Functional analysis of ORA47, a key regulator of jasmonate biosynthesis in arabidopsis

Khurshid, M.

CHAPTER 3

Identification of primary target genes of ORA47

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ABSTRACT

Upon herbivore or pathogen attack plants produce the jasmonate (JAs) hormones, which set in motion defense gene expression programmes. JAs signaling also induces all known JAs biosynthesis genes in what is considered a positive feedback loop. Overexpression of the AP2/ERF-domain transcription factor ORA47 leads to elevated expression of all JAs biosynthesis genes and to elevated levels of JAs, indicating that ORA47 controls the positive feedback loop. The work described here aimed to identify direct target genes of ORA47 by comparing plants containing an inducible expression cassette under induced and non-induced conditions and quantifying the transcript abundance of 24,000 genes using the *Arabidopsis thaliana* whole genome Affymetrix gene chip (ATH1). To distinguish between direct target genes and secondary genes responding to JAs we applied the inducible ORA47 strategy in the wildtype background and in *aos* mutant plants, which are unable to produce JAs. Unexpectedly, most JAs biosynthesis genes responded to ORA47 overexpression to a much lesser degree in the mutant background. The JAs biosynthesis gene *JASMONATE RESISTANT 1* (*JAR1*) responded equally strong in the wildtype and mutant background, making it a strong candidate for an ORA47 target gene. We hypothesize that the other JAs biosynthesis genes are also direct target genes of ORA47, but that they are subject to a second layer of JAs-responsive regulation.

INTRODUCTION

Upon insect herbivory or attack by microbial pathogens plants produce jasmonate (JAs) hormones (Pieterse et al., 2009). The bioactive amino acid conjugate 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.

JAs are lipid-derived compounds that are synthesized via a series of enzymatic reactions taking place in different subcellular compartments. 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) followed by three rounds of beta-oxidation involving three enzymes to yield jasmonic acid (JA). Subsequently, JA can be further metabolized in the cytoplasm. Methylation yields the volatile methyl-jasmonate (MeJA). Conjugation to the amino acid isoleucine by JA amino acid synthase (JAR1; Staswick and Tiryaki, 2004) yields the bioactive form JA-Ile (Fonseca et al., 2009).

In Arabidopsis genes involved in defense against wounding and insect herbivory are controlled by the JAs-responsive basic Helix-Loop-Helix transcription factor MYC2 (Lorenzo et al., 2004) and the related proteins MYC3 and MYC4 (Fernandez-Calvo et al., 2011; Niu et al., 2011). JAZ can bind to MYC (Chini et al., 2007; Chini et al., 2009; Fernandez-Calvo et al., 2011). JAZ1 was shown to repress the activity of MYC2 (Hou et al., 2010) and JAZ can bind to the corepressors TOPLESS (TPL) and TPL-related proteins either directly (Shyu et al., 2012) or via the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA; Pauwels et al., 2010). More recently a variety of transcription factors were reported to interact with members of the JAZ family (Pauwels and Goossens, 2011).

JAs signaling also induces all known JAs biosynthesis genes including *LOX2*, *AOS*, *AOC2* and *OPR3* (Sasaki et al., 2001; Pauwels et al., 2008) in what is considered a positive feedback loop. Overexpression of the AP2/ERF-domain transcription factor ORA47 induces the expression of all JAs biosynthesis genes, leads to elevated levels of JA and JA-Ile and causes severe growth inhibition (Chapter 2). ORA47 can activate the *LOX3* promoter in a transient assay in tobacco protoplasts (Pauwels et al., 2008), indicating that *LOX3* is a direct target gene of ORA47.

The aim of the work described here was to identify direct target genes of ORA47 by comparing plants containing an inducible expression cassette under induced and non-induced conditions and quantifying the transcript abundance of 24,000 genes using the *Arabidopsis thaliana* whole genome Affymetrix gene chip (ATH1). Our working hypothesis was that two types of genes would be switched on by ORA47 overexpression, the direct target genes and genes that respond to JAs produced in response to ORA47 overexpression. To distinguish between these two types we applied the inducible ORA47 strategy in the wildtype background and in *aos* mutant plants, which are unable to produce JAs. Unexpectedly, most JAs biosynthesis genes responded to ORA47 overexpression to a much lesser degree in the mutant background. The *JASMONATE RESISTANT 1* (*JAR1*) gene encoding the JA-Ile conjugating enzyme was a JAs biosynthesis gene that responded equally strong in the wildtype and mutant background, making it a strong candidate for an ORA47 target gene. We hypothesize that the other JAs biosynthesis genes are also direct target genes of ORA47, but that they are subject to a second layer of JAs-responsive regulation.

RESULTS

Validation of transgenic lines containing the inducible XVE-ORA47 cassette

AOS is a single-copy gene encoding a cytochrome P450 enzyme essential for the biosynthesis of JAs. An *aos* T-DNA knockout mutant does not produce any JAs (Park et al., 2002). To be able to distinguish between primary target genes of ORA47 and secondary JAs-responsive genes, transgenic plants containing the estradiol-responsive XVE-ORA47 cassette were crossed with the *aos* mutant.

