12-Oxo-Phytodienoic Acid Triggers Expression of a Distinct Set of Genes and Plays a Role in Wound-Induced Gene Expression in Arabidopsis[1][w]

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Jasmonic acid (JA) and methyl jasmonate (MeJA), collectively known as JAs, regulate diverse physiological processes in plants, including the response to wounding. Recent reports suggest that a cyclopentenone precursor of JA, 12-oxo-phytodienoic acid (OPDA), can also induce gene expression. However, little is known about the physiological significance of OPDA-dependent gene expression. We used microarray analysis of approximately 21,500 Arabidopsis (*Arabidopsis thaliana*) genes to compare responses to JA, MeJA, and OPDA treatment. Although many genes responded identically to both OPDA and JAs, we identified a set of genes (OPDA-specific response genes [ORGs]) that specifically responded to OPDA but not to JAs. ORGs primarily encoded signaling components, transcription factors, and stress response-related genes. One-half of the ORGs were induced by wounding. Analysis using mutants deficient in the biosynthesis of JAs revealed that OPDA functions as a signaling molecule in the wounding response. Unlike signaling via JAs, OPDA signaling was CORONATINE INSENSITIVE 1 independent. These results indicate that an OPDA signaling pathway functions independently of JA/MeJA signaling and is required for the wounding response in Arabidopsis.

Plants synthesize various fatty acid derivatives having biological activity. Among these, jasmonic acid (JA) and methyl jasmonate (MeJA), collectively known as jasmonates (JAs), are the best characterized. JAs are cyclopentanone compounds derived from linolenic acid via an octadecanoid pathway consisting of several enzymatic steps (Fig. 1). The early steps of this pathway occur in chloroplasts, where linolenic acid is converted to 12-oxo-phytodienoic acid (OPDA) by means of the three enzymes, lipoxygenase, allene oxide synthase (AOS), and allene oxide cyclase (Bell et al., 1995; Laudert et al., 1996; Stenzel et al., 2003). OPDA is subsequently reduced in a cyclopentenone ring by a peroxisome-localized enzyme, 12-oxo-phytodienoic acid reductase 3 (OPR3). The reaction product then undergoes three cycles of β-oxidation in the peroxisome, generating JA (Stintzi and Browse, 2000; Strassner et al., 2002; Reumann et al., 2004).

There have been numerous physiological analyses of the function of JAs. The role of JAs in the response to biotic stresses, such as insect or fungal attack (McConn et al., 1997; Ozawa et al., 2000), and abiotic stresses, such as mechanical wounding (Baldwin et al., 1997; Creelman and Mullet, 1997; Reymond et al., 2000; Richard et al., 2000), has been well documented. JAs also play important roles in anther development (Feys et al., 1994; McConn and Browse, 1996; Xie et al., 1998) and in the regulation of many other plant developmental processes (Creelman and Mullet, 1997). Several components are involved in signaling via JAs. One such component, CORONATINE INSENSITIVE 1 (COI1), contains an F-box motif that has similarity to F-box proteins involved in targeting proteins for removal by

ubiquitination (Xie et al., 1998; del Pozo and Estelle, 2000). Thus, COI1 is thought to be involved in protein ubiquitination and degradation regulated by JAs. COI1 is now thought to be a central component of signaling pathways involving JAs.

Recently, OPDA, an intermediate in the biosynthesis of JAs, has been shown to be the biologically active molecule. An *opr3* Arabidopsis (*Arabidopsis thaliana*) mutant that could not produce JA was identified that exhibits delayed anther dehiscence, resulting in male sterility (Sanders et al., 2000; Stintzi and Browse, 2000). Studies using this mutant showed that JA, and not its precursors, is the active signaling molecule that regulates anther development, since the application of JA, but not OPDA, restored fertility (Stintzi and Browse, 2000). However, *opr3* plants have shown to be resistant to the dipteran *Bradysia impatiens*, as are wild-type plants, suggesting that OPDA may be also the active signaling molecule and have a role in the induction of defense response genes (Stintzi et al., 2001). Moreover, using a mini-array system consisting of 150 defense genes, the same investigators showed that OPDA not only up-regulates COI1-dependent genes, which are induced by JA, but also several COI1-independent genes that do not respond to JA (Stintzi et al., 2001). These investigators suggested that OPDA may function cooperatively with JAs to regulate the expression of defense response genes. It was shown that OPDA was more active than JA in eliciting the tendril coiling response in *Bryonia dioica* (Blechert et al., 1999). More recently, OPDA was shown to induce stomatal opening (Ohashi et al., 2005). However, little is known about OPDA-dependent gene expression or other possible functions of OPDA.

In this study, we analyzed OPDA-dependent gene expression by comparing responses to OPDA and JAs using DNA microarrays covering 80% of the Arabidopsis genome. We identified a group of genes, designated ORGs (OPDA-specific response genes), which responded to OPDA but not to JAs. OPDA treatment of *coi1* mutants demonstrated that ORG expression is independent of the COI1-dependent JA signaling pathway. Using the OPDA and JAs biosynthetic mutants *aos* and *opr3* (Fig. 1), we showed that the normal response to wounding was impaired in *aos* but not in *opr3* mutants. These results demonstrate that OPDA is a lipid signal mediator in vivo, regulating ORGs that function during the wounding response.

RESULTS

Identification of Response Genes for OPDA and JAs by Microarray Analysis

To comprehensively evaluate the effect of OPDA, JA, and MeJA on the transcription of Arabidopsis genes in general, we used an oligonucleotide array covering 21,500 genes, corresponding to 80% of the genome. A time-course experiment was performed to analyze gene expression at 0, 30, and 180 min after treatment with OPDA, JA, or MeJA. After eliminating error spots, as described in "Materials and Methods," we calculated the normalized fold expression of 20,095 genes for each treatment. Scatter plots comparing expression between the three treatments were drawn on a logarithmic scale using the normalized fold expression of each gene. A higher correlation between JA and MeJA treatment was observed at 30 and 180 min (0.64 and 0.85, respectively) than that observed between OPDA and JAs treatment. Most of the genes that responded to JA treatment responded equivalently to MeJA treatment. The correlation coefficients of OPDA versus JA and OPDA versus MeJA were, respectively, 0.48 and 0.47 at 30 min and 0.77 and 0.76 at 180 min. As shown in Figure 2, A, B, D, and E, spots corresponding to up- or down-regulated genes (red or green spots, respectively) were observed specifically along axial lines. This result demonstrates that a group of genes responded to OPDA but not to JA or MeJA; these genes were termed OPDA-specific response genes, or ORGs. The analysis also revealed a large number of genes that responded to OPDA as well as JA and MeJA. From this comprehensive gene expression analysis, we concluded that OPDA regulates the expression of a distinct set of genes that are not regulated by JAs, in addition to genes that respond to all three compounds.

Validation of ORGs by Northern-Blot Analysis

Based on the microarray results, ORGs were defined as follows (for a detailed description, see "Materials
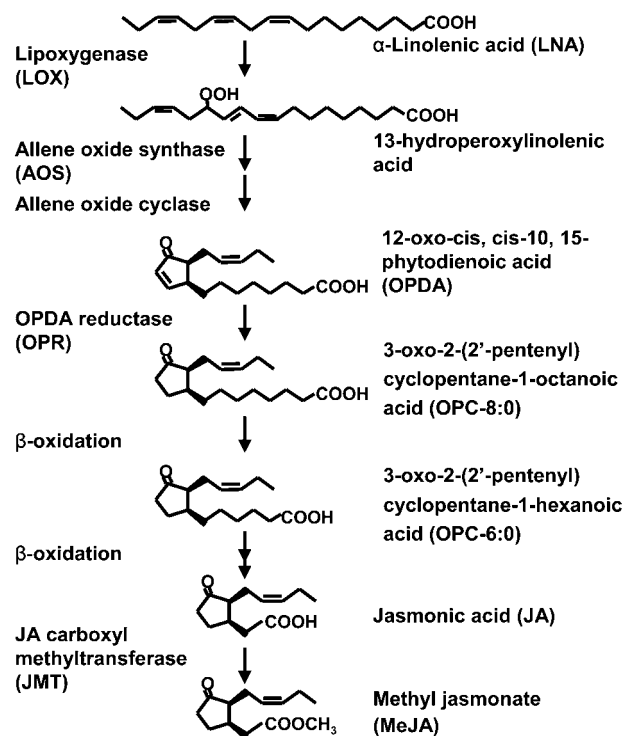

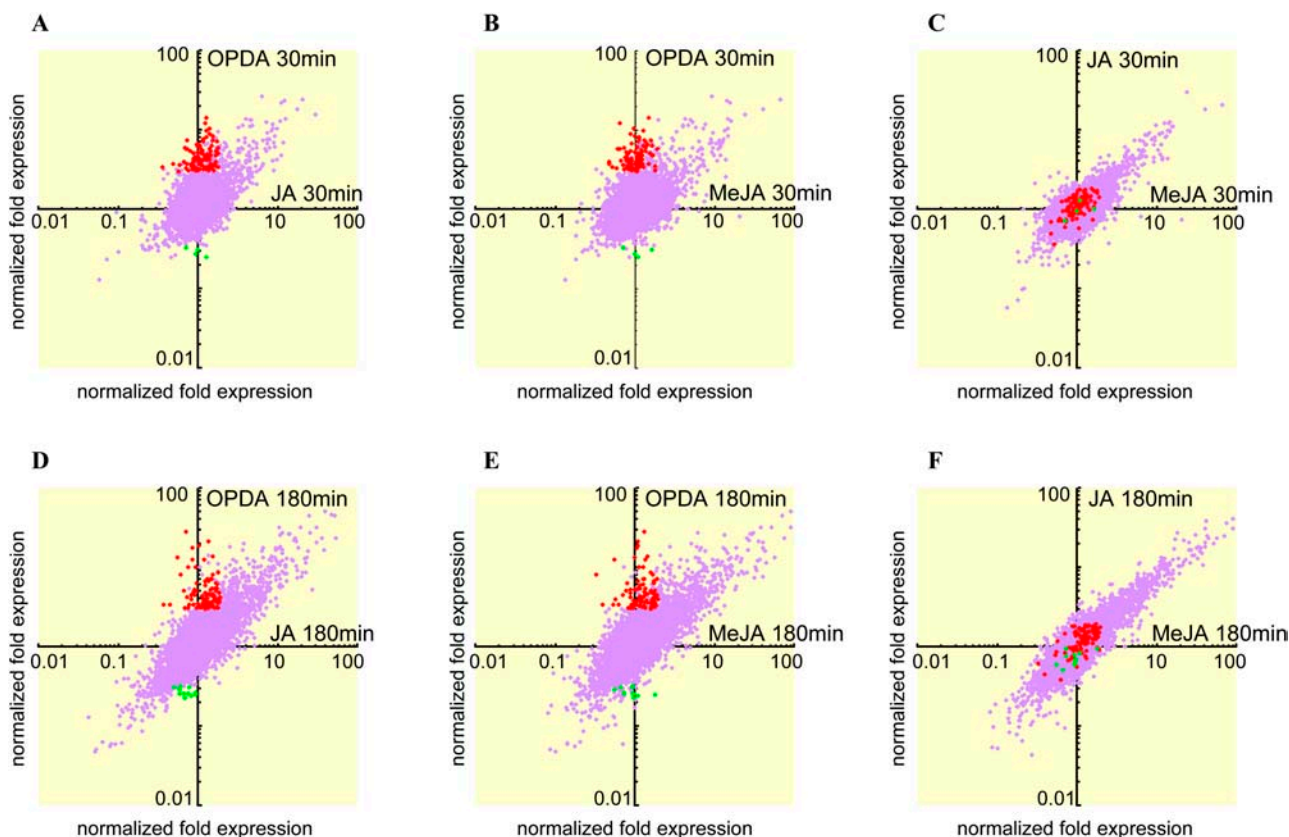**Figure 1.** Jasmonate biosynthetic pathway.

