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Monoterpene-induced molecular responses in *Arabidopsis thaliana* ☆

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ABSTRACT

Terpenoid volatiles mediate various forms of chemical communications of plants with other organisms. In this paper we demonstrate that exposure of intact *Arabidopsis thaliana* plants to monoterpene volatiles results in substantial changes of the plant transcriptome and induction of methyl jasmonate (MeJA) accumulation. We used a heterologous pinII::*GUS* reporter system to test monoterpenes for their potential to induce a response in *A. thaliana*. Plants showed increased pinII-promoter activity upon exposure to different monoterpene volatiles, similar to the response induced by MeJA, mechanical wounding, or insect feeding. Microarray gene expression profiling indicated induced changes in the abundance of several hundred transcripts in wild-type plants upon either exposure to myrcene volatiles or exposure to a blend of ocimene volatiles consisting of (*E*)-β-ocimene, (*Z*)-β-ocimene, and *allo*-ocimene. Many of the monoterpene-induced transcripts are annotated as either transcription factors or as stress or defense genes including several steps in the octadecanoid pathway. Metabolite analysis showed that exposure of *Arabidopsis* for 2 h to myrcene or ocimene induced increased tissue levels of MeJA. Octadecanoid biosynthesis (*aoc*) and signaling (*coi1*) mutants showed some reduced ocimene-induction of gene expression.

1. Introduction

Plants emit a large variety of volatile organic compounds, including a diverse array of low molecular weight terpenoids (Dudareva et al., 2006; Pichersky et al., 2006). Some of these volatile emissions function as chemical communication signals between plants and other organisms, for example in the attraction of pollinators, attraction of fruit dispersing animals, host recognition by herbivores, or signaling in multi-trophic plant-arthropod defence interactions (e.g., Paré and Tumlinson, 1999; De Moraes et al., 1998, 2001; Dicke and Van Loon, 2000; Kessler and Baldwin, 2001; Kappers et al., 2005; Rasmann et al., 2005). Whether or not plants themselves can receive biologically relevant information by detecting volatiles emitted from other plants, or from distant parts of the same plant, is a question of active research that dates back more than two decades (Baldwin and Schultz, 1983; Rhoades, 1983). The topic has been critically addressed in several recent experimental studies and reviews (e.g., Arimura et al., 2000; Birkett et al., 2000; Dolch and Tscharntke, 2000; Karban et al., 2000; Karban, 2001; Dicke et al., 2003; Engelberth et al., 2004; Baldwin et al., 2006; Paschold et al., 2006; Runyon et al., 2006; Heil and Bueno, 2007). For example, Runyon et al. (2006) showed that the parasitic dodder plant (*Cuscuta pentagona*) responds to terpenoid volatiles emitted from tomato plants for successful host targeting. This and other work that provide evidence for volatiles in plant–plant communication (reviewed in Pichersky and Gershenzon, 2002; Dicke et al., 2003; Baldwin et al., 2006) suggest that detection of volatiles may result in a substantial molecular response in the receiving plant. Recently, Kishimoto et al. (2005, 2006a,b) showed that exposure of *Arabidopsis thaliana* to the monoterpene *allo*-ocimene caused increased abundance of several gene transcripts and increased plant resistance against the pathogen *Botrytis cinerea*, but no large-scale transcriptome analysis assessing the extend of the response to terpenoid volatiles has been reported.

The objective of the present study was to assess the extent to which exposure to monoterpene volatiles affects the transcriptome of *A. thaliana* using a genome-wide transcriptome analysis. We report results from (1) testing of several acyclic and cyclic monoterpene volatiles for their potential to induce heterologous promoter

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activity in *A. thaliana*, (2) genome-wide expression profiling of *A. thaliana* tissues exposed to the volatile monoterpenes myrcene (**1**) and ocimene (**2**), (3) jasmonate (**3**) metabolite analysis of ocimene-treated plants, and (4) mutant analysis of ocimene-treated *A. thaliana*. Together, the results obtained demonstrate that monoterpene volatiles have a substantial effect on the *A. thaliana* transcriptome. Octadecanoids may play a role in the monoterpene-induced response.

2. Results

2.1. Monoterpene volatiles induce a heterologous pinII promoter in A. thaliana

To establish a system that allows for rapid screening of volatiles that may induce a plant response, we transformed *A. thaliana* with a *GUS*-reporter gene under the control of the potato proteinase inhibitor II promoter (pinII) (Thornburg et al., 1987; Godard et al., 2007). This promoter is known to drive wound-, herbivore-, or octadecanoid-induced gene expression in several plant species. In initial tests, we found pinII-dependent GUS expression induced in *A. thaliana* in response to mechanical wounding, insect-feeding, or exposure to MeJA volatiles (Godard et al., 2007) (Fig. 1). Using an enclosed system, we then assessed several different monoterpene volatiles for induction of pinII-dependent GUS activity. Potted plants were placed under a 1-L glass beaker containing a cotton ball with 0.4 or 4 μL of the monoterpene compound (0.4 μL myrcene (**1**) is equivalent to approximately 2 μmoles of the monoterpene). As a negative control, plants were treated in the same fashion without any compounds added to the cotton ball. Plants were exposed to the volatiles for 2 h. The monoterpene volatiles tested individually were myrcene (**1**), (+)-α-pinene (**4a**), (−)-α-pinene (**4b**), (−)-β-pinene (**5**), racemic limonene (**6**), racemic linalool (**7**), and a blend of 70% (*E*)-(β)-ocimene (**2a**), 10% (*Z*)-(β)-ocimene and 15% *allo*-ocimene. GUS tissue staining assays of rosette leaves showed that all monoterpenes tested caused the induction of the pinII promoter (Fig. 1). A GUS response was detected with as little as 0.4 μL of monoterpene applied to a cotton ball, of which less than half (approximately 0.15–0.2 μL) had been released into the vapor phase after 2 h. For comparison, methyl jasmonate, MeJA (containing approximately 10% of the putatively active *cis*-isomer) evaporating from a volume of 0.4 μL applied to a cotton ball did not result in detectable GUS tissue staining, but an induced response was detected with 2 μL MeJA (Fig. 1). In control experiments without volatiles added, enclosure of *A. thaliana* for 2 h under a 1-L glass container did not lead to GUS activation (Fig. 1), demonstrating that the pinII promoter response observed with monoterpenes or MeJA is triggered by the volatiles present in the gas phase.