As a validation of the hypothesis that expression of the XVE-ORA47 cassette in the *aos* mutant background would not lead to JAs biosynthesis we observed the phenotypes of seedlings after estradiol treatment. As shown in Figure 1, germination of wildtype seedlings containing the XVE-ORA47 cassette on medium containing estradiol was severely inhibited. Mutant *aos* seedlings containing the cassette developed normally on estradiol-containing medium.

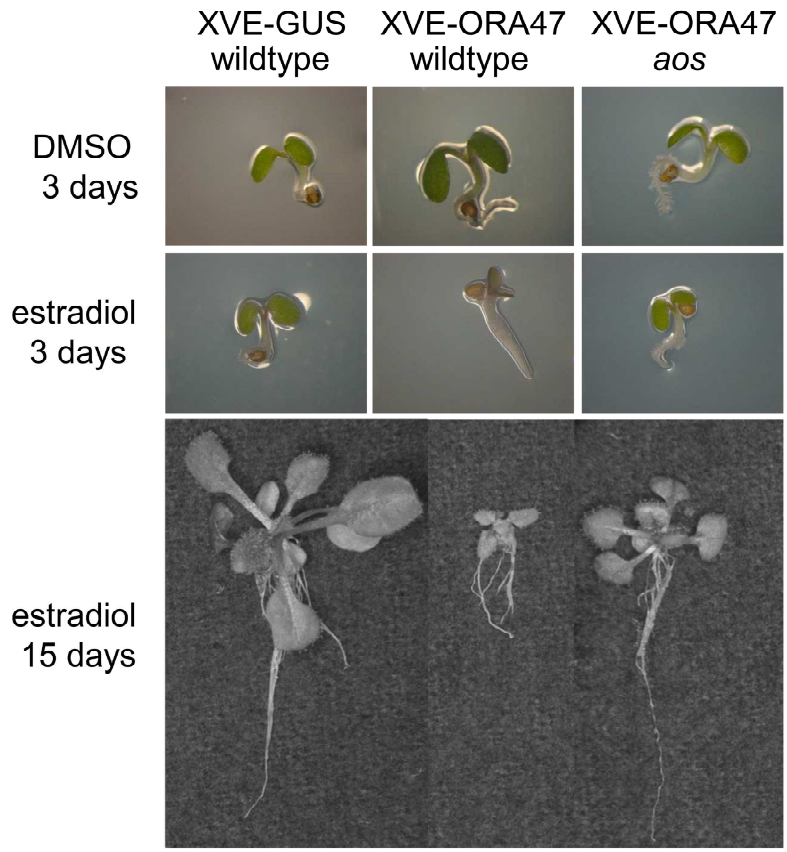

Figure 1. The *aos* mutation counteracts growth inhibition caused by ORA47 overexpression. Seedlings with the genotypes indicated at the top were germinated for 3 days on medium supplemented with 0.1% of the solvent DMSO or 5 µM estradiol. Seedlings germinated on DMSO-containing medium were then transferred to estradiol-containing medium and grown for another 15 days. Experiments were carried out with 2 XVE-GUS lines, 3 XVE-ORA47 lines in the wild-type background and 3 XVE-ORA47 lines in the *aos* mutant background. Representative pictures are shown (WT-GUS line 2.1; WT-ORA47 line 16C; *aos*-ORA47 line 16C2).

When seedlings germinated on control medium were transferred 3 days after germination to estradiol-containing medium, wildtype seedlings containing the XVE-ORA47 cassette showed severe growth retardation compared to control seedlings. Mutant *aos* seedlings containing the XVE-ORA47 cassette grew largely the same as control seedlings. However in general they were slightly

smaller, indicating that the dwarf phenotype of wildtype seedlings overexpressing ORA47 is largely but not entirely due to JAs production and that overexpression of ORA47 has additional minor growth-retarding effects.

To further validate the lines for use in genome-wide expression analysis, the functionality of the inducible cassette and the expression of selected JAs biosynthesis and JAs-responsive genes in response to JA and estradiol treatments were tested in the wildtype and *aos* mutant backgrounds (Fig. 2). Wildtype and *aos* seedlings that did not contain the XVE-ORA47 cassette showed the same responses to JA treatment and did not respond to estradiol treatment. The *aos* mutant seedlings still expressed an *aos* transcript in a JA-responsive manner. The amount of transcript was lower and its size was smaller than the wildtype transcript. Wildtype seedlings containing the XVE-ORA47 cassette showed leaky expression of the estradiol-inducible cassette. This resulted in elevated expression of several JA-responsive genes after treatment with the solvent DMSO. Treatment with estradiol resulted in elevated expression of all selected genes to levels that were similar to those after JA treatment. The *aos* seedlings expressed the *ORA47* gene at a high level after estradiol treatment, indicating that the XVE-ORA47 cassette was functional. However all selected genes except *AOC2* were not induced to levels detectable in this analysis. *AOC2* was induced by estradiol treatment but at a much lower level than in wildtype seedlings containing the XVE-ORA47 cassette. All genes responded to JA at a similar level as in non-transgenic *aos* seedlings.