Figure 2. Scatter plots of OPDA and JAs response genes. A and D, Normalized fold expression at 30 and 180 min after OPDA treatment plotted against the normalized fold expression at 30 and 180 min after JA treatment. B and E, Normalized fold expression of OPDA versus MeJA treatment at 30 and 180 min. C and F, Normalized fold expression of JA versus MeJA treatment at 30 and 180 min. Red spots denote genes whose expression was induced more than 3-fold by OPDA treatment at 30 min (A, B, and C) or 180 min (D, E, and F), and less than 2-fold by JAs at 30 and 180 min. Green spots denote genes whose expression was repressed more than 3-fold by OPDA treatment after 30 min (A, B, and C) or 180 min (D, E, and F), and responded less than 2-fold by treatment with JAs at 30 and 180 min.

and Methods"). Up-regulated ORGs were induced more than 3-fold by OPDA but less than 2-fold by JA and MeJA (Table I; Supplemental Table I). Down-regulated ORGs were repressed more than 3-fold by OPDA but less than 2-fold by JA and MeJA (Table II). Genes corresponding to 172 loci (157 induced and 15 repressed) satisfied these criteria. For identification of ORGs, we also applied hierarchical clustering for 1,078 genes whose expression responded more than 3-fold to any of three treatments. By this clustering, 214 ORG genes were also found (193 induced 21 repressed; see Supplemental Table II and Supplemental Fig. 1). Basically, most of genes identified in former criteria were included in latter criteria (144 induced and 11 repressed). We therefore used the gene list obtained by the former criteria for further analysis.

To verify that OPDA modulates ORG expression independently of JAs, we assessed the expression profile of selected putative ORGs by northern blotting. Consistent with the microarray data, northern analysis showed that the expression of *ZAT10* (At1g27730), *AtERF5* (At5g47230), *DREB2A* (At5g05410), *GST6* (At2g47730), and *FAD-OXR* (FAD-linked oxidoreductase; At4g20860) increased upon OPDA treatment but

did not respond to JAs (Fig. 3A). The expression of *AtVSP2* (At5g24770), a known jasmonate-responsive gene (Berger et al., 1995), increased similarly in response to each treatment. *ZAT10*, *AtERF5*, and *DREB2A* are known transcription factors related to the response to various abiotic stresses (Liu et al., 1998; Fujimoto et al., 2000; Ohta et al., 2001; Lee et al., 2002). *GST6* and *FAD-OXR* are also induced in stress responses (Dittrich and Kutchan, 1991; Chen et al., 1996). Expression of the transcription factor genes *ZAT10* and *AtERF5* was detected within 30 min of OPDA treatment and decreased thereafter. On the other hand, expression of *GST6* and *FAD-OXR* was induced at a later time (around 180 min).

To confirm the induction of ORGs, we used an *opr3* mutant, which lacks an enzyme catalyzing the reduction of the cyclopentenone ring of OPDA (Fig. 1) and thus is deficient in JAs (Stintzi and Browse, 2000; Stintzi et al., 2001). As shown in Figure 3B, the induction of *ZAT10* and *FAD-OXR* by OPDA was equivalent in the *opr3* mutant and wild-type strain (Wassilewskija [Ws] background). Similar results were obtained for *AtERF5*, *DREB2A*, and *GST6* (data not shown). These results demonstrate that OPDA, but

Table I. *List of up-regulated ORGs*

Up-regulated ORGs are shown. Microarray data for normalized fold expression are calculated from two biologically independent experiments. OPDA, JA, and MeJA were treated for plants grown in liquid culture, whereas wounding was performed for plants grown in solid medium. Normalized fold expression more than 3 is in bold. See also Supplemental Table I.

Annotation	Gene Name	Normalized Fold Expression							
		OPDA		JA		MeJA		Wounding	
		30 min	180 min	30 min	180 min	30 min	180 min	30 min	180 min
Group 1: Signaling Component									
Phophatase									
Protein phosphatase 2C (*PP2C*), putative	At3g27140	**3.0**	1.5	1.4	1.3	0.9	0.5	1.6	0.4
Protein kinase									
Protein kinase family (*OXI1*)	At3g25250	**12.1**	**3.1**	1.3	1.8	0.6	0.9	**10.0**	1.1
Protein kinase family	At4g25390	1.9	**3.5**	1.0	1.5	1.1	1.8	2.2	2.2
Protein kinase family (*MAPKKK18*)	At1g05100	**3.7**	**3.0**	1.1	1.1	1.0	1.6	**5.7**	2.6
Protein kinase, putative	At2g05940	**4.2**	1.6	1.2	1.5	1.3	1.2	1.5	0.7
Receptor-related protein kinase like	At5g25930	**9.6**	**7.1**	1.3	1.4	1.3	1.3	**7.4**	0.9
Calcium-binding protein									
Calcium-binding EF-hand family protein	At4g27280	**3.5**	0.8	0.6	0.3	0.7	0.4	0.4	0.4
Calcium-binding EF-hand family protein	At5g39670	**6.0**	2.8	1.1	0.8	1.4	0.8	1.5	1.1
Calcium-binding protein, putative (*PBP1*)	At5g54490	**3.7**	0.7	0.5	0.5	0.5	0.4	1.0	0.4
Calcium-transporting ATPase, putative	At3g63380	1.2	**3.4**	0.7	1.1	1.1	1.0	2.4	**8.1**
Calmodulin-binding protein	At5g26920	**4.4**	0.9	1.0	0.6	0.8	0.7	1.1	0.3
Calmodulin-related protein, putative	At3g01830	**8.1**	0.9	1.3	0.6	1.1	0.7	**12.2**	1.0
Calmodulin-related protein, putative	At5g42380	**5.5**	**5.0**	1.1	0.7	1.2	0.8	**6.3**	1.3
Other signaling component									
F-box protein family	At1g61340	**4.4**	0.9	1.0	0.6	1.1	0.7	**6.9**	1.2
Transcription factor inhibitor I κ B like	At5g45110	**3.4**	1.4	1.4	1.0	1.1	0.9	2.2	0.7
Response regulator 2 (*ATRR2*)	At3g48100	**3.7**	0.6	1.5	0.7	1.5	0.9	1.0	0.3
SigA-binding protein (*SIB1*)	At3g56710	**7.5**	1.5	1.2	0.7	1.1	0.6	1.2	0.7
Group 2: Transcription Factor									
AP2-EREB-type transcription factor									
AP2 domain transcription factor TINY, putative	At1g22810	**13.9**	1.5	1.3	1.2	1.5	1.1	**58.8**	**5.2**
AP2 domain transcription factor, putative	At1g19210	**6.1**	1.2	1.0	1.0	1.1	1.2	1.3	0.3
C-repeat/DRE-binding protein, putative	At5g52020	2.1	**6.3**	1.4	1.0	0.9	0.8	1.9	1.0
DRE-binding protein (*DREB2A*)	At5g05410	**7.3**	**4.4**	0.7	0.9	1.6	1.9	**7.9**	2.8
Ethylene responsive element binding factor 5 (*AtERF5*)	At5g47230	**4.3**	0.9	0.9	0.5	0.9	0.5	0.3	0.3
Transcription factor TINY, putative	At1g33760	**3.6**	0.6	0.9	0.7	1.0	0.5	0.6	0.1
WRKY-type transcription factor									
WRKY family transcription factor (*WRKY30*)	At5g24110	**5.8**	1.0	0.7	0.5	0.7	0.5	**9.7**	0.3