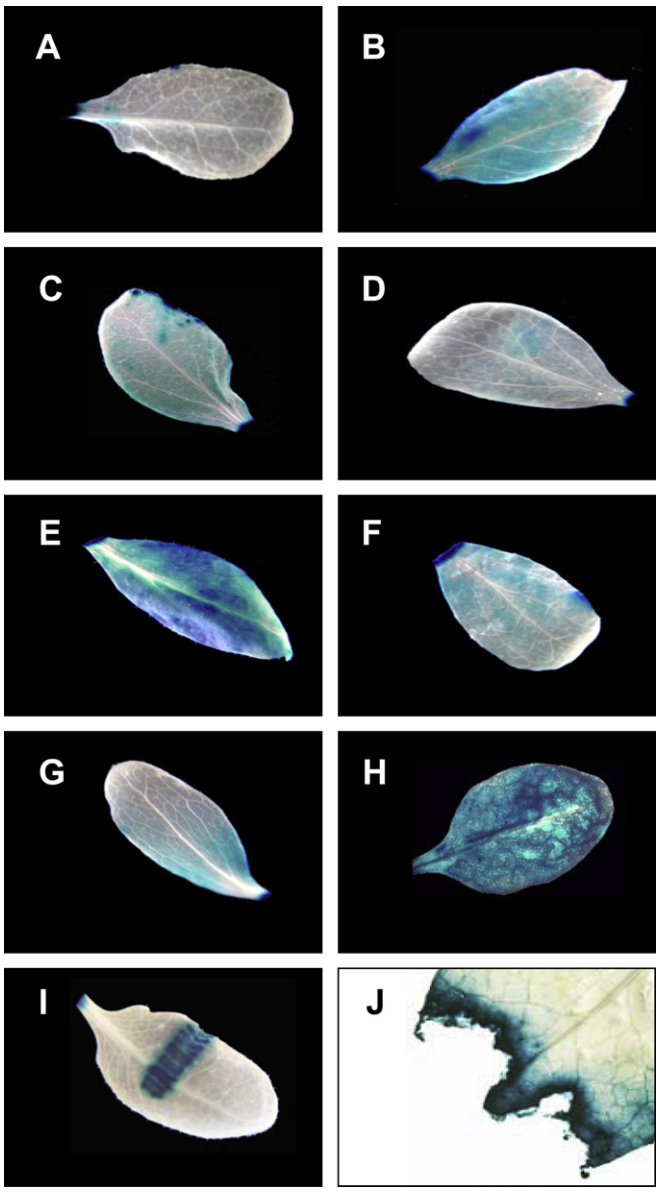

Fig. 1. Monoterpenes, MeJA, wounding or insect feeding induce heterologous pinII promoter activity in *Arabidopsis*. Plants transformed with a pinII::*GUS* construct were either (A) untreated; exposed for 2h to the following monoterpene volatiles (B) ocimene, (C) myrcene, (D) limonene, (E) α-pinene, (F) β-pinene, (G) linalool; exposed for 2 h to (H) MeJA volatiles; treated by (I) mechanical wounding or (J) feeding insect larvae. Note that intensity of tissue staining is only a qualitative but not a quantitative means of assessment of the response. (I) and (J) served as positive controls previously shown in Godard et al. (2007).

2.2. Transcriptome response of A. thaliana exposed to monoterpene volatiles

Following our observation that monoterpene volatiles induced heterologous pinII promoter activity, a chemical genomics approach was used to test the effect of monoterpene volatiles on gene expression in *A. thaliana*. Microarray analyses were done on a 70-mer oligo-nucleotide platform representing 29,110 different transcripts (Ehlting et al., 2005). Plants were exposed for 2 h to ocimene or myrcene volatiles released from 4 μL applied to a cotton ball as described above. The ocimene-induced transcriptome response was also measured over a time course with plants exposed to volatiles for 0.5, 2, 6 or 24 h. Transcriptome profiles of treated plants were compared with those of controls which were handled in exactly the same fashion as the treated plants, except no monoterpenes were added to the experimental system (Supplementary Figure 1). Microarray analyses were performed with above-ground tissues including rosette leaves, stems, and cauline leaves. Experiments were repeated with at least two and in most cases four independent biological replicates per treatment and timepoint.

Transcripts corresponding to 6243 different array elements were reproducibly detected with parametric p values $\leqslant 0.05$ (Student's t-test) (Supplementary Table 1 in the online version of this paper). From this set, differentially regulated genes were defined as those with at least 2-fold monoterpene-induced increase or decrease in transcript abundance relative to the corresponding non-treated control. Using these criteria, no statistically significant differences in transcript abundance were found with plants exposed for 0.5 h to ocimene volatiles. At 2 h of exposure

to ocimene, 468 genes showed differences in transcript abundance, of which 398 (85%) were up- and 70 (15%) were down-regulated (Fig. 2). The overall response to ocimene was reduced by 6 h and 24 h with 249 and 340 genes, respectively, showing differences in transcript abundance, the majority of which (211 and 308, respectively) were up-regulated. The transcriptome response to ocimene volatiles showed a dynamic temporal pattern with partial overlap of differentially regulated genes for the timepoints tested (Supplementary Figure 2). The most strongly up- and down-regulated transcripts for each timepoint and treatment are listed in Table 1, which prominently features methyl transferases as strongly up-regulated genes in the early response at 2 h after myrcene or ocimene treatment. Only 35 genes were up-regulated over the entire time course (Table 2). Seventy six genes showed up-regulation and four showed down-regulation both at the 2h- and 6h-timepoints. Sixty-one genes were commonly up-regulated, and three were commonly down-regulated for the 6h- and 24h-response. Common to the 2h- and 24h-timepoints were 77 genes that were up-regulated and one that was down-regulated.

Similar to the response induced with ocimene at 2 h, a 2-h treatment with myrcene volatiles resulted in a significant change of transcript abundance for 986 elements on the array, of which 730 (74%) were up- and 256 (26%) were down-regulated (Fig. 2). Sixty percent (237) of the genes that were up-regulated by ocimene at 2 h were also up-regulated at the same timepoint by myrcene, which provides additional validation for the response observed, and 29% (20) of the genes down-regulated by ocimene were commonly down-regulated by myrcene (Supplementary Figure 2).

2.3. Stress-related genes, membrane proteins, and transcription factors are over-represented in the monoterpene-induced transcriptome

To gain insights into the qualitative changes of the transcriptome affected by myrcene or ocimene, we identified associations of differentially regulated genes (detected upon ocimene or myrcene exposure at the 2-h timepoint) with functional categories using the TAIR gene ontology (GO) system (www.arabidopsis.org). Differentially regulated genes of known or putative functions were sorted by GO categories for "biological processes", "cellular components", or "molecular functions" (Fig. 3). The relative abundance of monoterpene-regulated transcripts for a particular GO category was compared to the relative abundance of that GO category within the *A. thaliana* genome. Several gene categories were identified as over- or under-represented in the transcriptome of plants exposed to monoterpene volatiles relative to their genome abundance (Fig. 3).