Based on the observations that the XVE-ORA47 cassette was responsive to estradiol and that the *AOC2* gene was upregulated, we concluded that our *aos* lines were suitable for micro-array analysis.

Genome-wide analysis of gene expression in response to ORA47 overexpression

The micro-array analysis was designed such that three independent wildtype lines and three independent *aos* lines containing the XVE-ORA47 construct were treated with the solvent DMSO or with 5 µM estradiol (Fig. 3). ATH1 micro-arrays were used to evaluate the effect of estradiol treatment and hence ORA47

overexpression on gene expression. In the wildtype background 367 genes were upregulated and 27 genes were downregulated. In the *aos* mutant background 65 genes were upregulated and 4 genes were downregulated (Fig. 6; Table 1). When the genes were grouped according to gene ontology (GO) annotation, it appeared that in the wildtype the largest groups of genes with annotated functions were involved in metabolic processes and in responses to stimuli (Fig. 4). Zooming in on the metabolic processes group, it turned out that relatively

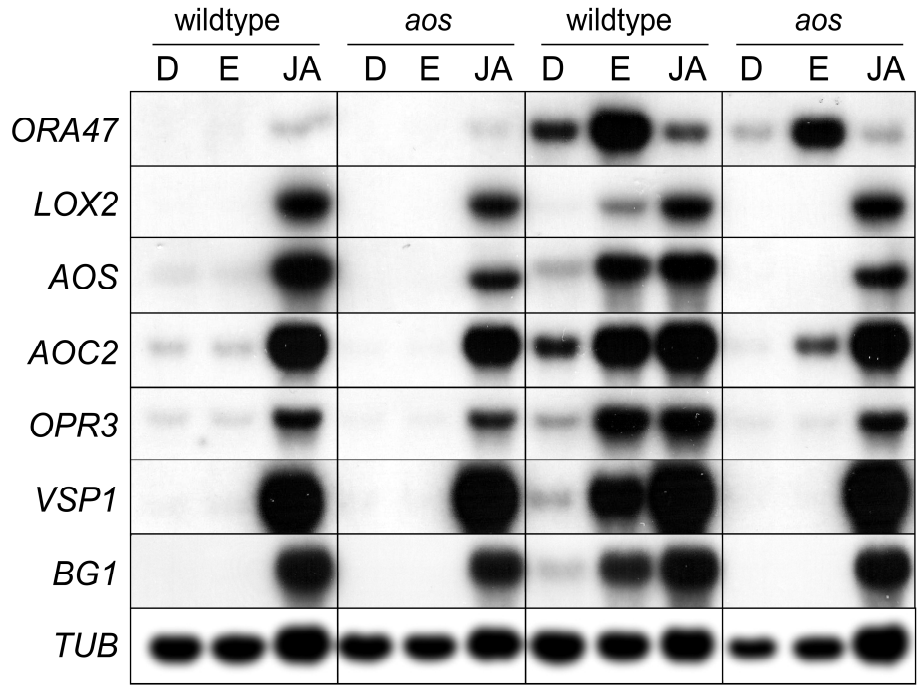

Figure 2. Expression of selected JAs biosynthesis and JAs-responsive genes in response to ORA47 overexpression in the wildtype and *aos* mutant background. Two-week-old wildtype or *aos* seedlings that did or did not contain the estradiol-responsive XVE-ORA47 cassette were treated for 8 hrs with 0.1 % of the solvent DMSO (D), 50 µM JA or 5 µM estradiol (E). The RNA gel blots were hybridized with the indicated probes. The *Tubulin* (*TUB*) probe was used to verify RNA loading. All panels for each probe were on the same blot and exposed to film for the same time allowing direct comparison of expression levels.

large parts were involved in amino acid, phenylpropanoid and oxylipin metabolism. Zooming in on the response to stimulus group, oxylipin metabolism surfaced again as an important process. In addition the *JAZ* family formed an important group and a large group of genes was annotated as involved in defense including wounding responses.

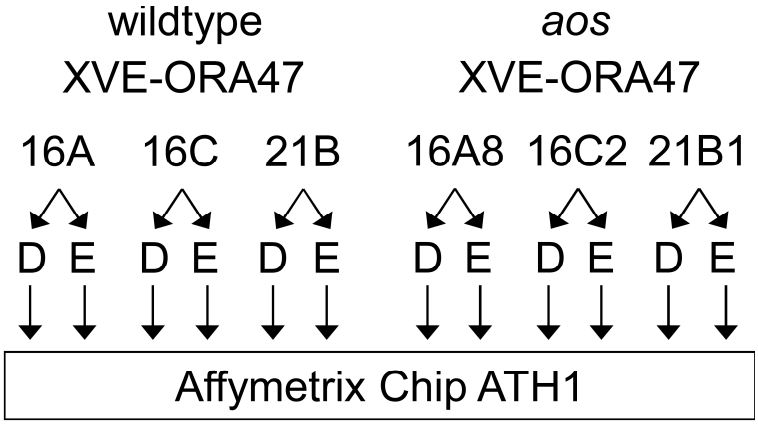

Figure 3. Micro-array design. The *aos* mutant was crossed with two T3 offspring from XVE-ORA47 line 16 and with T3 offspring from line 21. Homozygous *aos* mutants containing the XVE-ORA47 cassette were selected. Two-week-old wildtype or *aos* seedlings containing the estradiol-responsive XVE-ORA47 cassette were treated for 8 hrs with 0.1 % of the solvent DMSO (D) or 5 µM estradiol (E) and RNA was isolated.