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

| Annotation | Gene Name | Normalized Fold Expression | | | | | | | |
| | | OPDA | | JA | | MeJA | | Wounding | |
		30 min	180 min	30 min	180 min	30 min	180 min	30 min	180 min
WRKY family transcription factor (*WRKY46*)	At2g46400	**3.7**	0.7	1.0	0.6	1.1	0.8	1.3	0.5
WRKY family transcription factor (*WRKY53*)	At4g23810	**3.1**	0.2	0.4	0.2	0.3	0.2	2.0	0.4
MYB-type transcription factor									
Myb family transcription factor (*MYB15*)	At3g23250	**5.1**	1.5	1.8	1.5	1.5	1.2	**34.6**	**4.5**
Transcription factor (*MYB4*)	At4g38620	**5.6**	0.9	0.7	1.1	0.7	0.8	1.0	0.9
Zinc-finger-type transcription factor									
C2H2-type zinc-finger protein family	At2g28710	2.6	**4.5**	0.7	0.8	0.7	0.7	0.3	0.7
C2H2-type zinc-finger protein related	At2g37430	**10.2**	1.1	1.4	1.5	1.0	1.0	**4.5**	0.7
C2H2-type zinc-finger protein related (*FZF*)	At2g24500	1.2	**3.1**	0.9	1.1	0.9	1.4	2.0	**4.7**
CONSTANS B-box zinc-finger family protein	At2g47890	2.2	**3.1**	1.0	1.0	0.9	0.9	1.3	1.5
CONSTANS B-box zinc-finger family protein	At3g21150	**3.3**	1.0	0.9	0.7	0.8	1.2	1.4	0.5
RING-H2 zinc-finger protein related	At5g27420	**3.4**	0.5	0.4	0.3	0.5	0.3	1.3	0.2
Salt-tolerance zinc-finger protein (*ZAT10*)	At1g27730	**7.0**	1.7	1.2	0.7	1.3	0.7	1.6	0.7
Zinc-finger protein (*PMZ*)	At3g28210	**3.7**	**17.4**	0.7	0.8	0.9	1.0	**14.5**	**23.6**
Zinc-finger protein Zat12 (*RHL41*)	At5g59820	**6.4**	**3.5**	1.0	1.2	1.4	1.8	1.9	1.1
Zinc-finger-related protein	At3g46080	**5.1**	1.7	0.9	0.7	0.8	1.0	2.9	1.7
Other-type transcription factor									
bHLH protein family	At2g28160	**4.0**	**3.7**	1.7	1.5	1.0	1.4	1.5	1.1
Transcription factor GT-3a	At5g01380	1.8	**4.0**	1.0	1.2	0.9	0.6	**15.7**	**12.3**
Group 3: Stress Response									
Pathogenesis related									
Disease resistance protein (TIR class), putative	At2g20145	**3.2**	1.5	1.3	0.9	1.0	0.9	1.6	2.0
Disease resistance protein (TIR-NBS class), putative	At1g66090	**5.3**	0.9	0.8	0.6	0.9	0.7	2.4	0.5
Disease resistance protein family (*LRR*)	At3g05360	1.0	**3.3**	0.9	1.3	1.0	1.1	1.7	**3.9**
Seven transmembrane MLO protein family (*MLO6*)	At1g61560	**3.1**	**3.9**	1.6	1.8	1.8	1.4	**11.0**	0.7
Gly-rich protein	At3g04640	**4.1**	**3.4**	1.2	0.9	1.1	0.9	1.0	0.7
Stress-related protein related	At1g67360	**3.1**	2.0	1.1	0.8	1.1	0.9	**3.6**	1.6
Thaumatin family	At4g36010	2.3	**4.5**	1.6	1.6	1.2	1.8	**14.1**	**3.9**
Cell wall modification									
BON1-associated protein 1 (*BAP1*)	At3g61190	**3.3**	2.5	1.4	1.8	0.8	0.9	1.8	0.8
Cellulose synthase family (*ATCSLE1*)	At1g55850	1.6	**3.1**	1.0	1.7	0.8	1.8	1.1	1.1
Glucosyltransferase related	At2g15480	**4.4**	**6.7**	0.9	1.2	1.1	1.7	**7.2**	**4.9**
Glycosyltransferase family	At1g73880	0.9	**3.6**	0.9	1.8	0.8	1.3	0.9	1.0

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Table I. (*Continued from previous page.*)

Annotation	Gene Name	Normalized Fold Expression							
		OPDA		JA		MeJA		Wounding	
		30 min	180 min	30 min	180 min	30 min	180 min	30 min	180 min
UDP-glycosyltransferase family	At1g07260	1.2	**3.2**	0.8	1.1	1.0	1.1	0.5	0.4
Cinnamyl-alcohol dehydrogenase (CAD) family	At1g09500	1.5	**7.2**	0.6	1.3	0.5	1.3	0.5	1.8
Xyloglucan endotransglycosylase (*TCH4*)	At5g57560	**3.5**	0.4	0.8	0.3	0.8	0.4	0.9	0.5
Heat-shock response									
17.6-kD heat-shock protein (AA 1-156)	At1g53540	**6.3**	**13.5**	1.5	1.5	1.3	1.4	**20.6**	**32.3**
Class I heat-shock protein (*HSP 17.4*)	At3g46230	**3.3**	**12.4**	1.3	1.2	0.8	1.3	**3.5**	**13.8**
Class II heat-shock protein	At5g12020	**3.7**	**12.5**	0.8	0.8	0.9	0.5	2.0	**10.0**
Endomembrane-localized small heat-shock protein	At4g10250	**5.9**	**5.4**	1.8	1.6	1.1	1.2	1.0	**4.9**
Heat-shock protein 17.6A (*AT-HSP17.6A*)	At5g12030	**4.4**	**13.2**	1.0	0.6	0.8	1.0	2.7	**15.1**
Heat-shock protein family	At1g52560	**8.7**	**28.1**	1.4	0.7	0.9	1.3	1.6	**18.2**
Heat-shock protein family	At5g37670	**4.6**	**3.0**	1.3	0.8	0.8	0.9	2.6	**6.0**
Heat-shock protein family (*HSP18.2*)	At5g59720	2.4	**4.7**	1.3	1.8	1.1	0.9	1.7	**12.1**
Heat-shock protein hsp70b	At1g16030	**4.1**	**10.0**	1.3	0.8	1.2	1.3	**5.5**	**10.5**
Heat-shock protein hsp70t-2	At2g32120	**3.5**	**3.9**	1.6	0.8	1.2	1.2	**5.3**	**10.6**
Heat-shock protein, putative	At1g59860	**5.4**	**4.8**	1.4	0.9	1.3	1.3	**20.2**	**8.8**
Heat-shock protein, putative	At2g20560	**4.1**	**7.2**	1.1	1.5	0.8	1.9	**7.5**	**5.9**
Mitochondrion-localized small heat-shock protein	At4g25200	**5.2**	2.2	1.1	0.6	1.0	1.8	1.6	**7.3**
Small heat-shock protein related	At2g19310	2.3	**3.0**	0.9	1.2	0.8	1.6	1.3	1.2
Small heat-shock protein, chloroplast precursor (*HSP21*)	At4g27670	**5.3**	3.0	1.7	1.5	1.2	1.4	1.6	**14.7**
Oxidative burst stress response									
Glutathione peroxidase, putative (*AtGPX6*)	At4g11600	1.5	**3.2**	0.8	1.1	1.0	1.4	3.0	**4.5**
Glutathione *S*-conjugate ABC transporter (*AtMRP1*)	At1g30400	1.1	**3.8**	0.8	1.2	0.9	1.2	1.1	1.7
Glutathione transferase, putative (*GST21*)	At2g29470	1.5	**4.2**	1.0	1.6	1.2	1.1	1.0	0.7
Glutathione transferase, putative (*GST6*)	At2g47730	1.7	**5.1**	0.8	1.0	1.0	1.8	2.3	**3.4**
Iron superoxide dismutase (*FSD1*)	At4g25100	**3.1**	2.5	1.0	0.9	0.9	1.0	1.0	0.6
Group 4: Secondary Metabolism									
FAD-linked oxidoreductase family	At1g30700	1.6	**7.9**	0.4	0.6	0.3	0.3	**4.0**	**3.7**
FAD-linked oxidoreductase family	At4g20860	**3.7**	**6.6**	1.1	1.4	1.1	1.1	**6.0**	2.3
FAD-linked oxidoreductase family	At5g44360	2.5	**4.5**	1.1	1.4	1.0	0.7	1.0	1.1

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Table I. (*Continued from previous page.*)

Annotation	Gene Name	Normalized Fold Expression							
		OPDA		JA		MeJA		Wounding	
		30 min	180 min	30 min	180 min	30 min	180 min	30 min	180 min
Cytochrome P450, putative (*CYP72A15*)	At3g14690	1.8	**4.0**	1.0	1.3	1.1	1.7	1.4	**3.8**
Cytochrome P450, putative (*CYP707A3*)	At5g45340	**3.2**	0.3	0.5	0.3	1.0	0.9	0.5	0.2
Group 5: Hormonal Response									
Auxin									
Auxin-induced protein family	At3g09870	**4.5**	0.7	1.0	0.8	1.1	0.9	0.6	0.4
Auxin-induced protein family	At5g35735	**4.1**	**3.4**	1.0	1.0	0.9	1.0	**6.0**	1.8
JA									
12-Oxo-phytodienoate reductase (*OPR2*)	At1g76690	2.7	**6.2**	1.1	1.7	1.2	1.8	**3.1**	**6.7**
Abscisic acid									
ABA-responsive protein related	At5g13200	2.8	**3.2**	1.6	1.1	1.6	1.3	**8.6**	**4.1**
Group 6: Transporter									
ABC transporter family protein	At1g15520	1.7	**18.7**	0.9	1.0	0.9	1.1	**3.0**	**16.1**
Ammonium transporter, putative (*ATAMT1;2*)	At1g64780	1.6	**4.2**	1.5	1.9	1.3	1.3	1.6	1.2
High-affinity sulfate transporter related (*SEL1*)	At1g78000	0.7	**10.1**	0.9	0.9	1.2	1.1	0.4	1.2
Mitochondrial carrier protein family	At2g22500	**4.7**	1.1	1.4	1.0	1.3	0.8	**4.1**	0.8
Mitochondrial carrier protein family	At4g24570	**3.9**	0.4	0.7	0.3	0.5	0.3	1.1	0.4
Nucleoporin 98-related protein	At1g59660	**4.7**	0.9	1.7	0.7	1.6	1.0	0.8	0.5
Zinc transporter, putative (*MTPa2*)	At3g58810	1.2	**3.6**	0.8	1.4	1.0	1.2	2.3	2.7
Others									
60S ribosomal protein L10 (*RPL10C*)	At1g66580	1.2	**3.8**	1.0	1.3	0.9	1.6	**3.5**	**10.2**
AAA-type ATPase family	At3g50930	**6.3**	1.2	1.1	0.8	1.7	1.1	1.3	0.7
Carbonic anhydrase related	At2g28210	**3.2**	**4.2**	1.2	0.7	1.0	0.9	1.7	2.3
DnaJ protein family	At3g08970	**4.0**	**5.0**	0.9	1.7	1.1	1.6	2.4	**4.3**
Glycosyl hydrolase family 1	At2g44460	0.9	**6.1**	1.3	1.7	0.9	1.3	1.3	1.6
Glycosyl hydrolase family 1, *β*-glucosidase (*DIN2*)	At3g60140	1.2	**3.1**	1.5	1.3	1.0	1.3	0.2	0.3
Heavy-metal-associated domain-containing protein	At5g52760	**3.2**	0.8	1.0	0.5	1.0	0.7	1.5	0.8
Hydrolase, *α/β* fold family	At4g24160	**4.2**	**4.1**	1.2	1.3	1.1	1.8	**6.1**	**3.2**
Lemir (miraculin) related	At1g17860	1.8	**3.4**	1.3	1.5	1.1	0.9	**6.3**	2.7
Lys decarboxylase-related protein	At5g06300	1.3	**5.3**	0.8	1.7	0.8	1.1	1.0	1.4
Nitrilase 4 (sp P46011) (*NIT4*)	At5g22300	1.2	**3.9**	1.1	1.7	1.0	1.9	1.2	1.8
Phospholipid/glycerol acyltransferase family	At4g01950	**3.2**	0.7	0.7	0.7	0.6	0.5	1.9	0.6
Pyridine nucleotide-disulfide oxidoreductase family	At3g44190	**3.6**	**4.3**	1.1	1.1	1.0	1.2	**12.7**	**3.2**
Transferase family	At5g07870	**7.0**	**4.7**	1.7	1.6	1.1	1.5	2.0	1.8