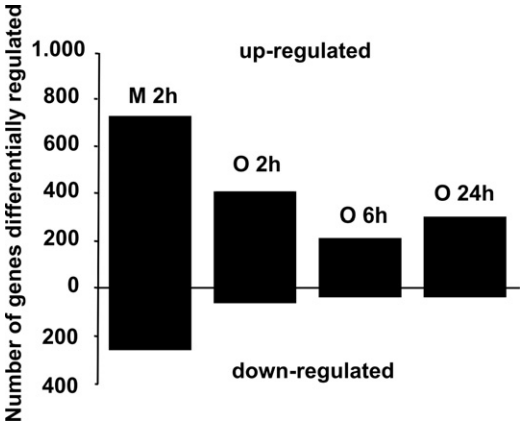

Fig. 2. Transcriptome profiling of *Arabidopsis* exposed to myrcene or ocimene volatiles. Number of different transcripts showing change in transcript abundance of at least 2-fold ($p \leqslant 0.05$). Plants were exposed to myrcene volatiles for 2 h (M2h), or to ocimene volatiles for 2 h (O2h), for 6 h (O6h), or 24 h (O24h).

In the "biological processes" categories, transcripts associated with abiotic and biotic stress and transcripts associated with general stress were significantly over-represented in the transcriptome affected by myrcene or ocimene relative to the account of these categories in the *A. thaliana* genome (Fig. 3). This finding is further supported by data showing that 25% of genes identified as up-regulated by ocimene at more than one time point were annotated as stress-related. In contrast, transcripts associated with protein metabolism, developmental processes, cell organization, and DNA/RNA metabolism were significantly under-represented in the transcriptomes of ocimene- or myrcene-treated plants. In the cellular components categories, only transcripts associated with membranes were significantly over-represented in myrcene treated plants.

In the categories referred to as "molecular functions", the abundance of transcription factors was significantly higher in monoterpene-induced transcriptomes relative to their genomic abundance (Fig. 3). At 2 h of plant exposure to volatiles, transcription factors accounted for 7.3% and 6.3% of the differentially regulated transcriptome in plants treated with ocimene or myrcene, respectively. This induced over-representation of transcription factors was an early and transient response to the treatment with volatiles. At 24 h, only 4.4% of the transcriptome corresponded to transcription factors, without being significantly different from the 4.2% representation of transcription factors in the *Arabidopsis* genome.

At 2 h of treatment with myrcene or ocimene, 83 different transcription factors were shown to be differentially expressed in at least one treatment (Fig. 4A; Supplementary Table 1). Most prominent among the differentially expressed transcription factors were members of the AP2/ERF family with 19% of the transcription factors in the monoterpene-induced transcriptome, followed by bHLH elements (17%), and WRKY and NAM factors (13%) (Fig. 4B). Other volatile-induced transcription factors included MYB and bZIP factors, as well as transcription factors containing zinc finger elements. Most of the differentially expressed transcription factors were up-regulated and only about 25% showed down-regulation. Comparative analysis of *A. thaliana* gene expression data available in the public domain (https://www.genevestigator.ethz.ch/at/) showed that several of the transcription factors that respond to monoterpenes do not show a similar response to other stress signaling molecules such as ethylene, MeJA, salicylic acid, or abscissic acid (Fig. 5).

2.4. Monoterpene volatiles induce octadecanoid responses

Given the prominence of stress-related transcripts in the response of *A. thaliana* to myrcene or ocimene volatiles, we further dissected this segment of the monoterpene-induced transcriptome. Genes of the octadecanoid pathway and genes known to respond to octadecanoids (biosynthesis or response to JA) were among the most prevalent within the stress-gene category up-regulated both by ocimene or myrcene (Fig. 6). Other highly abundant genes in the myrcene-affected transcriptome were genes associated with responses to biotic stress and known defence genes. In the 2-h exposure experiments, octadecanoid pathway and octadecanoid response genes represented 21% and 12% of regulated stress genes in the ocimene- and myrcene-treated plants, respectively. At this timepoint, several known steps in the biosynthesis of JA and MeJA were detected by microarray analysis as up-regulated by ocimene ($\geqslant$2-fold increase; $p \leqslant 0.05$) (Table 3). Transcripts for most of the JAZ proteins, which include the recently discovered JAZ regulators of jasmonate signaling (Chini et al., 2007; Thines et al., 2007), were also up-regulated following monoterpene treatment (Table 4). In a general validation of microarray results,

Table 1
Most strongly up- or down-regulated transcripts for each treatment and timepoint; FC: fold change; *p* val: *p* value; *q* val: q value; gp val: global *p* value (encompassing of all array slides); gq val: global *q* value (encompassing of all array slides)