The genes induced in the *aos* background had a variety of functional annotations including several stress-responsive and defense genes. Two groups of genes involved in a common process emerged, one group involved in JAs biosynthesis and one group involved in biosynthesis of the defense metabolites glucosinolates. The JAs biosynthesis group included *LOX2*, *LOX3*, *AOC1*, *AOC2*, *OPR3* and *JAR1*. It also included *MES10*, encoding MeJA esterase converting MeJA to JA. The glucosinolate group included genes encoding the transcription factor MYB29, regulating aliphatic glucosinolate biosynthesis (Hirai et al., 2007), the transcription factor MYB34, regulating aromatic glucosinolate biosynthesis (Celenza et al., 2005), the enzyme flavin-

Table 1. List of genes differentially expressed in the *aos* background. Fold change in response to estradiol treatment is indicated in a linear scale.

AGI code	Gene symbol	Description	Fold
At1g74930	ORA47	ERF/AP2 transcription factor	17,4
At1g17420	LOX3	Lipoxygenase	15,2
At2g03020, At4g16540	-	Heat shock protein	13,0
At3g25760, At3g25770	AOC1, AOC2	Allene oxide cyclase	7,8
At4g22620	-	SAUR-like auxin-responsive protein	7,7
At5g47330	-	alpha/beta-Hydrolases	6,4
At2g33380	RD20	Putative calcium-binding EF-hand protein	5,5
At2g46370	JAR1	Jasmonate-amido synthetase	5,5
At3g45140	LOX2	Lipoxygenase	4,9
At2g34930	-	Disease resistance family protein	4,8
At2g42540	COR15A	Cold-regulated protein cor15a precursor	4,6
At1g32860	-	Glycosyl hydrolase	4,4
At5g54170	-	Polyketide cyclase/dehydrase	4,4
At4g30560	ATCNGC9	Cyclic nucleotide gated channel protein	4,3
At5g55180	-	O-Glycosyl hydrolase	3,9
At4g17350	-	Unknown protein	3,8
At4g36900	RAP2.10	ERF/AP2 transcription factor	3,8
At4g27570	-	UDP rhamnose--anthocyanidin-3-glucoside rhamnosyltransferase	3,7
At1g20070	-	Unknown protein	3,6
At5g09530	-	Periaxin	3,5
At5g50720	HVA22E	HVA22 homolog	3,5
At3g05640	-	Protein phosphatase 2C	3,4
At1g01470	LEA14	Late-embryogenesis abundant protein	3,3
At3g13310	-	Chaperone DnaJ-domain protein	3,3
At3g04010	-	O-Glycosyl hydrolases	3,1
At2g06050	OPR3	12-oxophytodienoate	3,1
At2g31940	-	Unknown protein	3,0

At4g24960	HVA22D	ABA- and stress-inducible protein	3,0
At5g07690	ATMYB29	Myb-like transcription factor	2,9
At5g44210	ERF9	ERF/AP2 transcription factor family	2,9
At2g15830	-	Unknown protein	2,8
At3g50440	MES10	Methyl jasmonate esterase	2,7
At5g19875	-	Unknown protein	2,7
At1g75040	PR5	Thaumatin-like protein	2,7
At5g47240	atnudt8	Nudix hydrolase homolog 8	2,6
At2g44130, At2g44140	-	Galactose oxidase	2,6
At5g09520	-	Hydroxyproline-rich glycoprotein	2,6
At1g43910	-	P-loop containing nucleoside triphosphate hydrolase	2,5
At2g36830	GAMMA-TIP	Tonoplast intrinsic protein	2,4
At4g38080	-	Hydroxyproline-rich glycoprotein	2,4
At5g22500	FAR1	Alcohol-forming fatty acyl-CoA reductase	2,3
At2g22080	-	Unknown protein	2,3
At4g38810	-	EF-Hand containing protein	2,3
At1g52400	BGLU18	Glycosyl hydrolase	2,3
At5g60890	MYB34	Myb-like transcription factor	2,3
At2g29450	ATGSTU5	TAU glutathione S-transferase	2,3
At3g22540	-	Unknown protein	2,3
At1g33790	-	Jacalin lectin protein	2,3
At4g15210	BAM5	Beta-amylase	2,3
At3g17130	-	Plant invertase/pectin methylesterase inhibitor protein	2,2
At5g64700	-	Nodulin MtN21	2,2
At3g21420	-	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	2,2
At2g22330	CYP79B3	Cytochrome P450	2,1
At1g21550	-	Calcium-binding EF-hand family protein	2,1
At1g65860	FMO GS-OX1	Flavin-monooxygenase	2,1
At2g32240	-	Unknown protein	2,1
At5g65300	-	Unknown protein	2,1
At3g47380	-	Plant invertase/pectin methylesterase inhibitor protein	2,1