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Annotation	Gene Name	Normalized Fold Expression							
		OPDA		JA		MeJA		Wounding	
		30 min	180 min	30 min	180 min	30 min	180 min	30 min	180 min
Pyridine nucleotide-disulphide oxidoreductase family	At5g22140	**6.6**	**20.9**	1.0	1.4	0.9	1.1	**6.9**	**24.2**
Aldo/keto reductase family	At1g60730	1.1	**4.6**	0.9	1.4	0.9	1.8	1.8	**4.1**
Aldo/keto reductase family	At1g60750	1.4	**5.8**	0.9	1.2	1.4	1.2	2.9	2.1
Ubiquinol-cytochrome-c reductase-related protein	At5g25450	2.5	**5.3**	1.2	1.5	0.8	1.3	**5.4**	**8.9**
Expressed and putative proteins are 49									

not JAs, regulates the expression of these representative ORGs. In contrast, the induction of *AtVSP2* by OPDA was abolished in the *opr3* mutant, but was induced by OPDA and JAs in wild-type plants (Fig. 3, A and B), indicating that *AtVSP2* responds specifically to JAs. This result also showed that exogenous OPDA could be converted to JA within 180 min under these experimental conditions, given that *AtVSP2* expres-

sion in OPDA-treated wild-type plants became evident after this time.

Functional Classification of ORGs

Of the 172 ORGs, 123 (70%) were annotated using locus annotation from The Arabidopsis Information Resource (Rhee et al., 2003). Forty-nine (30%) were

Table II. *List of down-regulated ORGs*

Down-regulated ORGs are shown. Microarray data for normalized fold expression are calculated from two biologically independent experiments. OPDA, JA, and MeJA were treated for plants grown in liquid culture, whereas wounding was performed for plants grown in solid medium. Normalized fold expression less than 0.33 is in bold.

Annotation	Gene Name	Normalized Fold Expression							
		OPDA		JA		MeJA		Wounding	
		30 min	180 min	30 min	180 min	30 min	180 min	30 min	180 min
Group 1: Signaling Component									
Protein kinases									
Ser/Thr kinase-like protein	At4g23190	0.9	**0.3**	1.1	0.9	1.1	0.7	2.0	**0.2**
Phosphatidylinositol 3- and 4-kinase family	At1g64460	0.6	**0.3**	0.5	0.5	0.8	0.7	0.5	0.9
Group 2: Transcription Factor									
AP2-EREB-type TF									
C-repeat/DRE binding factor 1 (*CBF1*) (*DREB1B*)	At4g25490	**0.3**	0.6	0.7	0.6	0.7	0.8	0.5	**0.3**
Group 3: Stress Response									
Oxidative burst stress response									
Copper/zinc superoxide dismutase (*CSD2*)	At2g28190	**0.2**	0.4	1.3	1.6	1.1	1.0	0.6	0.7
Copper/zinc superoxide dismutase, putative	At1g12520	**0.3**	0.4	0.9	1.1	1.0	0.9	**0.3**	0.7
Peroxidase, putative	At1g34510	0.4	**0.2**	0.8	0.9	1.3	1.8	1.0	1.0
Peroxidase, putative	At5g22410	0.4	**0.2**	0.7	0.8	1.3	1.1	1.8	1.0
Peroxidase, putative	At1g05240	0.5	**0.3**	0.7	0.6	0.7	0.6	0.9	1.2
Cell wall modification									
Expansin, putative	At1g62980	0.4	**0.3**	0.7	0.6	1.0	1.0	0.5	0.7
Xyloglucan endotransglycosylase, putative	At4g28850	0.4	**0.3**	0.7	0.6	1.2	0.9	0.6	0.6
Others									
Plasma membrane H+-ATPase like	At3g60330	0.7	**0.3**	1.0	0.8	1.0	0.9	1.1	1.0
Pro-rich protein	At3g62680	0.5	**0.3**	0.6	0.7	1.0	1.0	1.1	1.0
Ribosomal protein L13 homolog	At3g48130	**0.3**	1.0	1.0	0.5	1.6	0.7	1.0	1.0
Glycosyl hydrolase family 9	At1g48930	0.5	**0.2**	0.7	0.7	0.9	1.0	0.7	0.4
Invertase/pectin methylesterase inhibitor family	At5g62340	0.8	**0.3**	0.7	0.8	1.0	0.7	1.0	0.7

Figure 3. Northern-blot analysis of OPDA response genes in wild-type and *opr3* mutant plants. Five micrograms of total RNA (5 μg) were loaded per lane, and the blot was hybridized with the indicated probes; *actin8* served as a loading control. A, Ten-day-old wild-type (Col) plants treated with 30 μM OPDA, JA, or MeJA for 0 (control), 15, 30, 60, 180, or 360 min. B, Ten-day-old wild-type (Ws) and *opr3* mutant (Ws background) plants treated with 30 μM OPDA for 0, 15, 30, 60, 180, or 360 min.

"hypothetical," "expressed," or "putative" genes (Supplemental Table I). We classified the up-regulated ORGs into six groups (Table I) and the down-regulated ORGs into three groups (Table II). Among the up-regulated ORGs, Group 1 comprises genes related to signal transduction, including kinases, calcium-dependent signaling components, and calmodulins. The induction of genes in this group occurred within 30 min of treatment and their expression was transient. Group 2 comprises transcription factors and includes AP2/EREB (Gutterson and Reuber, 2004), WRKY (Eulgem et al., 2000), MYB (Stracke et al., 2001), and zinc-finger types (Tague and Goodman, 1995) of DNA-binding protein families. These genes responded rapidly and transiently to OPDA. Groups 1 and 2 contain 40 genes, accounting for 26% of the up-regulated ORGs we identified. Group 3 contains genes for various proteins involved in stress responses. For example, *AtGPX6* is induced under conditions of oxidative stress (Rodriguez Milla et al., 2003), and *TCH4* is involved in plant morphogenetic responses to environmental changes (Xu et al., 1995). Group 3 also contains a large number of heat shock protein genes that were expressed at 180 min rather than at 30 min, in contrast to the genes in Groups 1 and 2, which were mainly expressed at 30 min. Groups 4, 5, and 6 contain genes related to secondary metabolism, plant hormones, and transport systems for metals and ions, respectively. We also identified a number of other ORGs that do not fit

into the categories of Groups 1 to 6 (see Table I, Others). Compared with the up-regulated ORGs, we identified few that were down-regulated (Table II).

We also identified genes that responded to JAs (JRGs), according to the criteria described in "Materials and Methods." Briefly, genes that were induced or repressed more than 3-fold by both JA and MeJA were selected as JRGs. A total of 449 loci (371 induced and 78 repressed) satisfied these criteria (Supplemental Table III). This group mainly included genes involved in metabolic pathways, such as ascorbate and glutathione metabolism, JA biosynthesis, and indole glucosinolate synthesis, which are known to be regulated by JAs (Brader et al., 2001; Sasaki et al., 2001; Turner et al., 2002; Sasaki-Sekimoto et al., 2005).

ORG Function in the Wounding Response

Although the exact role of OPDA is unclear since it is a precursor of JA, it is expected to have a related physiological function. JAs have a pivotal role in the wounding response (Reymond and Farmer, 1998). To address the role of OPDA in this response, we performed microarray analysis using RNAs isolated from mechanically wounded plants at 0, 30, and 180 min after wounding. Forty-five percent of ORGs and 46% of JRGs responded to wounding treatment. These results suggest that OPDA is also a signaling molecule involved in the response to wounding.

To determine whether OPDA is required to induce expression of ORGs in wounded plants in vivo, the expression of *ZAT10*, *FAD-OXR*, and *AtERF5* in mechanically wounded plants was quantified for two JA biosynthesis mutants (Fig. 1): *opr3*, which has a defect in JA biosynthesis, and *aos*, which lacks both OPDA and JA (Stintzi and Browse, 2000; Park et al., 2002). We used northern blotting to quantify *ZAT10*, *FAD-OXR*, *AtERF5*, and *AtVSP2* mRNA expression in wounded *aos* and *opr3* mutants and plants with the corresponding wild-type backgrounds Columbia (Col) and Ws. In *aos* mutants, expression levels of *ZAT10*, *FAD-OXR*, and *AtERF5* were reduced by one-half compared with wild-type (Col) plants (Fig. 4A). However, expression levels in *opr3* mutants and wild-type plants (Ws) did not differ (Fig. 4B). *AtVSP2* expression was detected in wild-type plants but not in either mutant (data not shown). Thus, it is clear that OPDA signaling induces *ZAT10*, *FAD-OXR*, and *AtERF5* expression in wounded plants.

Figure 4A also indicates that, even in the *aos* mutant, about one-half of the wound-induced expression of *ZAT10*, *FAD-OXR*, and *AtERF5* was not abolished. Recently, various oxylipins were shown to function as signaling molecules that induce gene expression (Gerwick et al., 1991; Howe and Schilmiller, 2002; Weber, 2002). Since most oxylipins are derived from linolenic acid, it would be expected that the residual response to wounding seen in the *aos* mutant is mediated by oxylipins derived from linolenic acid. Therefore, we treated wild-type (Col) and mutant (*aos*) plants with linolenic acid and analyzed the expression levels of *ZAT10* and *AtVSP2* by northern blotting. *ZAT10* and *AtVSP2* mRNAs accumulated in Col (Fig. 4C). In *aos*, the *ZAT10* mRNA level was reduced compared with wild-type plants (Fig. 4C), and expression of *AtVSP2* expression was completely lost.

Expression of ORGs Is Independent of the COI1 Pathway

COI1 is required for the response to JAs (Feys et al., 1994). Because the structure of OPDA is similar to that of JA, responses to OPDA may also be regulated via the COI1 pathway. Therefore, we investigated whether COI1 is required for the induction of ORGs using a *coi1-16* mutant (Ellis and Turner, 2002). After application of OPDA or JA to *coi1-16*, the ORG expression profile was investigated by northern blotting. *ZAT10*, *FAD-OXR*, *AtERF5*, *DREB2A*, *GST6*, and *TCH4* (At5g57560) expression increased after OPDA treatment (Fig. 5). On the other hand, the induction of *AtVSP2* by OPDA or JA was abolished in this mutant. These results indicate that COI1 is not involved in the regulation of ORGs.