Treatment, timepoint/gene function (known or predicted)	Locus	FC	*p* val	*q* val	gp val	gq val
Myrcene, 2 h						
Methyl transferase	At3g44860	24.11	0.00	0.00	0.00	0.00
Methyl transferase	At1g66700	22.57	0.00	0.00	0.00	0.00
Glycosyl transferase	At3g11340	21.39	0.00	0.00	0.00	0.00
Methyl transferase	At5g38100	21.00	0.00	0.00	0.00	0.00
Methyl transferase	At1g66690	18.23	0.00	0.00	0.00	0.00
Methyl transferase	At3g44870	16.78	0.00	0.00	0.00	0.00
Expressed protein	At5g03350	16.40	0.00	0.00	0.00	0.00
WRKY family transcription factor	At1g80840	14.90	0.00	0.00	0.00	0.00
Expressed protein	At2g34600	9.75	0.00	0.00	0.00	0.00
IAA-Ala hydrolase (IAR3)	At1g51760	8.82	0.00	0.00	0.00	0.00
Nodulin MtN21 family protein	At3g28080	0.22	0.00	0.00	0.00	0.01
Senescence associated protein	At1g78020	0.21	0.00	0.00	0.00	0.00
Expressed protein	At3g62550	0.21	0.00	0.00	0.00	0.00
Myrosinase associated protein	At3g14210	0.20	0.00	0.00	0.00	0.02
Expressed protein	At5g35480	0.20	0.00	0.01	0.02	0.07
Expressed protein	At1g27020	0.19	0.00	0.00	0.00	0.03
Expressed protein	At1g27030	0.18	0.00	0.00	0.00	0.02
Expansin, putative (EXP8)	At2g40610	0.18	0.00	0.00	0.00	0.01
CONSTANS B-box zinc finger protein	At1g68520	0.15	0.00	0.00	0.00	0.01
GAST1-related protein	At1g74670	0.10	0.00	0.00	0.00	0.01
Ocimene, 2 h						
Small heat shock protein	At2g29500	13.30	0.00	0.00	0.00	0.01
Expressed protein	At2g34600	13.04	0.00	0.00	0.00	0.00
Methyl transferase	At5g38100	11.71	0.00	0.00	0.00	0.00
Methyl transferase	At3g44860	10.73	0.00	0.00	0.00	0.00
Methyl transferase	At3g44870	10.68	0.00	0.00	0.00	0.00
Glutathione transferase	At2g29460	10.15	0.00	0.00	0.00	0.00
Methyl transferase	At1g66700	9.63	0.00	0.00	0.00	0.00
Expressed protein	At1g19180	8.91	0.00	0.00	0.00	0.00
Calmodulin-related protein	At1g76640	8.10	0.00	0.00	0.00	0.01
WRKY family transcription factor	At1g80840	7.68	0.00	0.00	0.00	0.00
Photosystem II type I chl a/b binding protein	At2g34420	0.35	0.08	0.04	0.01	0.05
Glycosyl transferase	At2g16890	0.33	0.00	0.01	0.00	0.02
DnaJ protein	At1g80920	0.33	0.00	0.00	0.01	0.00
Heat shock protein	At4g21870	0.32	0.00	0.01	0.00	0.03
Expressed protein	At3g62550	0.31	0.00	0.00	0.00	0.01
Hypothetical protein	At3g24370	0.29	0.00	0.07	0.00	0.12
Hypothetical protein	At1g23650	0.27	0.00	0.06	0.00	0.07
Expressed protein	At1g09310	0.24	0.00	0.12	0.02	0.11
Light regulated protein	At3g26740	0.18	0.00	0.05	0.04	0.12
Ocimene, 6 h						
Glutathione transferase	At2g29460	5.70	0.50	0.81	0.00	0.02
12-Oxophytodienoate reductase (OPR1)	At1g76680	5.64	0.09	0.78	0.00	0.00
Ribonuclease, RNS1	At2g02990	5.59	0.18	0.51	0.00	0.00
Expressed protein	At2g34610	5.57	0.13	0.61	0.00	0.03
Oxidoreductase, 2OG-Fe(II) oxygenase family	At2g38240	5.38	0.28	0.56	0.00	0.01
Hypothetical protein	At5g33355	5.15	0.02	0.69	0.00	0.01
Lipoxygenase (LOX2)	At3g45140	5.03	0.59	0.33	0.00	0.00
Palmitoyl protein thioesterase precursor	At4g17470	4.72	0.14	0.80	0.00	0.04
Glutathione transferase, putative	At2g29490	4.65	0.50	0.56	0.00	0.04
12-Oxophytodienoate reductase (OPR2)	At1g76690	4.25	0.01	0.78	0.00	0.00
Ubiquitin extension protein (UBQ2)/60S	At2g36170	0.38	0.10	0.51	0.01	0.04
Gibberellin-regulated protein -related	At1g22690	0.38	0.04	0.40	0.00	0.01
Expressed protein	At1g09310	0.37	0.03	0.76	0.02	0.10
MGDG synthase (MGD2), putative	At5g20410	0.37	0.44	0.38	0.03	0.07
Photosystem II type I chl a/b binding protein	At2g34420	0.37	0.92	0.87	0.01	0.06
Polyubiquitin (UBQ3)	At5g03240	0.34	0.44	0.76	0.00	0.04
Dehydrin protein family	At1g54410	0.31	0.00	0.09	0.05	0.13
Expressed protein	At5g55390	0.31	0.48	0.77	0.03	0.10
Expressed protein	At1g65845	0.28	0.68	0.82	0.00	0.01
Glyceraldehyde 3-P dehydrogenase A (GapA)	At3g26650	0.25	0.86	0.86	0.02	0.08
Ocimene, 24 h						
Expressed protein	At1g10585	9.42	0.00	0.07	0.00	0.01
Disease resistance protein (LRR)	At2g33060	5.43	0.00	0.07	0.01	0.04
Lipoxygenase (LOX2)	At3g45140	4.84	0.00	0.15	0.00	0.04
Hypothetical protein	At5g24200	4.80	0.00	0.15	0.04	0.02
Leucine rich repeat protein	At2g25440	4.64	0.00	0.11	0.00	0.12
Expressed protein	At1g19180	4.58	0.00	0.08	0.00	0.03
Amino transferase	At2g24850	4.38	0.00	0.11	0.00	0.00
AIG1	At1g33960	4.31	0.00	0.09	0.01	0.00
AAA-type ATPase	At3g50930	4.22	0.00	0.07	0.00	0.05
Terpene synthase	At1g61120	4.14	0.00	0.06	0.00	0.01

(*continued on next page*)

Table 1 (*continued*)

Treatment, timepoint/gene function (known or predicted)	Locus	FC	*p* val	*q* val	gp val	gq val
Membrane related protein CP5	At1g64720	0.42	0.00	0.30	0.00	0.13
Myrosinase-associated protein	At3g14210	0.40	0.01	0.16	0.00	0.02
RNA recognition motif (RRM)-containing protein	At1g33470	0.39	0.00	0.06	0.00	0.01
Expressed protein	At2g26500	0.38	0.00	0.15	0.05	0.13
Glycine-rich protein	At2g32690	0.37	0.00	0.15	0.05	0.13
Expansin (EXP11)	At1g20190	0.36	0.00	0.08	0.01	0.05
Glycosyl transferase	At2g16890	0.36	0.00	0.07	0.00	0.01
RRM-containing RNA-binding protein	At5g07060	0.35	0.00	0.14	0.04	0.11
Ubiquitin extension protein (UBQ2)	At2g36170	0.25	0.00	0.07	0.01	0.04
Expressed protein	At2g27385	0.21	0.00	0.07	0.02	0.07

p and *q* values of less than 0.004 are given as 0.00.

quantitative RT-PCR analysis confirmed microarray results for 15 of 16 genes tested (Table 5), and the octadecanoid pathway genes *LOX2* (lipoxygenase; At3g45140), *AOS* (allene oxide synthase; At5g42560), and *OPR3* (oxophytodienoate reductase, At2g06050) were included in this qRT-PCR transcript analysis (Fig. 7). Over the 2–24 h time course, *LOX2* responded with 2.2–12.2-fold induction, *AOS* with 3.2–12.4-fold induction, *OPR3* with a 1.5–5.1-fold induction as detected by RT-PCR (Fig. 7).

To test if monoterpene-induced increase in transcript abundance for octadecanoid biosynthesis was translated into metabolic changes we measured levels of JA and MeJA in plant tissues exposed to ocimene volatiles (Fig. 8). Tissue levels of MeJA were below our detection limits in the untreated control plants. However, MeJA levels increased to approximately 0.4 ng/g fresh weight at 2 h or 6 h of treatment, and 0.2 ng/g at 24 h of treatment. The induced levels of MeJA were similar to those found with wound-treated plants. While changes in JA levels were not detected in any consistent fashion in ocimene-treated plants, JA was increased in wounded plants.