At5g44530	-	Subtilase	2,0
At5g59740	-	UDP-N-acetylglucosamine (UAA) transporter	2,0
At3g02230	RGP1	UDP-arabinose mutase	2,0
At2g28570	-	Unknown protein	2,0
At5g24420	-	6-phosphogluconolactonase 5	2,0
At4g15290	ATCSLB05	Cellulose synthase like protein	-2,1
At5g46900, At5g46890	-	Bifunctional inhibitor/LTP/seed storage 2S albumin	-2,3
At4g19030	NLM1	Aquaporin	-2,6

monooxygenase glucosinolate S-oxygenase 1 (FMO-GSOX1) involved in aliphatic glucosinolate biosynthesis, and the cytochrome P450 enzyme CYP79B3 involved in aromatic glucosinolate biosynthesis (Sonderby et al., 2010). In addition a gene encoding myrosinase-binding protein involved in the formation of toxic glucosinolate breakdown products was induced (Table 1).

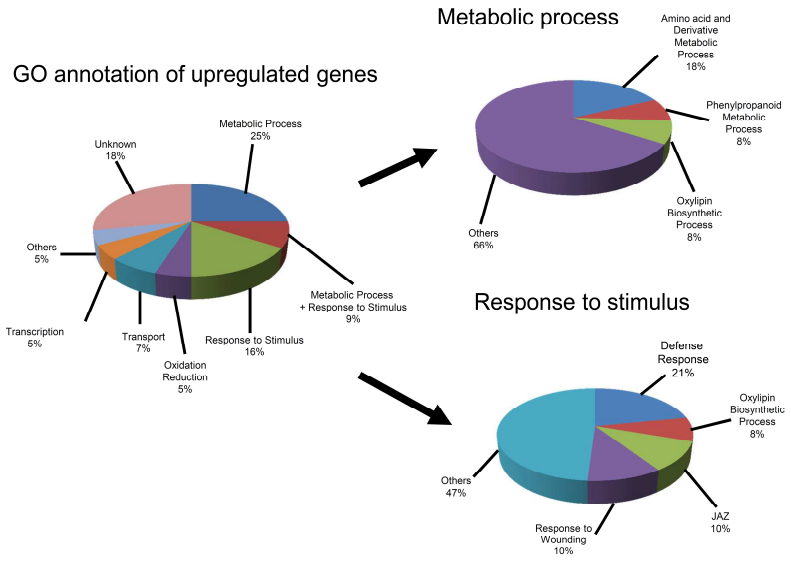

Figure 4. GO annotation of genes induced by ORA47 overexpression in the wildtype background.

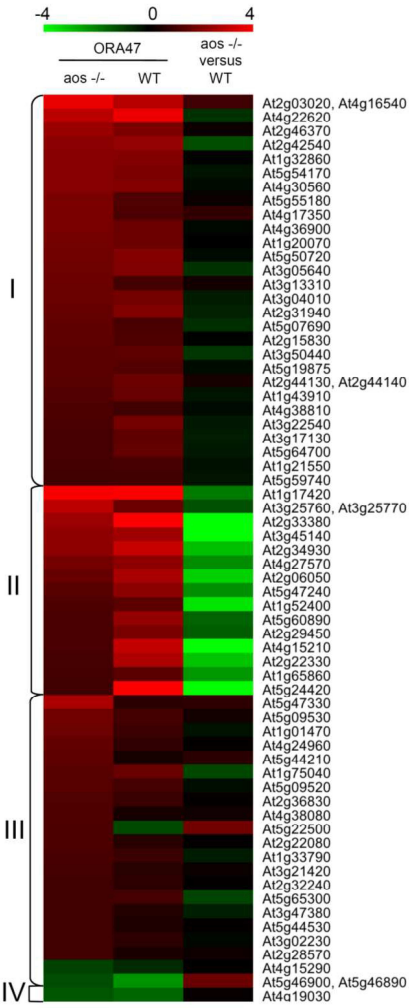

Figure 5. Heat maps of genes expressed differentially in the *aos* mutant with and without ORA47 overexpression. Relative expression of these genes in the wildtype background with and without ORA47 induction and the relative expression in the *aos* mutant versus the wildtype background both overexpressing ORA47 are also shown. Each individual square in the heat map is the average of the three lines analyzed for each condition. Color coding is shown in a log2 scale ranging from -4 to +4. Genes are grouped according to expression pattern in *aos* versus wildtype induced condition. Group I are genes that were similarly induced in both backgrounds. Group II were induced in both groups but to a lesser extent in the *aos* mutant. Group III are genes that were up- or down-regulated in the *aos* background but did not respond to ORA47 overexpression in the wildtype background. Group IV consists of a single gene that was downregulated in both backgrounds.