OPC8:0 and OPC6:0 Also Induce the Expression of ORGs

OPDA and JA have a similar structural backbone, a consequence of the fact that OPDA is the precursor of JA before reduction and β-oxidation. However, the

above results indicate that OPDA functions as a signaling molecule independently of JAs, suggesting that some structural peculiarity of OPDA is recognized in plants. As shown in Figure 1, there are two major differences in structure between OPDA and JA. First, OPDA has a double bond in the cyclopentenone ring, which forms an α, β-unsaturated carbonyl structure. Various compounds, such as acrolein, having this type of structure can induce gene expression in plants (Alméras et al., 2003). We therefore tested whether ORGs could be induced by acrolein (Fig. 6A). As shown in Figure 6B, acrolein induced the expression of ORGs. Secondly, we noted that the length of the carbon chain with the carboxyl group in OPDA is longer than that of JA. To determine whether this structural difference underlies the transcriptional activation function of OPDA, we analyzed the effect of 3-oxo-2-(2′-pentenyl)cyclopentane-1-octadecanoic acid (OPC8:0) and 3-oxo-2-(2′-pentenyl)cyclopentane-1-hexanoic acid (OPC6:0; downstream intermediates of JA biosynthesis; Fig. 1) on the expression of ORGs. Figure 6C shows that OPC8:0 and OPC6:0, like OPDA, induced the expression of ORGs, although JA with a shorter carbon chain has no effect on the gene expression. These results suggest that the peculiar induction activity of OPDA not only derives from the α, β-unsaturated carbonyl structure but also from the presence of a longer carbon chain than in JA.

DISCUSSION

OPDA Regulation of Gene Expression Is Distinct from JAs

JAs are important regulators of plant responses to environmental stresses, such as wounding, insect attack, and infection (Creelman and Mullet, 1997; Reymond and Farmer, 1998). Under these conditions, the expression of genes regulated by JAs is altered (Farmer and Ryan, 1992; Reymond et al., 2004). OPDA also induces gene expression (Stintzi et al., 2001), but the nature of this activity remains largely unknown.

We used genome-wide expression analysis to identify genes that respond to OPDA, JA, and MeJA, and identified a large number of OPDA response genes, ORGs distinct from those that respond to JAs, or JRGs (Fig. 2; Supplemental Fig. 1). The characteristics of ORGs and JRGs differ greatly (Tables I and II; Supplemental Table III). A significant proportion of ORGs are signaling components and transcriptional factors, whereas JRGs mainly include enzymes involved in metabolic pathways for jasmonates, ascorbate, glutathione, and indole glucosinolate (Brader et al., 2001; Sasaki et al., 2001; Turner et al., 2002; Sasaki-Sekimoto et al., 2005). COI1 is an important signaling component of JA-mediated response (Feys et al., 1994; Benedetti et al., 1995). As shown in Figure 5, expression of JRGs is regulated via a COI1-dependent pathway. In contrast, expression of ORGs was COI1-independent,
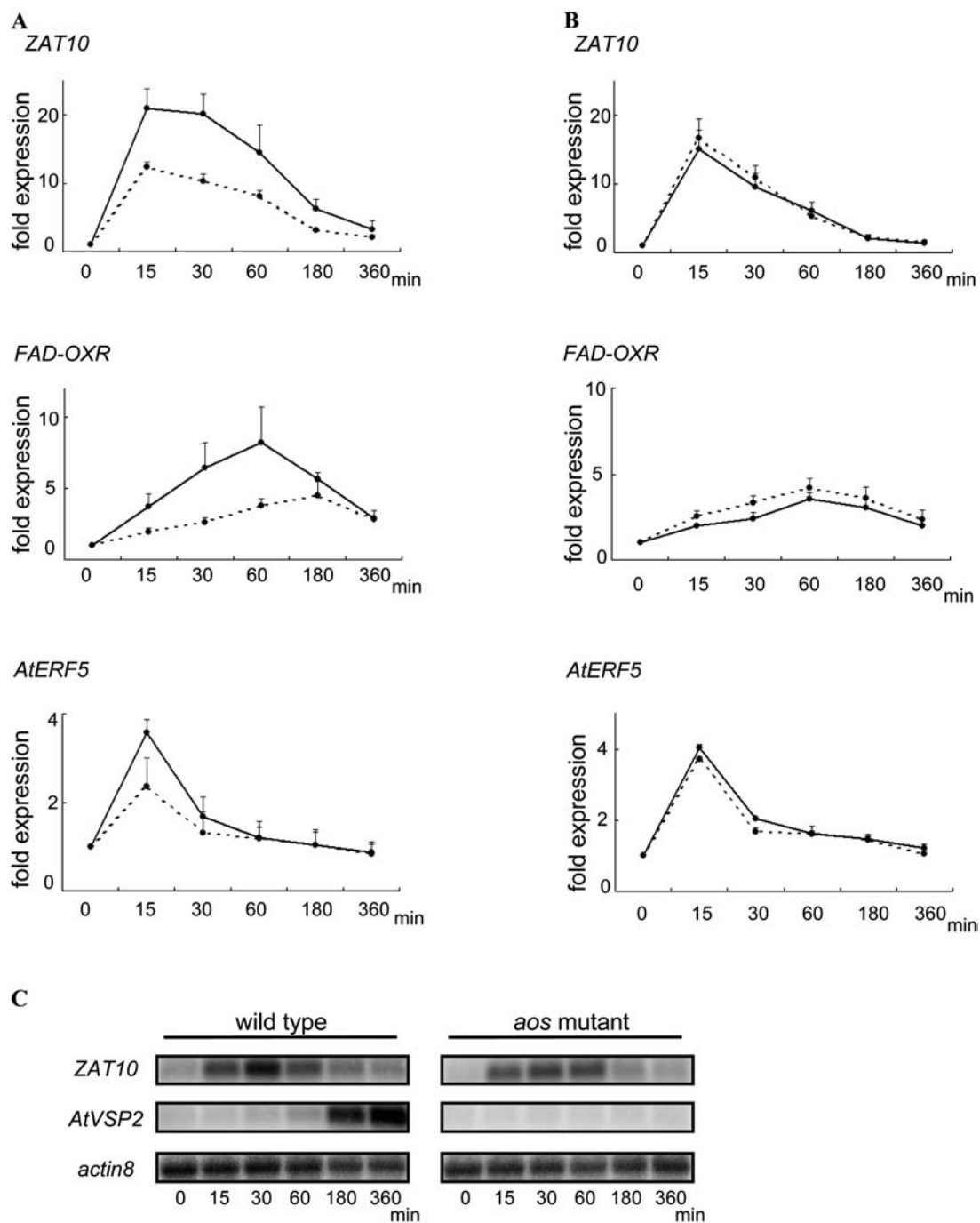
Figure 4. Expression of ORGs in wounded wild-type plants and in mutants deficient in jasmonate biosynthesis. A and B, Leaves of 21-d-old wild-type (Col) and *aos* mutant (Col background) plants (A) or wild-type (Ws) and *opr3* mutant (Ws background) plants (B) were wounded several times across the mid-vein with tweezers. RNA was isolated 0 (control), 15, 30, 60, 180, or 360 min after wounding. Five micrograms of total RNA were loaded per lane, and the blots were hybridized with the indicated probes. *actin8* served as a loading control. Graphs show fold expression of *ZAT10*, *FAD-OXR*, and *AtERF5*. Fold expression was calculated as the ratio of wounded-to-control band intensity. Solid lines indicate wild-type plants Col (A) and Ws (B), and dotted lines indicate the mutants *aos* (A) and *opr3* (B). Average values ± SEM represent the average of four (*ZAT10* and *FAD-OXR*) or three (*AtERF5*) independent experiments. C, Ten-day-old wild-type (Col) plants and *aos* mutants (Col background) were treated with 30 μM linolenic acid for 0 (control), 15, 30, 60, 180, or 360 min. Northern blotting was performed as described above.
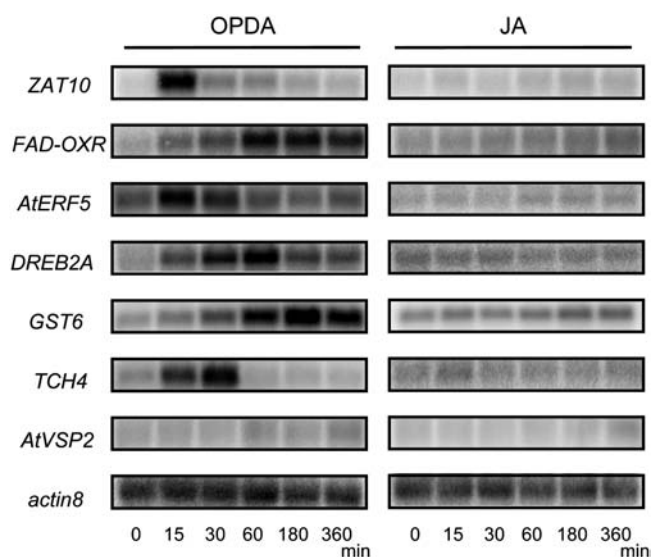
Figure 5. Expression of ORGs in *coi1* mutants. Ten-day-old *coi1-16* mutants (Col background) were treated with 30 μM OPDA or JA, and total RNA was isolated after 0 (control), 15, 30, 60, 180, or 360 min. For northern blots, 5 μg RNA was loaded per lane, and blots were hybridized with the indicated probes; *actin8* served as a loading control.

indicating that the OPDA signaling pathway is independent of the signaling pathway(s) for JAs.

The above results indicate that OPDA and JAs have distinct functions in gene regulation, suggesting that OPDA itself functions as a signaling molecule. The α, β-unsaturated carbonyl structure and the length of carbon chain with the carboxyl group in OPDA appear to be important for its signaling function. Various compounds having an α, β-unsaturated carbonyl group are strong inducers of gene expression in plants (Alméras et al., 2003). Consistent with these findings, acrolein, which has an α, β-unsaturated carbonyl group, induced expression of ORGs (Fig. 6B). Membrane fatty acid-derived prostaglandin plays a signaling role in mammals. Among them described to date, the prostaglandin A and J$_2$ series share structural similarity with OPDA in that they have both an α, β-unsaturated carbonyl group and a long fatty acid chain. In prostaglandin J$_2$, the α, β-unsaturated carbonyl group is critical for its biological activities, which include Michel addition of thiol groups of cellular constituents, namely, glutathione, Cys, and proteins (Atsmon et al., 1990). Therefore, the presence of the α, β-unsaturated carbonyl group may also be important for the specific signaling role of OPDA. Interestingly, our data show that OPC8:0 and OPC6:0 also induce ORG expression (Fig. 6C), indicating that the longer carbon chain length than JA is also important. Although the distinct recognition mechanism of OPDA-mediated signaling remains unclear, the α, β-unsaturated carbonyl group and the length of carbon chain might provide OPDA specificity in plants. Differences in the subcellular localization of OPDA and JA may also influence their signaling functions.