2.5. Octadecanoid mutants coi1 and aoc have a diminished response to ocimene volatiles

Since gene expression profiling and metabolite analysis suggest that octadecanoids are part of the induced response to monoterpene volatiles, we tested the effect of ocimene with mutants in octadecanoid biosynthesis (*aoc*, allene oxide cyclase At3g25780) and octadecanoid signaling (*coi1*, coronatine insensitive At2g39940). Using a set of 13 genes which are induced by ocimene in wild-type (wt) plants, we measured changes of transcript abundance in treated (2 h ocimene) relative to untreated plants in wt, *coi1* and *aoc* plants, and for comparison in the ethylene response (*coi1*, At3g23150)

Table 2
Transcripts that were up-regulated at all time points (2 h, 6 h, 24 h) of exposure of *A. thaliana* to ocimene volatiles

Locus	Gene function	2 h			6 h			24 h		
		FC	*p*	*q*	FC	*p*	*q*	FC	*p*	*q*
At1g10585	Expressed protein	6.3	0.00	0.05	4.0	1.00	0.87	9.4	0.00	0.07
At3g45140	Lipoxygenase (LOX2)	3.5	0.02	0.29	5.0	0.59	0.80	4.8	0.00	0.15
NA	BAC T10O24 from Chromosome 1	6.9	0.00	0.04	2.2	0.61	0.81	4.8	0.00	0.15
At2g24850	Aminotransferase, putative	7.4	0.00	0.00	3.7	0.00	0.14	4.4	0.00	0.09
At2g02990	Ribonuclease, RNS1	3.0	0.02	0.28	5.6	0.18	0.61	4.0	0.00	0.15
At4g17470	Palmitoyl protein thioesterase precursor, putative	2.8	0.03	0.36	4.7	0.14	0.56	4.0	0.01	0.16
NA	Chromosome 4, contig fragment No. 46	2.9	0.03	0.38	5.8	0.65	0.81	3.9	0.01	0.18
At1g15520	ABC transporter family protein	5.4	0.00	0.02	2.1	0.72	0.83	3.8	0.00	0.14
At5g42050	Expressed protein	2.0	0.06	0.47	2.3	0.08	0.48	3.8	0.00	0.12
At2g29460	Glutathione transferase, putative	10.1	0.00	0.00	5.7	0.50	0.78	3.4	0.00	0.15
At3g14620	Cytochrome P450, putative	3.1	0.00	0.01	2.0	0.09	0.50	3.3	0.00	0.06
NA	BAC F9L11 genomic sequence	3.9	0.00	0.06	2.2	0.06	0.45	3.2	0.00	0.15
At2g34610	Expressed protein	3.4	0.00	0.08	5.6	0.13	0.56	3.1	0.00	0.15
At1g43160	AP2 domain transcription factor RAP2.6	3.2	0.00	0.15	2.1	0.90	0.86	3.1	0.01	0.16
At1g32950	Subtilisin-like serine protease	2.2	0.08	0.55	2.5	0.88	0.86	3.0	0.02	0.23
At5g06870	Polygalacturonase inhibiting protein (PGIP2)	2.5	0.00	0.14	2.0	0.01	0.22	2.8	0.00	0.14
At2g29490	Glutathione transferase, putative	2.9	0.00	0.05	4.6	0.50	0.78	2.7	0.00	0.14
At5g33355	Hypothetical protein	2.0	0.02	0.32	5.1	0.02	0.33	2.7	0.00	0.14
At3g11340	Glycosyltransferase family	3.8	0.00	0.00	2.5	0.01	0.22	2.7	0.00	0.09
At1g66690	Methyltransferase-related	5.9	0.00	0.02	2.0	0.92	0.87	2.6	0.02	0.23
At2g38240	Oxidoreductase, 2OG-Fe(II) oxygenase family	5.3	0.00	0.03	5.4	0.28	0.69	2.6	0.02	0.22
At3g21480	Expressed protein	2.4	0.01	0.20	2.4	0.28	0.68	2.5	0.01	0.17
At3g46660	Glucosyltransferase-related protein	2.7	0.00	0.07	2.4	0.71	0.83	2.5	0.00	0.15
At1g76690	12-Oxophytodienoate reductase (OPR2)	4.4	0.00	0.00	4.3	0.01	0.27	2.4	0.00	0.06
At5g26340	Hexose transporter, putative	2.2	0.00	0.11	2.3	0.48	0.77	2.3	0.00	0.14
At4g01870	Expressed protein	4.6	0.00	0.02	2.4	0.29	0.69	2.3	0.02	0.21
At3g50770	Calmodulin-related protein, putative	2.5	0.01	0.25	2.2	0.96	0.87	2.3	0.02	0.21
At4g39670	Expressed protein	2.4	0.03	0.35	2.8	0.57	0.80	2.2	0.04	0.27
At2g18690	Expressed protein	2.2	0.00	0.13	2.3	0.95	0.87	2.2	0.00	0.15
At2g29500	Small heat shock protein -related	13.3	0.00	0.00	3.7	0.38	0.74	2.1	0.09	0.36
At5g16990	Allyl alcohol dehydrogenase, putative	4.1	0.00	0.00	2.1	0.62	0.81	2.1	0.00	0.10
At5g61820	Expressed protein	2.3	0.00	0.00	4.3	0.75	0.84	2.1	0.00	0.06
At5g63790	No apical meristem (NAM) protein family	2.8	0.00	0.05	3.2	0.88	0.86	2.1	0.01	0.19
At1g76680	12-Oxophytodienoate reductase (OPR1)	5.0	0.00	0.00	5.6	0.09	0.51	2.0	0.00	0.09
At4g08870	Arginase-related	2.5	0.01	0.25	2.4	0.00	0.11	2.0	0.05	0.28

FC: fold change; *p*: *p* value; *q*: *q* value. *p* and *q* values of less than 0.004 are given as 0.00.