A more detailed look at the genes differentially expressed in the *aos* background showed that around 70% are also differentially expressed in the wildtype background (Fig. 6). Four patterns of gene expression could be distinguished (Fig. 5). Group I containing 30 genes was upregulated to a similar level in the wildtype and *aos* background by estradiol treatment. Within this group are the JA-Ile biosynthesis genes *JAR1* and *MES10*. Group II consisting of 16 genes was also upregulated in both backgrounds but at a lower level in the *aos* mutants. It contains the JAs biosynthesis genes *LOX2*, *LOX3*, *AOC1*, *AOC2* and *OPR3*. Group III contains 22 genes showing altered expression in the *aos* background but not in the wildtype with 19 upregulated and 3 downregulated genes. Finally, 1 gene (At4g19030), encoding an aquaporin, was downregulated by ORA47 overexpression in both backgrounds (group IV).

Validation of the ATH1 micro-array data

The expression levels determined from ATH1 arrays were confirmed by Northern blot hybridization expression analysis. Figure 7 shows the expression of 2 genes selected from group I (genes equally upregulated in both backgrounds), 3 group II genes (upregulated in both backgrounds but stronger in the wildtype) and 2 genes from the group that were only upregulated in the wildtype background. The expression of *MES10* and *JAR1* was induced very similarly by estradiol in both backgrounds confirming the microarray result. The expression of the JAs biosynthesis genes *LOX2*, *AOC2* and *OPR3* responded to estradiol treatment in the *aos* mutant, but to a much lower level than in the wildtype. The expression of the *MYC2* gene and of its likely target gene *VSP1* responded strongly in the wildtype but not in the mutant background. It can be concluded that the Northern blot results confirmed the micro-array data.

DISCUSSION

The goal of the studies described here was to identify candidate direct target genes of ORA47. To this end we used an inducible ORA47 overexpression strategy. Since ORA47 overexpression leads to JAs biosynthesis (Chapter 2), we

reasoned that upregulated genes in a wildtype background would probably consist of direct ORA47 target genes and secondary JAs-responsive genes. Therefore we applied the inducible strategy using the *aos* mutant which is unable to make any JAs (Park et al., 2002).

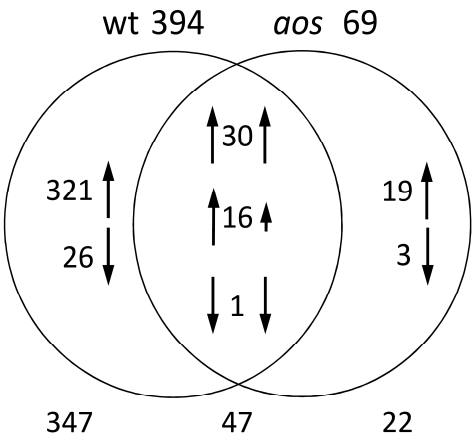

Figure 6. Venn diagram of numbers of genes differentially expressed upon ORA47 induction in wildtype and *aos* backgrounds. Different arrow sizes indicate a response differing in amplitude between the two genotypes. Arrows pointing up and down indicate genes up and down regulated by ORA47 induction, respectively.

With 69 genes differentially expressed in the *aos* mutant background we found a relatively small number of candidate direct target genes. For example in a comparison of wildtype plants versus plants constitutively overexpressing the AP2/ERF-domain transcription factor ERF1 (Lorenzo et al., 2003), 164 genes were found to be differentially expressed using a gene chip that contained 3-fold less gene probes than the ATH1 chip used in our study. Using a gene chip that contained 4-fold less gene probes than the ATH1 micro-array, 268 genes were found to be differentially expressed in a comparison of wildtype plants and plants constitutively overexpressing the transcription factor AtMYB102 (de Vos et al., 2006). The fact that we found 10-20-fold less differentially expressed genes is likely due to two reasons. We eliminated a large number of secondary genes by using a JAs-negative mutant. Transcription factors studied by others may have a similar but unknown effect on the generation of signaling

molecules causing secondary effects. The second reason is that we used an inducible system to overexpress the transcription factor for a relatively short time instead of constitutively overexpressing it. In a study using a similar inducible approach with 4 different AP2/ERF-domain transcription factors that all caused strong upregulation of the *PDF1.2* gene when constitutively overexpressed, it was observed that using inducible overexpression only two transcription factors caused increased *PDF1.2* expression (Pré et al., 2008). Those same two transcription factors were able to trans-activate the *PDF1.2* promoter in a transient assay in protoplasts, whereas the two others were not. This indicates that the inducible approach preferentially increases the expression of direct target genes. Consistent with this notion only 86 differentially expressed genes were found using plants inducibly overexpressing the AP2/ERF-domain transcription factor ORA59 using the Agilent Arabidopsis 3 Oligo array carrying 26,000 gene probes (Pré et al., 2008). This indicates that less than 100 direct target genes for a single transcription factor is a more realistic estimate than the around 1000 genes suggested by other studies. The insight that the use of a constitutive overexpression strategy results in a group of upregulated genes of which 90% are secondary response genes may also explain the lack of correlation between upregulation by transcription factor overexpression and the presence of consensus binding sites for that transcription factor in the promoters reported by some studies (Chakravarthy et al., 2003).