OPDA is synthesized in chloroplasts (Bell et al., 1995; Laudert et al., 1996; Stenzel et al., 2003), and it has been reported that OPDA also occurs in an esterified form attached to a plastid-specific galactolipid (Stelmach et al., 2001). OPDA is converted to JA via subsequent action by OPR3 and β-oxidation in peroxisomes (Stintzi and Browse, 2000; Strassner et al., 2002). Therefore, the final subcellular site for emission of each signaling compound is different. In addition, OPDA and JA are differentially distributed in tomato (*Lycopersicon esculentum*) flowers (Hause et al., 2000), suggesting the different role of OPDA other than the intermediate of JA biosynthesis. Therefore, differences in their synthesis and accumulation sites may explain the significance of the two different signals existing in the same plant cell.

OPDA Function in the Wounding Response

JAs are essential in vivo regulators of defense responses (Reymond and Farmer, 1998), particularly
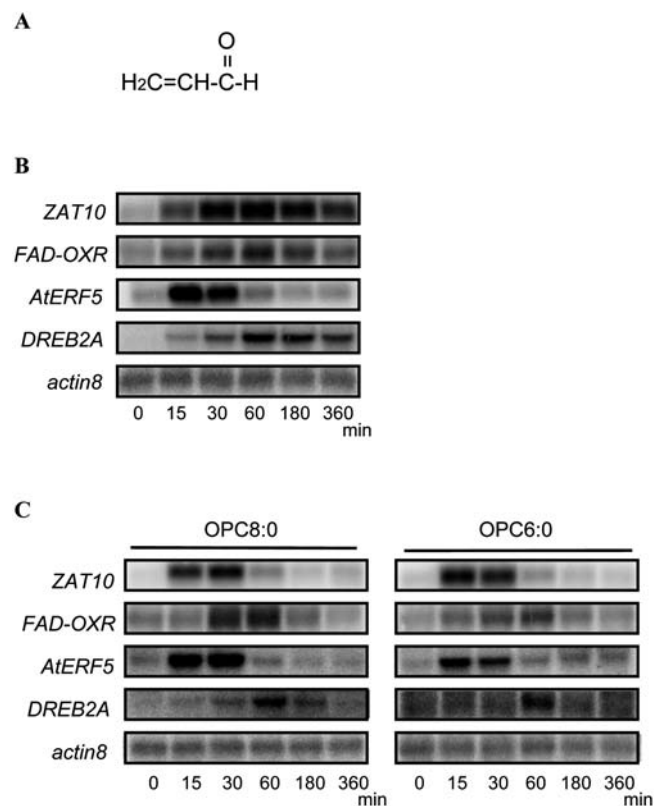

Figure 6. Response of ORGs to structural analogs of OPDA. A, Structure of acrolein, showing the α, β-unsaturated carbonyl group. B, Ten-day-old wild-type (Col) plants were treated with 30 μM acrolein for 0 (control), 15, 30, 60, 180, or 360 min. Five micrograms of total RNA was loaded per lane, and the blots were hybridized with the indicated probes; *actin8* served as a loading control. C, Ten-day-old wild-type (Col) plants were treated with 30 μM OPC8:0 or OPC6:0 for 0 (control), 15, 30, 60, 180, or 360 min. Five micrograms of total RNA was loaded per lane, and the blots were hybridized with the indicated probes; *actin8* served as a loading control.

the wounding response. Numerous reports have shown that mechanical wounding of leaves causes JAs to accumulate (Creelman et al., 1992; Peña-Cortés et al., 1995; Parchmann et al., 1997; Reymond et al., 2000), which may be the primary signal that activates a set of wound-inducible genes (León et al., 2001). Consistent with these reports, wounding altered the expression of almost one-half of the JRGs in this study. As described in "Results" section, similar proportions of ORGs and JRGs responded to wounding. Moreover, OPDA also accumulates after mechanical wounding of leaves (Reymond et al., 2000), suggesting its involvement in the wounding response. We showed that wound-induced ORG expression decreases by one-half in the *aos* mutant (Fig. 4A), whereas this was not the case for the *opr3* mutant (Fig. 4B). These results indicate that OPDA functions as a signaling mediator in the wounding response through a mechanism that is distinct from that of JAs. Because *AtVSP2* activation requires JA synthesis (shown in Fig. 3B), the *AtVSP2* mRNA was not detected in either of the mutants that does not accumulate JA (data not shown).

In the *aos* mutant, approximately 50% of ORG expression remained. Recently, various oxylipins derived from linolenic acid were shown to accumulate and induce gene expression in plants (Howe and Schilmiller, 2002). Therefore, this residual response to wounding in *aos* could be mediated by other oxylipins derived from linolenic acid. As shown in Figure 4C, ORG expression was observed in the *aos* mutant treated with linolenic acid, suggesting the action of an oxylipin(s) derived from the linolenic acid without the AOS reaction. There are several candidate oxylipin compounds in this regard. 13-Hydroperoxylinolenic acid, a precursor of OPDA in the JA biosynthetic pathway (Fig. 1), is not only the substrate of AOS but also of enzymes such as hydroperoxide lyase. Moreover, hydroperoxide lyase- or lipoxygenase-derived types of oxylipins accumulate after wounding or upon infection (Croft et al., 1993; Vollenweider et al., 2000; Vancanneyt et al., 2001; Feussner and Wasternack, 2002). Furthermore, free radical-catalyzed, nonenzymatic reactions can produce phytoprostane, which is similar in structure to OPDA (Krischke et al., 2003). Phytoprostane is also known to induce gene expression in plants, although its induction activity is not as strong as OPDA (Iqbal et al., 2004). These oxylipins may account for the ORG expression observed in the *aos* mutant.

Wounding induced the expression of 74 ORGs. These included many signal transduction components and transcription factors (Tables I and II), suggesting that OPDA is required upstream of the wounding response. Wounding causes the accumulation of reactive oxygen species, resulting in oxidative stress (Olson and Varner, 1993; Orozco-Cárdenas and Ryan, 1999). Other environmental factors, such as drought, heat, cold, and salinity, also cause oxidative stress (Cheong et al., 2002; Fowler and Thomashow, 2002; Xiong et al., 2002). Remarkably, the expression of many

of the ORGs identified in this study is regulated by oxidative stress. The MAPK signaling cascade is activated by oxidative stress (Waskiewicz and Cooper, 1995; Jonak et al., 1996; Bögre et al., 1997; Meskiene et al., 1998; Baudouin et al., 1999). Genes responsible for the MAPK cascade, *OXI1* (At3g25250), protein kinase family (At4g25390), *MAPKKK18* (At1g05100), and protein phosphatase 2C (At3g27140), were among the ORGs we identified. In particular, *OXI1* is an essential component of the signal transduction pathway linked to oxidative burst signals in Arabidopsis (Rentel et al., 2004). Oxidative stress also induces calcium signaling in Arabidopsis (Yang and Poovaiah, 2003; Rentel and Knight, 2004). The up-regulated ORGs (Table I) included a large number of calcium signaling components such as the calcium-binding EF-hand family protein (At4g27280, At5g39670), a putative calcium-binding protein (At5g54490), a calmodulin-binding protein (At5g26920), and a putative calmodulin-related protein (At3g01830, At5g42380). The induction profiles of ORGs involved in these signaling pathways were generally rapid and transient (Table I), suggesting that OPDA plays an important role in the early response to oxidative stress in plants. Moreover, several transcription factors identified as ORGs, *ZAT10* (At1g27730), *AtERF5* (At5g47230), *DREB2A* (At5g05410), *MYB4* (At4g38620), and *ZAT12* (At5g59820), are induced under oxidative stresses such as cold, wounding, dehydration, and salinity (Liu et al., 1998; Fujimoto et al., 2000; Ohta et al., 2001; Lee et al., 2002; Vannini et al., 2004; Davletova et al., 2005). Induction of these genes began within 30 min of OPDA treatment. These results suggest that OPDA signaling and subsequent gene expression occur rapidly under oxidative stress conditions such as those occurring after wounding.

We found that OPDA modulates a distinct set of genes via a COI1-independent signaling pathway. We also propose that OPDA plays a role in the wounding response. The precise function of OPDA in physiological events is still largely unknown. In further analyses of OPDA, isolation of mutants in which OPDA signaling or OPDA level is specifically altered will be a key step to complete our understanding of oxylipin signaling in plant cells.

MATERIALS AND METHODS

Plant Materials, Chemical Treatments, and Wounding

For chemical treatments, Arabidopsis (*Arabidopsis thaliana*; Col Ws, *aos*, *opr3*, and *coi1-16*) were grown in Murashige and Skoog liquid medium (Murashige and Skoog, 1962) containing 1% (w/v) Suc on an orbital shaker under continuous light at 22°C. The jasmonate signaling mutant *coi1-16* was kindly supplied by Dr. John Turner (University of East Anglia). After 10 d, the plants were treated with 30 μM OPDA, JA, MeJA, OPC8:0, OPC6:0, linolenic acid, or acrolein. OPDA, OPC8:0, and OPC6:0 were synthesized and characterized for purity as previously described (Ainai et al., 2003). JA, MeJA, linolenic acid, and acrolein were purchased from Sigma-Aldrich. Plants were harvested at 0 (nontreated), 15, 30, 60, 180, and 360 min after treatment. For wounding studies, Arabidopsis (Col, Ws, *aos*, and *opr3* mutants) were grown on Murashige and Skoog medium plates containing 0.8% agarose under

continuous light at 22°C. The OPDA biosynthetic mutant *aos* was a kind gift from Dr. Joon-Hyun Park and Dr. Kenneth A. Feldmann (Ceres), and the JA biosynthetic mutant *opr3* was a kind gift from Dr. John Browse (Washington State University). All rosette leaves of 21-d-old plants were wounded several times across the mid-vein with tweezers. The plants were harvested at 0 (unwounded), 15, 30, 60, 180, and 360 min after the wounding treatment.

RNA Isolation

For northern-blot analyses, total RNA was extracted from Arabidopsis by the phenol/SDS method (Chirgwin et al., 1979). For microarray experiments, the extracted RNA was additionally purified using an RNeasy mini kit (Qiagen). Total RNA was purified from wounded plants using the RNeasy mini kit for both microarray and northern-blot analyses.