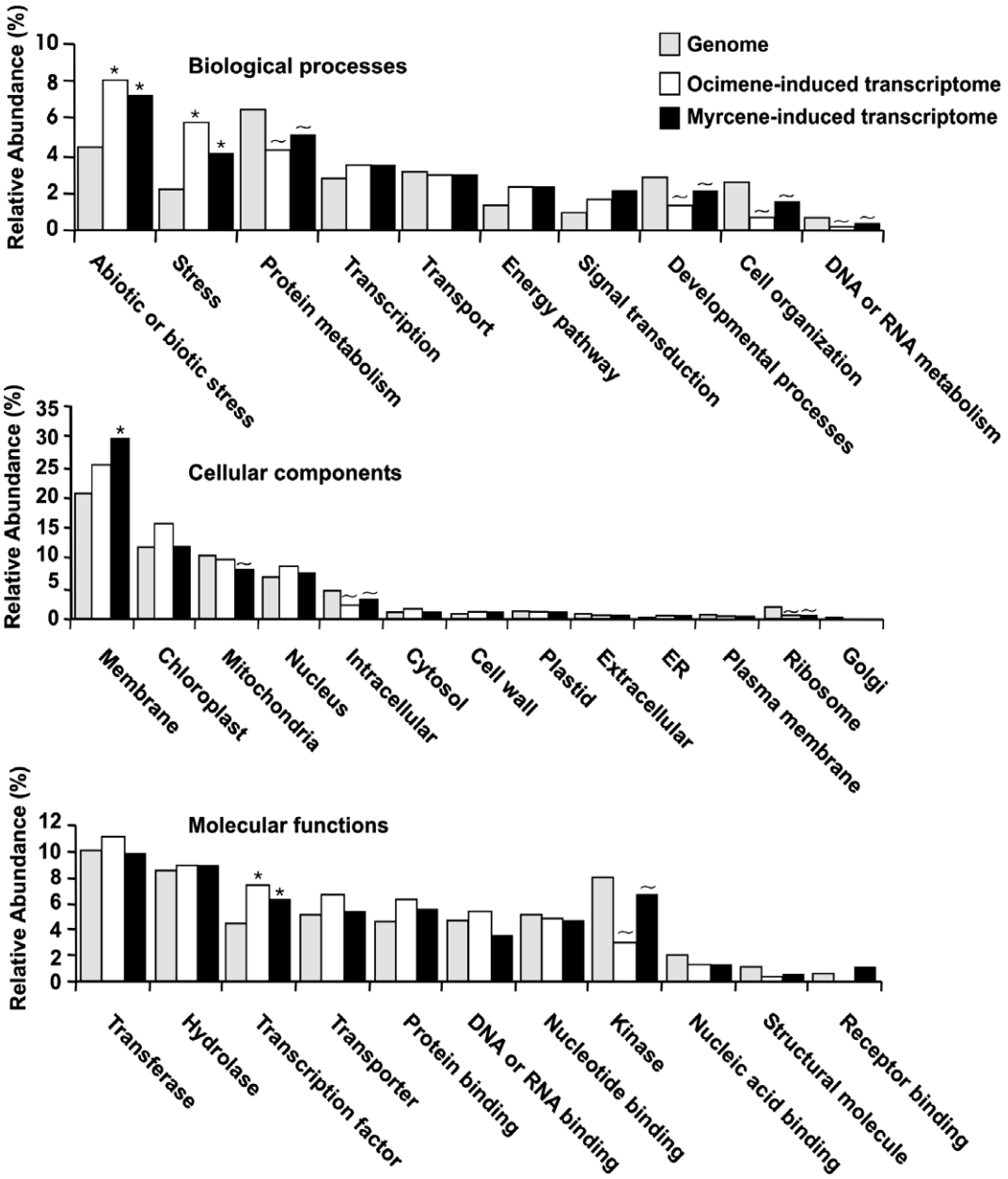

Fig. 3. Relative abundance of genes organized by GO categories in the *Arabidopsis* genome and in the transcriptome of plants treated for 2 h with ocimene or myrcene volatiles. Distribution of differentially regulated transcripts ($\geqslant$2-fold change abundance; $p \leqslant 0.05$) across GO categories for biological processes, cellular components and molecular functions. Black bars represent the differentially regulated transcriptome in myrcene-treated plants. Open bars represent the differentially regulated transcriptome in ocimene-treated plants. Grey bars represent genome abundance. Only genes with known or putative functions are represented. A test for quality of proportions ($p \leqslant 0.001$) was used to assess differential representation between transcriptome and genome abundance. Asterisks indicate categories significantly over-represented in the transcriptome; squiggles indicate categories significantly under-represented in the transcriptome.

mutant (Fig. 9). The *aoc* plants showed significantly (90% confidence interval) reduced ocimene-induction for two ethylene response factors (*RAP2.6*, *ERF1*), *RD26* transcription factor, octadecanoid response elements *COR1*, *IAR3* and *OPR1*, and for two stress-induced proteins (*TPS*, *DR*). In addition, other genes (*ERF11*, *WRKY40*, *STI*) also appeared to have reduced ocimene-induction in the *aoc* plants, but the effect was not statistically significant within a 90% confidence interval. Only two ocimene-inducible genes tested, *VSP1* and *HS*, were not affected by the *aoc* mutant. The octadecanoid signaling mutant *coi1* showed a very similar effect of reduced ocimene-induced gene expression with an additional reduced repression of *VSP1*, but a lack of effect on *RD26* and *OPR1*. Several ocimene-induced genes (*RAP2.6*, *ERF1*, *ERF11*, *COR1*, *IAR3*) also showed a reduced response in the *etr2* mutant.

3. Discussion

Monoterpenes are among the most commonly produced plant volatiles. Their formation and emission is often increased in response to wounding or attack by arthropod herbivores. While monoterpenes emitted from flowers, fruits, and wounded vegetative tissues are well known to elicit responses in a wide array of animal species, including vertebrates and invertebrates, recent work has also established that some plants respond to monoterpene volatiles as well. For example, treatment of *A. thaliana* with the monoterpene *allo*-ocimene, similar to treatment with C6-volatiles, can enhance resistance against *B. cinerea* (Kishimoto et al., 2005, 2006a,b; Shiojiri et al., 2006). A recent study on host targeting by the parasitic dodder plant also demonstrated the biological