As expected we found the JAs biosynthesis genes among the genes differentially expressed in the *aos* background, suggesting that they are direct target genes. However whereas *JAR1* and *MES10* were upregulated to a similar level in the wildtype and *aos* background, most JAs biosynthesis genes were upregulated much less in the *aos* mutant. We hypothesize that genes equally responding to ORA47 overexpression in both backgrounds are bona fide candidates for being direct target genes of ORA47. We think that genes that respond in both backgrounds but at a lower level in the *aos* background are also serious candidates for being ORA47 target genes. For example *AOC2* and *LOX3* are among the Group II genes. The *LOX3* promoter was found to be activated

by ORA47 in a transient assay in tobacco protoplasts (Pauwels et al., 2008), strongly suggesting that it is a direct target gene. The *AOC2* gene is induced by JA in a cycloheximide-independent manner (Zarei, 2007), and a GCC-box like sequence from the *AOC2* promoter is bound in vitro by recombinant ORA47 protein (Zarei et al., 2011), suggesting that *AOC2* is also a direct target gene.

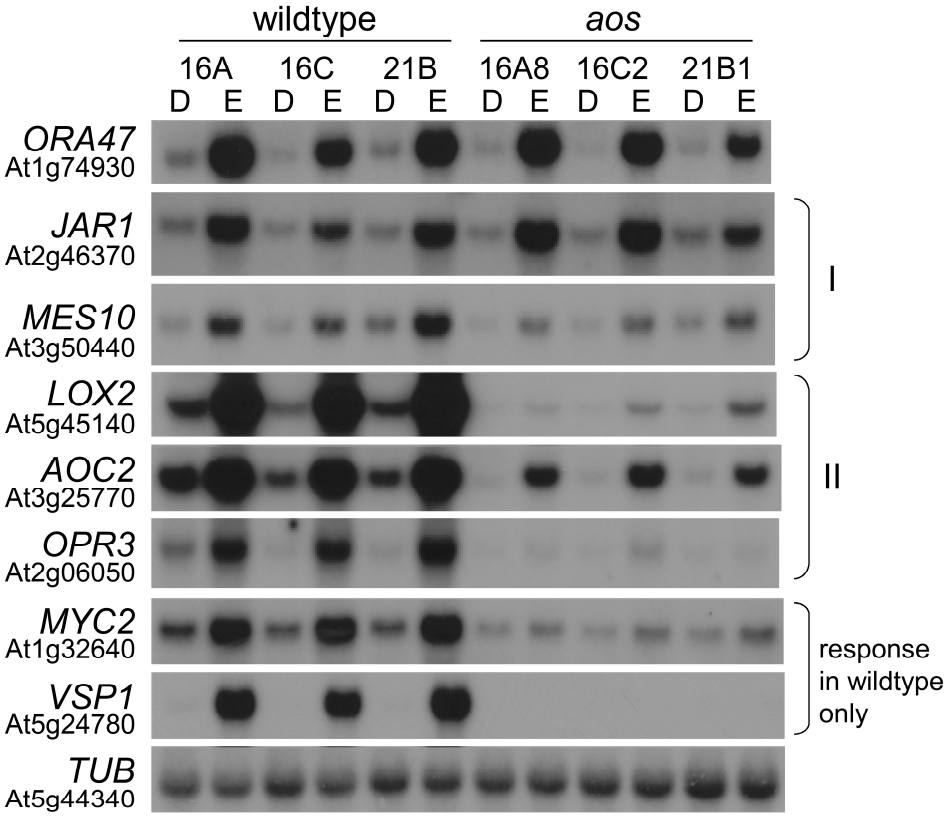

Figure 7. Expression of selected JAs biosynthesis and JAs-responsive genes in response to ORA47 overexpression in the wildtype and *aos* mutant background. Two-week-old wildtype or *aos* seedlings that did or did not contain the estradiol-responsive XVE-ORA47 cassette were treated for 8 hrs with 0.1 % of the solvent DMSO (D) or 5 µM estradiol (E). The RNA gel blots were hybridized with the indicated probes. The *TUB* probe was used to verify RNA loading.

We consider genes that respond to ORA47 overexpression in the wildtype background but not in the *aos* background as bona fide secondary response genes which are expressed as a result of JAs production. An unexpected category of genes were those that were only upregulated in the *aos* background. We hypothesize that these genes are direct target genes of ORA47. A reason why these genes are not upregulated in the wildtype background might be that they are repressed because of secondary effects due to JAs production.

An interesting group of genes with common annotations upregulated in the *aos* background are related to glucosinolate biosynthesis. The glucosinolate-myrosinase system is a major defense mechanism in Arabidopsis (Burow et al., 2010; Müller et al., 2010). Genes involved in regulation and biosynthesis of both aliphatic and indole glucosinolates were upregulated in the *aos* background. In the wildtype background an additional 6 genes involved both in aliphatic and aromatic glucosinolate biosynthesis were upregulated bringing the total number of upregulated genes involved in glucosinolate biosynthesis or its regulation up to 10. Therefore it seems likely that ORA47 overexpressing wildtype plants have increased levels of both aliphatic and indole glucosinolates and it would be interesting to confirm this experimentally and to test the effect of ORA47 overexpression on insect feeding behavior.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

