about 50 mg) was extracted with 500 µL methanol containing 0.1 ng/µL of each stable isotope-labeled internal standard (\(^2\)H\(_6\)-JA, \(^2\)H\(_2\)-(-)JA-Ile and \(^3\)H\(_5\)-OPDA) using a bead mill (FastPrep24 instrument, MP Biomedicals LLC, Santa Ana, CA, USA). After centrifugation at 20,000 x g (2 min, 0°C), 450 µL of supernatant was diluted with water to 4.5 mL and subjected to solid-phase extraction (SPE) on HR-XC material (Macherey & Nagel, Düren, Germany). The fraction containing phytohormones was eluted using 900 µL acetonitrile and subjected directly to
LC-MS analysis using a 3200 Q TRAP® LC/MS/MS System hybrid QqLIT mass spectrometer (AB Sciex, Darmstadt, Germany).

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