DNA Microarray Analysis

The Arabidopsis 2 oligo microarray and the Agilent Linear Amplification/Labeling kit (Agilent Technologies) were used for DNA microarray analyses, all of which were conducted in biological duplicate. The quality of total RNA samples was verified using the RNA 6000 Nano Assay (Agilent Technologies). Sample amplification, labeling, and hybridization essentially followed the protocol recommended by Agilent Technologies. Briefly, 500 ng of each total RNA sample was reverse transcribed into cDNA using the T7 promoter primer. Labeled cRNA was synthesized from the cDNA. The reaction was performed in a solution containing dNTP mix, cyanine 3-dCTP (for treated samples) or cyanine 5-dCTP (for untreated samples; Perkin-Elmer), and T7 RNA Polymerase, and incubated at 40°C for 2 h. To remove unincorporated nucleotides, the labeled cRNA was purified using the RNeasy mini kit (Qiagen). Hybridization was performed in 500 μL of a hybridization mixture containing cRNA probes, the labeled orientation marker (Deposition Control SP300; Operon Technologies), and Cot-1 DNA (Invitrogen) at 60°C for 17 h. The glass slides were then washed and scanned using an Agilent microarray scanner (Agilent Technologies). See also Supplemental File 1, MIAME-compliant description.

Normalization and Analysis of Microarray Data

Spot intensities were quantified, background subtracted, and dye normalized by Agilent Feature Extraction software. Using the spot intensity, a base-10 logarithmic value of relative expression to control (RE) of each gene was determined by the same software (data available in Supplemental Table IV). Then we calculated "normalized fold expression" from RE to unify the duplicate data and to compare between treatments using Microsoft Excel as below.

For treatment tr = {OPDA, JA, MeJA }, replicate r = {1,2}, time t = {30 min, 180 min}, and gene g:

$$NormalizedRelativeExpression\ (NRE)_{tr,r,t,g} = \frac{\bar{\sigma}_t}{\sigma_{tr,t}} \times RE_{tr,r,t,g},$$

where σ is SD. Since OPDA, JA, and MeJA were applied in the same way for liquid cultured plants, for all these data:

$$\bar{\sigma}_t = \frac{1}{6}\sum_{tr,r}\sigma_{tr,r,t} = \frac{\sigma_{OPDA,1,t} + \sigma_{OPDA,2,t} + \sigma_{JA,1,t} + \sigma_{JA,2,t} + \sigma_{MeJA,1,t} + \sigma_{MeJA,2,t}}{6}.$$

Wounding treatment was performed on 21-d-old seedlings grown on MS medium containing agarose. Thus, wounding data were treated independently.

$$\bar{\sigma}_t = \frac{\sigma_{wound,1,t} + \sigma_{wound,2,t}}{2}$$

We obtained biologically duplicate data for every treatment. To unify the duplicate data, we calculated weighted averages based on a flag (PosAndSignif flag) in Agilent microarray. In the Agilent microarray, if genes showed significantly higher expression than microarray background, the genes were flagged as 1; otherwise 0 for each Green and Red channel. We did not use the data if at least one of the PosAndSignif flags for Green or Red channels was 0. And if the flags for both of the duplicate data were 0, the gene (locus) was removed from all subsequent analyses. For the remaining 20,095 loci, normalized fold expression was determined.

$$Flag_{tr,r,t,g} \equiv PosAndSignif_{tr,r,t,g}(Green) \times PosAndSignif_{tr,r,t,g}(Red) = \{0,1\}$$

$$NRE_{tr,t,g} = \frac{\sum_r (Flag_{tr,r,t,g} \times NRE_{tr,r,t,g})}{\sum_r (Flag_{tr,r,t,g})}$$

$$Normalized\ Fold\ Expression = 10^{NRE}$$

Selection of ORGs, JRGs, and WRGs

To screen ORGs, JRGs, and wounding response genes (WRGs), normalized fold expression of each gene was calculated as described above. Then, maximum normalized fold expression (MAF) and minimum normalized fold expression (MIF) were calculated as follows.

$$\log_{10}(MAF_{tr,g}) = \max_t(NRE_{tr,t,g})$$

$$\log_{10}(MIF_{tr,g}) = \min_t(NRE_{tr,t,g})$$

The MAF and MIF of OPDA, JA, MeJA, and wound treatment were calculated for each gene (MAF_{OPDA}, MIF_{OPDA}, MAF_{JA}, MIF_{JA}, MAF_{MeJA}, MIF_{MeJA}, MAF_{wound}, and MIF_{wound}, respectively). For ORGs, we selected genes using the following criteria: For up-regulated ORGs, MAF_{OPDA} was more than 3 and both MAF_{JA} and MAF_{MeJA} were less than 2; for down-regulated ORGs, MIF_{OPDA} was less than 0.33 and both MIF_{JA} and MIF_{MeJA} were more than 0.5. A total of 172 genes (157 induced, 15 repressed) fulfilled the criteria. For JRGs, we selected genes having both MAF_{JA} and MAF_{MeJA} of more than 3 or both MIF_{JA} and MIF_{MeJA} of less than 0.33. A total of 449 genes (371 induced, 78 repressed) fulfilled these criteria. For WRGs, we selected genes having MAF_{wound} of more than 3 or MIF_{wound} of less than 0.33; 1,721 genes (1,035 genes induced, 696 repressed, 10 genes overlapped in both categories) fulfilled these criteria.

Northern Blotting

Total RNA (5 μg) was prepared from untreated or treated plants as described. The RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane. Probes were prepared from plasmid DNAs of AV544343, AV521889, AV546998, AV537643, AV544278, and AV532124 (accession nos. reported by Asamizu et al. [2000]) for *AtERF5*, *ZAT10*, *FAD-OXR*, *GST6*, *TCH4*, and *AtVSP2*, respectively. The primer sets DREB2AF (5′-gtgttgttgtattgtagattgtgtttg-3′) and DREB2AR (5′-gtcttctc-tattgtcatatcactgtttcg-3′), and ACTIN8F (5′-cttaggtattgcagaccgtatgagc-3′) and ACTIN8R (5′-gtttttatccgagtttgaagaggct-3′), were used for *DREB2A* and *ACTIN8* genes, respectively. Probes were labeled with [α-^{32}P]dCTP. Hybridization was performed as described (Sasaki et al., 2001). Signals were measured using Image Quant (version 5.1; Molecular Dynamics).

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Supplementary figure 1. Clustered display of genes responding to OPDA, JA, and MeJA treatment.

Data from chemical treatments (OPDA, JA, and MeJA) were analyzed by hierarchical clustering. Result of hierarchical clustering is represented as heat map of gene expression profiles, where red indicates induced and blue indicates repressed. Hierarchical clusters of selected genes are shown. In Cluster 1, genes preferentially responding to OPDA but not to JA and MeJA are shown. Cluster 2 is comprised of genes repressed by OPDA but not JA and MeJA (supplementary file 2). Cluster 1 contains most of ORGs presented in Table 1. On the other hand, Cluster 2 contains most of down-regulated ORGs in Table 2.

Calculation was performed as follows. We first selected 1078 genes for clustering, which include all 172 ORGs and 449 JRGs according to a following criterion;

$$MxAbNRE_g \equiv \max_{tr,t} | NRE_{tr,t,g} | > \log_{10}(3)$$

Then $NRE_{tr,r,t,g}$, the value before unification of duplicate data (see Materials and Methods), was normalized by the $MxAbNRE_g$ and the normalized value was used for clustering.

$$MxAbNNRE_{tr,r,t,g} = \frac{NRE_{tr,r,t,g}}{MxAbNRE_g}$$

The normalized value, $MxAbNNRE_{tr,r,t,g}$, of 1078 genes were hierarchically clustered with their Euclidean distances using ward method in statistical language R.

Almost identical result was obtained when we calculated from $NRE_{tr,t,g}$, the NRE value after unification of duplicate data (data not shown).

Supplementary file 4. The MIAME Checklist

Experimental Design:

1. Type of experiment:
   - Comparison of untreated vs. OPDA-, JA-, and MeJA-treated Arabidopsis.
   - Comparison of control (no wounding) vs. wounded Arabidopsis.

2. Experimental factors:
   - Time course (0 min, 30 min and 180 min)
   - Treatment (OPDA, JA, MeJA, and wounding)

3. Number of hybridizations performed in the experiment: 16

4. Hybridization design:

	Label	Strain	Condition		Harvested	Tissue
			Growth	Treatment		
1	C1	wild-type	MS medium, 10 days	none	0 min	whole plants
	OP11	wild-type	MS medium, 10 days	OPDA	30 min	whole plants
2	C2	wild-type	MS medium, 10 days	none	0 min	whole plants
	OP12	wild-type	MS medium, 10 days	OPDA	180 min	whole plants
3	C3	wild-type	MS medium, 10 days	none	0 min	whole plants
	OP21	wild-type	MS medium, 10 days	OPDA	30 min	whole plants
4	C4	wild-type	MS medium, 10 days	none	0 min	whole plants
	OP22	wild-type	MS medium, 10 days	OPDA	180 min	whole plants
5	C5	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA11	wild-type	MS medium, 10 days	JA	30 min	whole plants
6	C6	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA12	wild-type	MS medium, 10 days	JA	180 min	whole plants
7	C7	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA21	wild-type	MS medium, 10 days	JA	30 min	whole plants
8	C8	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA22	wild-type	MS medium, 10 days	JA	180 min	whole plants
9	C9	wild-type	MS medium, 10 days	none	0 min	whole plants
	MA11	wild-type	MS medium, 10 days	MeJA	30 min	whole plants
10	C10	wild-type	MS medium, 10 days	none	0 min	whole plants
	MJ12	wild-type	MS medium, 10 days	MeJA	180 min	whole plants
11	C11	wild-type	MS medium, 10 days	none	0 min	whole plants
	MJ21	wild-type	MS medium, 10 days	MeJA	30 min	whole plants
12	C12	wild-type	MS medium, 10 days	none	0 min	whole plants
	MJ22	wild-type	MS medium, 10 days	MeJA	180 min	whole plants
13	C13	wild-type	MS medium, 21 days	none	0 min	Rosette leaf
	WOD11	wild-type	MS medium, 21 days	wounding	30 min	Rosette leaf
14	C14	wild-type	MS medium, 21 days	none	0 min	Rosette leaf
	WOD12	wild-type	MS medium, 21 days	wounding	180 min	Rosette leaf
15	C15	wild-type	MS medium, 21 days	none	0 min	Rosette leaf
	WOD21	wild-type	MS medium, 21 days	wounding	30 min	Rosette leaf
16	C16	wild-type	MS medium, 21 days	none	0 min	Rosette leaf
	WOD22	wild-type	MS medium, 21 days	wounding	180 min	Rosette leaf

5. Quality control steps taken: Confirmation of mRNA levels by RNA 6000 RNA Nano Assay (Agilent
   Technologies, Inc., U.S.A.)