Arabidopsis thaliana wild-type plants, the *aos* mutant, and all transgenic plants are in the genetic background of ecotype Col-0. *aos* seeds were obtained from the Nottingham Arabidopsis Stock Center. Pollen from XVE-ORA47 transgenic plant (Chapter 2) flowers was used to pollinate flowers from male-sterile *aos* mutant plants (Park et al., 2002). F1 plants were selected on solid MA medium (Masson and Paszkowski, 1992) containing 25 µg/ml hygromycin, were selfed, and hygromycin-resistant F2 plants were screened by PCR to select for homozygous *aos* mutants. To obtain large amounts of F3 seeds flowers of F2

plants were treated with MeJA to restore fertility. Surface-sterilized seeds were grown for 10 days at 21°C in a growth chamber (16 h light/8 h dark photoperiod at 200 µE m^{-2} s^{-1} at 70% relative humidity) on solid MA medium containing 25 µg/ml hygromycin. Fifteen to 20 seedlings per sample were transferred to 50 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 mL MA medium and incubated on a shaker at 120 rpm for 4 additional days before treatment. Seedlings were treated with 5 µM estradiol (Sigma-Aldrich, St. Louis, MO) or 50 µM JA (Sigma). As control, seedlings were treated with the solvent DMSO (0.1%).

RNA extraction and Northern blot analyses

Total RNA was extracted from pulverized frozen tissue by phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride, washed with 70 % ethanol, and resuspended in water. For RNA-blot analysis, 10 µg RNA samples were subjected to electrophoresis on 1.5% agarose/1% formaldehyde gels, and blotted to GeneScreen nylon membranes (Perkin-Elmer Life Sciences, Boston, MA). All probes were ^{32}P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed on X-ray films. DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5′-GAA GAT CTC AAT GGA AGA AGA ATC GGG TTT AGT A-3′ and 5′-GAA GAT CTC ATC AAA AAT CCC AAA GAA TCA-3′ for *ORA47* (*At1g74930*), 5′- ATG GCT TCT ATT TCA ACC CC- 3′ and 5′- CTA AAA GCT AGC TTT CCT TAA CG- 3′ for *AOS* (*At5g42650*), 5′-ATG TTG GAG AAG GTT GAA AC-3′ and 5′- TCA AAA CGC TGT GCT GAA G-3′ for *JAR1* (*At2g46370*), 5′-ACA CCA CAT GCA GCA ACA AC-3′ and 5′-TGA GGC TTA GTG AGC ATA GC-3′ for *MES10* (*At3g50440*), 5′-GTC GAC TTC ATG AAA TTA AAA TGT TTC TC-3′ and 5′-GTC GAC CCA AAA GAT TAC AAA GAC TTT TC-3′ for *AOC2* (*At3g25770*), 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 *LOX2* (*At3g45140*), 5′-ATG ACG GCG GCA CAA GGG AAC-3′ and 5′-TCA GAG

GCG GGA AGA AGG AG-3′ for *OPR3* (*At2g06050*), 5′-ATG ACT GAT TAC CGG CTA CAA-3′ and 5′-CCG ATT TTT GAA ATC AAA CTT G-3′ for *MYC2* (*At1g32640*), 5′-CGG GAT CCA TGA AAA TCC TCT CAC TTT-3′ and 5′-CCC TCG AGT TAA GAA GGT ACG TAG TAG G-3′ for *VSP1* (*At5g24780*), 5′-ATG GTG AGG TTC GAG AAG-3′ and 5′-CTA GAG TTC TTC CCT CAG-3′ for *BG1* (*At1g52400*) and 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 *TUB* (*At5g44340*).

Micro-array hybridization and data analysis

For micro-array analysis, total RNA was extracted using the RNeasy plant mini kit (Qiagen) according to manufacturer's protocol. The Affymetrix micro-arrays (*Arabidopsis* ATH1 genome array) contain probe sets representing around 24,000 genes on a single array. RNA labeling and hybridization on the ATH1 micro-arrays (one sample per chip) were performed according to the manufacturer's instructions (www.affymetrix.com/support/technical/manual/expression_manual.affx) by ServiceXS (www.servicexs.com). The probe arrays were scanned and data analysis was performed using matlab software (v.7.6). The probe level intensities from the CEL files were normalized using the Robust Multichip Average algorithm. A test statistic was computed for each gene to evaluate the evidence for differential expression. P-values were generated by the permuted two-tailed t-test using the "mattest" function of matlab. The "mavolcanoplot" function was used to identify genes with expression changes. A cutoff value of 2-fold and a p-value < 0.05, which are commonly used for micro-array analysis, were adopted to discriminate genes that were differentially expressed. Annotation of the genes represented on the micro-array was obtained from The Arabidopsis Information Resource (TAIR). The heat map was built using the MultiExperiment Viewer software (v4.8). To test the hybridization quality, "Arabidopsis control genes" coding for GAPDH, actin, tubulin, ubiquitin, and several ribosomal RNAs (25S, 5S), spotted by the manufacturer, were verified. The expression ratios [DMSO/estradiol] of the control genes were consistently in the range of 0.81–1.29.

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