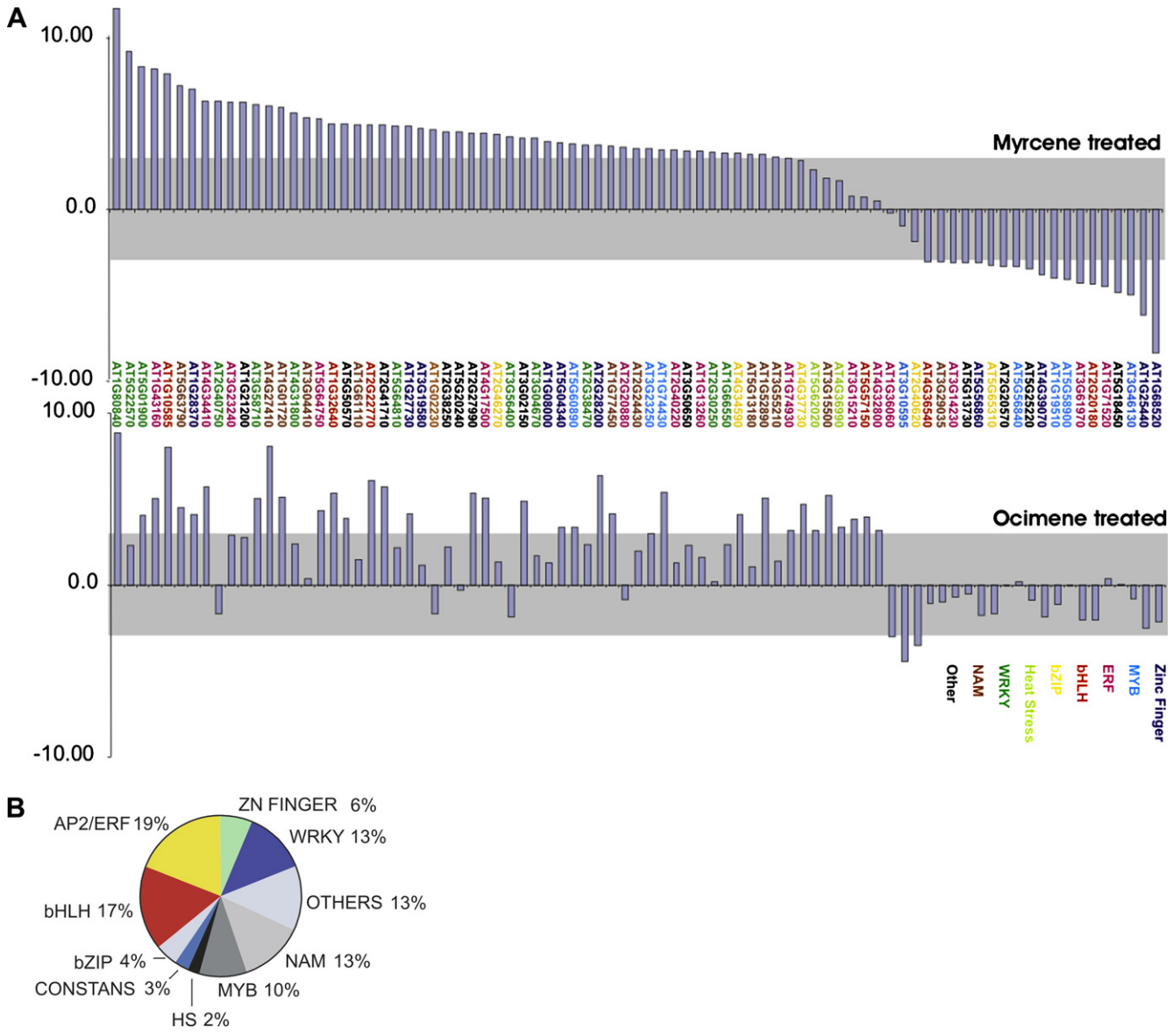

Fig. 4. Transcription factor expression profile of *Arabidopsis* exposed for 2 h to myrcene or ocimene volatiles. (A) Fold up- (positive scale) or fold down-regulation (negative scale) of transcripts encoding transcription factor. Transcripts with $\geqslant$2-fold change of abundance ($p \leqslant 0.05$) are shown outside of the grey shaded zone. (B) Categories and distribution of monoterpene-regulated transcription factors. Standard acronyms are used. HS, heat shock transcription factors; NAM, no apical meristem transcription factors.

relevance of monoterpene volatile signaling in the plant-to-plant interaction with tomato host plants (Runyon et al., 2006).

A. thaliana has the genomic, biochemical, and physiological capacities to produce and emit monoterpene volatiles, including myrcene and (*E*)-β-ocimene (Bohlmann et al., 2000; Aubourg et al., 2002; Aharoni et al., 2003; Fäldt et al., 2003). Here we have shown that *A. thaliana* also has the capacity to mount a substantial molecular and transcriptome response to monoterpene volatiles. Octadecanoid formation and possibly octadecanoid signaling appear to be involved in this response. These findings are supported by results from several different experiments showing monoterpene-induced activation of a heterologous promoter, change of transcriptome as assessed by genome-wide microarray profiling and RT-PCR, increased MeJA tissue levels, and reduced volatile-induced response in the *aoc* and *coi1* mutants.

We found that the monoterpene-inducible pinII-promoter (Thornburg et al., 1987), as a reporter system in *A. thaliana*, provides a useful tool to assess plant responses to the environment with applications in chemical genomics screenings. Given that sev-

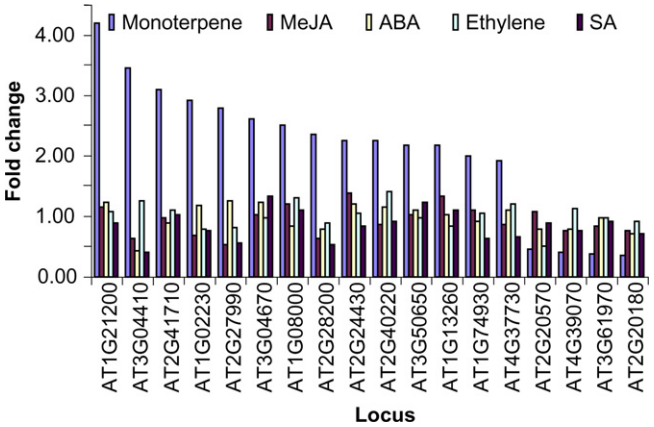

Fig. 5. Differential expression of monoterpene-induced transcription factors. Gene expression data for treatments with MeJA, ethylene, salicylic acid (SA), or abscissic acid (ABA) were obtained from the Gene Express Viewer database (https://www.genevestigator.ethz.ch/at/).

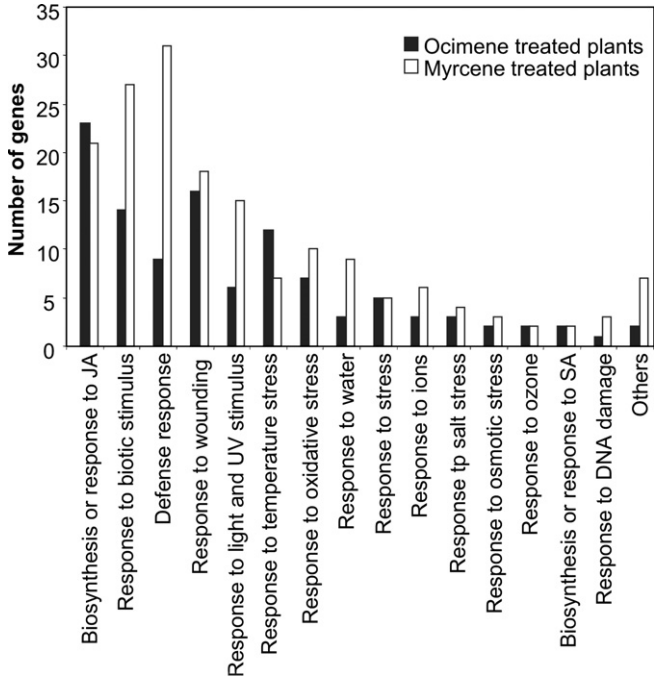

Fig. 6. Abundance of monoterpene-induced (at 2 h) transcript species annotated as stress-related. Categories of stress-related genes that showed differential expression at 2 h after treatment with myrcene or ocimene ($\geqslant$2-fold change transcript abundance; $p \leqslant 0.05$) are based on the GO from the TAIR database.