<u>Samples used, extract preparation and labeling:</u>

1. The origin of the biological sample:

 - *Arabidopsis thaliana* ecotype Columbia

2. Manipulation of biological samples and protocols used:

 - *Arabidopsis thaliana* cultured in a growth chamber controlled at 22ºC

 - Continuous light

 - Sterile culture

 - MS medium (Murashige and Skoog, (1962) Physiol. Plant. 15, 493-497)

    For comparison of untreated vs. OPDA-, JA-, and MeJA-treated Arabidopsis, liquid medium was used. On the other hand, solid medium was applied to compare wounded Arabidopsis to the control (no wounding).

3. Protocols or preparation of the hybridization extract:

 - RNA extraction with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany)

4. Labeling Protocols:

 - Double-stranded cDNA synthesis using Low RNA Input Linear Amplification Kit (Agilent Technologies, Inc.)

 - Synthesis of Cy3-labeled (for treated sample) or Cy5-labeled (for untreated sample) cRNA by in vitro transcription using the Low RNA Input Linear Amplification Kit (Agilent Technologies, Inc.)

 - Purification of labeled cRNA with the RNeasy column (Qiagen)

 - Fragmentation of labeled cRNA: 60ºC, 30 min using the In Situ Hybridization Kit Plus (Agilent Technologies, Inc.)

<u>Hybridization procedures and parameters:</u>

1. Hybridization of fragmented cRNA to Arabidopsis 2 Microarray (Agilent Technologies, Inc.)

 - 60ºC, 17 hr hybridization in a hybridization oven (Agilent Technologies, Inc.)

 - Hybridization Cocktail

   1.0 µg fragmented cRNA labeled with Cy3 and Cy5

   Hybridization Buffer (Agilent Technologies, Inc.)

   Deposition Control (Operon Technologies, Inc., U.S.A.)

   Human Cot-1 DNA (Invitrogen Corporation, U.S.A.)

2. Washing

 - Wash Buffer 1

   6× SSPE (Agilent Technologies, Inc.)

0.005% N-lauroylsarcosine (Agilent Technologies, Inc.)

 - Wash Buffer 2

  0.06× SSPE

  0.005% N-lauroylsarcosine

 - Wash Buffer 3

  Stabilization and Drying Solution (Agilent Technologies, Inc.)

 - Wash Buffer 1, 1 min, room temperature

 - Wash Buffer 2, 1 min, room temperature

 - Wash Buffer 3, 30 sec, room temperature

Measurement data and specifications:

1. Scanning: DNA Microarray Scanner (Agilent Technologies, Inc.)

2. Image analysis: Agilent Feature Extraction software (Agilent Technologies, Inc.)

3. Fold expression of signal intensities (Relative Expression) were calculated from the following comparisons. Signal intensities of the probes and Relative Expression are presented in an Excel file (supplementary file 5).

A	OP11 *vs.* C1
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J	MJ12 *vs.* C10
K	MJ21 *vs.* C11
L	MJ22 *vs.* C12
M	WOD11 *vs.* C13
N	WOD12 *vs.* C14
O	WOD21 *vs.* C15
P	WOD22 *vs.* C16

Array Design:

Arabidopsis 2 oligo microarray (Agilent Technologies, Inc.)

The content of this microarray was derived from the ATH1 v. 3 database of The Institute for Genomic Research (TIGR) and represents 21,500 genes.

Supplementary figure 1. Clustered display of genes responding to OPDA, JA, and MeJA treatment.

Data from chemical treatments (OPDA, JA, and MeJA) were analyzed by hierarchical clustering. Result of hierarchical clustering is represented as heat map of gene expression profiles, where red indicates induced and blue indicates repressed. Hierarchical clusters of selected genes are shown. In Cluster 1, genes preferentially responding to OPDA but not to JA and MeJA are shown. Cluster 2 is comprised of genes repressed by OPDA but not JA and MeJA (supplementary file 2). Cluster 1 contains most of ORGs presented in Table 1. On the other hand, Cluster 2 contains most of down-regulated ORGs in Table 2.

Calculation was performed as follows. We first selected 1078 genes for clustering, which include all 172 ORGs and 449 JRGs according to a following criterion;

$$MxAbNRE_g \equiv \max_{tr,t} | NRE_{tr,t,g} | > \log_{10}(3)$$

Then $NRE_{tr,r,t,g}$, the value before unification of duplicate data (see Materials and Methods), was normalized by the $MxAbNRE_g$ and the normalized value was used for clustering.

$$MxAbNNRE_{tr,r,t,g} = \frac{NRE_{tr,r,t,g}}{MxAbNRE_g}$$

The normalized value, $MxAbNNRE_{tr,r,t,g}$, of 1078 genes were hierarchically clustered with their Euclidean distances using ward method in statistical language R.

Almost identical result was obtained when we calculated from $NRE_{tr,t,g}$, the NRE value after unification of duplicate data (data not shown).

Supplementary file 4. The MIAME Checklist

<u>Experimental Design:</u>

1. Type of experiment:

  - Comparison of untreated vs. OPDA-, JA-, and MeJA-treated Arabidopsis.

  - Comparison of control (no wounding) vs. wounded Arabidopsis.

2. Experimental factors:

  - Time course (0 min, 30 min and 180 min)

  - Treatment (OPDA, JA, MeJA, and wounding)

3. Number of hybridizations performed in the experiment: 16

4. Hybridization design:

	Label	Strain	Condition		Harvested	Tissue
			Growth	Treatment		
1	C1	wild-type	MS medium, 10 days	none	0 min	whole plants
	OP11	wild-type	MS medium, 10 days	OPDA	30 min	whole plants
2	C2	wild-type	MS medium, 10 days	none	0 min	whole plants
	OP12	wild-type	MS medium, 10 days	OPDA	180 min	whole plants
3	C3	wild-type	MS medium, 10 days	none	0 min	whole plants
	OP21	wild-type	MS medium, 10 days	OPDA	30 min	whole plants
4	C4	wild-type	MS medium, 10 days	none	0 min	whole plants
	OP22	wild-type	MS medium, 10 days	OPDA	180 min	whole plants
5	C5	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA11	wild-type	MS medium, 10 days	JA	30 min	whole plants
6	C6	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA12	wild-type	MS medium, 10 days	JA	180 min	whole plants
7	C7	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA21	wild-type	MS medium, 10 days	JA	30 min	whole plants
8	C8	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA22	wild-type	MS medium, 10 days	JA	180 min	whole plants
9	C9	wild-type	MS medium, 10 days	none	0 min	whole plants
	MA11	wild-type	MS medium, 10 days	MeJA	30 min	whole plants
10	C10	wild-type	MS medium, 10 days	none	0 min	whole plants
	MJ12	wild-type	MS medium, 10 days	MeJA	180 min	whole plants
11	C11	wild-type	MS medium, 10 days	none	0 min	whole plants
	MJ21	wild-type	MS medium, 10 days	MeJA	30 min	whole plants
12	C12	wild-type	MS medium, 10 days	none	0 min	whole plants
	MJ22	wild-type	MS medium, 10 days	MeJA	180 min	whole plants
13	C13	wild-type	MS medium, 21 days	none	0 min	Rosette leaf
	WOD11	wild-type	MS medium, 21 days	wounding	30 min	Rosette leaf
14	C14	wild-type	MS medium, 21 days	none	0 min	Rosette leaf
	WOD12	wild-type	MS medium, 21 days	wounding	180 min	Rosette leaf
15	C15	wild-type	MS medium, 21 days	none	0 min	Rosette leaf
	WOD21	wild-type	MS medium, 21 days	wounding	30 min	Rosette leaf
16	C16	wild-type	MS medium, 21 days	none	0 min	Rosette leaf
	WOD22	wild-type	MS medium, 21 days	wounding	180 min	Rosette leaf

5. Quality control steps taken: Confirmation of mRNA levels by RNA 6000 RNA Nano Assay (Agilent

  Technologies, Inc., U.S.A.)

Samples used, extract preparation and labeling:

1. The origin of the biological sample:
 - *Arabidopsis thaliana* ecotype Columbia

2. Manipulation of biological samples and protocols used:
 - *Arabidopsis thaliana* cultured in a growth chamber controlled at 22ºC
 - Continuous light
 - Sterile culture
 - MS medium (Murashige and Skoog, (1962) Physiol. Plant. 15, 493-497)
     For comparison of untreated vs. OPDA-, JA-, and MeJA-treated Arabidopsis, liquid medium
     was used. On the other hand, solid medium was applied to compare wounded Arabidopsis to the
     control (no wounding).

3. Protocols or preparation of the hybridization extract:
 - RNA extraction with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany)

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 - Double-stranded cDNA synthesis using Low RNA Input Linear Amplification Kit (Agilent
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 - Fragmentation of labeled cRNA: 60ºC, 30 min using the In Situ Hybridization Kit Plus (Agilent
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Hybridization procedures and parameters:

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   Deposition Control (Operon Technologies, Inc., U.S.A.)
   Human Cot-1 DNA (Invitrogen Corporation, U.S.A.)

2. Washing
 - Wash Buffer 1
   $6\times$ SSPE (Agilent Technologies, Inc.)

0.005% N-lauroylsarcosine (Agilent Technologies, Inc.)

 - Wash Buffer 2

   0.06× SSPE

   0.005% N-lauroylsarcosine

 - Wash Buffer 3

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 - Wash Buffer 1, 1 min, room temperature

 - Wash Buffer 2, 1 min, room temperature

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K	MJ21 vs. C11
L	MJ22 vs. C12
M	WOD11 vs. C13
N	WOD12 vs. C14
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	JA12	wild-type	MS medium, 10 days	JA	180 min	whole plants
7	C7	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA21	wild-type	MS medium, 10 days	JA	30 min	whole plants
8	C8	wild-type	MS medium, 10 days	none	0 min	whole plants
	JA22	wild-type	MS medium, 10 days	JA	180 min	whole plants
9	C9	wild-type	MS medium, 10 days	none	0 min	whole plants
	MA11	wild-type	MS medium, 10 days	MeJA	30 min	whole plants
10	C10	wild-type	MS medium, 10 days	none	0 min	whole plants
	MJ12	wild-type	MS medium, 10 days	MeJA	180 min	whole plants
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	MJ21	wild-type	MS medium, 10 days	MeJA	30 min	whole plants
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