Table 3
Monoterpene-induced change of transcript abundance for the octadecanoid pathway detected by microarray analysis

Gene function	Locus	O2		M2	
		FC	*p*	FC	*p*
Lipoxygenase (LOX2)	At3g45140	3.5	0.06	2.6	0.02
Allene oxide synthase (AOS)	At5g42650	3.4	0.10	1.6	0.00
Allene oxide cyclase (AOC)	At3g25780	3.0	0.00	2.2	0.00
12-Oxophytodienoate reductase (OPR3)	At2g06050	5.0	0.00	2.5	0.00
OPC-8:0 CoA Ligase1 (OPCL1)	At1g20510	3.1	0.00	4.7	0.00
JA carboxyl methyltransferase (JMT)	At1g19640	2.2	0.00	2.4	0.00

O2: Exposure to ocimene volatiles for 2 h; M2: exposure to myrcene volatiles for 2 h; FC: fold change; *p*: *p* value.

eral structurally different cyclic and acyclic monoterpenes induced the pinII-promoter activity in transgenic *A. thaliana* plants, it is possible that the response to monoterpenes could be the result of lipophilic monoterpenes interacting in a non-specific fashion with cell membranes. Since the monoterpene *allo*-ocimene and

Table 5
RT-PCR transcript profiling of select genes used for validation of microarray analysis; mean fold change (FC) at 2h after ocimene treatment over control, SE ($n = 3$) standard error

Gene	Locus	FC	SE
RAP2.6	At4g77410	15.1	3.5
TPS	At1g61120	13.2	6.3
WRKY40	At1g80840	12.7	5.9
ERF1	At3g23240	12.5	0.5
DR	At2g34930	8.8	3.1
VSP1	At5g24780	5.0	0.9
HS	At2g29500	4.8	2.3
COR1	At1g19670	4.6	2.1
IAR3	At1g51760	4.0	0.9
ERF11	At1g28370	3.9	1.4
OPR1	At1g76680	3.6	0.5
RD26	At4g77410	3.1	0.4
STI	At1g62740	2.6	1.0
LOX2	At3g45140	2.2	0.4
AOS	At5g42650	3.1	0.6
OPR3	At2g06050	1.5	0.1

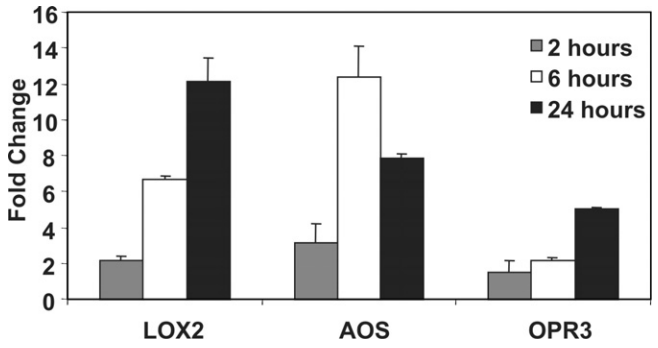

Fig. 7. Ocimene-induced transcript levels for selected steps of the octadecanoid pathway measured by RT-PCR. Exposure to ocimene volatiles for 2, 6 or 24 h. Mean with standard error ($n = 3$ biological replicates each with 2-fold technical replication). LOX2: lipoxygenase2, AOS: allene oxide synthase, OPR3: 12-oxophytodienoate reductase.

C6-volatiles induced similar resistance in *A. thaliana* to *B. cinerea* (Kishimoto et al., 2005, 2006a,b; Shiojiri et al., 2006), it will be interesting to test in future work if monoterpene volatiles and other lipophilic volatiles such as C6-aldehydes induce similar overall transcriptome responses.

The transcriptome response of *A. thaliana* to monoterpene volatiles is dominated by genes associated with response to biotic or abiotic stress, defence, and transcription factors. This response is similar to the response that we observed when *A. thaliana* plants were exposed to insect feeding and the leaf transcriptome was analyzed using the same oligonucleotide array platform (Ehlting

Table 4
Effect of monoterpene volatiles on JAZ transcripts detected with *p* value $\leqslant 0.05$

Description	Locus	M2	*p*	O2	*p*	O6	*p*	O24	*p*
JAZ1	At1g19180	7.76	0.00	8.91	0.00	1.84	0.93	4.58	0.00
JAZ2	At1g74950	3.26	0.00	3.76	0.00	1.17	0.32	1.52	0.04
JAZ3	At1g17860	2.32	0.00	1.21	0.20	1.22	0.63	0.91	0.51
JAZ5	At1g17380	3.06	0.01	4.53	0.00	1.51	0.52	2.13	0.06
JAZ6	At1g72450	2.74	0.00	4.37	0.00	0.90	0.07	1.12	0.56
JAZ7	At2g34600	9.75	0.00	13.04	0.00	1.10	0.04	1.50	0.13
JAZ8	At1g30135	3.89	0.00	6.20	0.00	1.14	0.58	1.24	0.53
JAZ9	At1g70700	3.97	0.00	3.01	0.00	2.00	0.66	1.88	0.02
JAZ10	At5g13220	8.19	0.00	6.45	0.00	1.06	0.87	1.76	0.07
JAZ11	At3g43440	0.73	0.02	0.87	0.25	0.91	0.46	1.32	0.04

M2: Exposure to myrcene volatiles for 2 h; O2, O6, O24: exposure to ocimene volatiles for 2 h, 6 h, or 24 h, respectively; FC: fold change; *p*: *p* value.

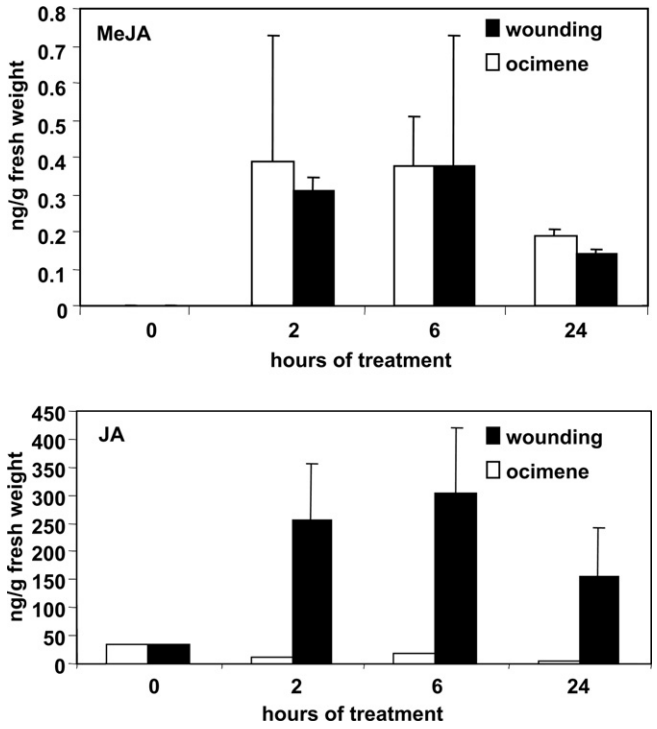

Fig. 8. Levels of MeJA (upper panel) and JA (lower panel) in wounded and ocimene exposed plants. Each bar represents the mean of three or four biological replicates with standard error. When no error bars are shown, metabolite levels were below detection limit in all but one replicate.

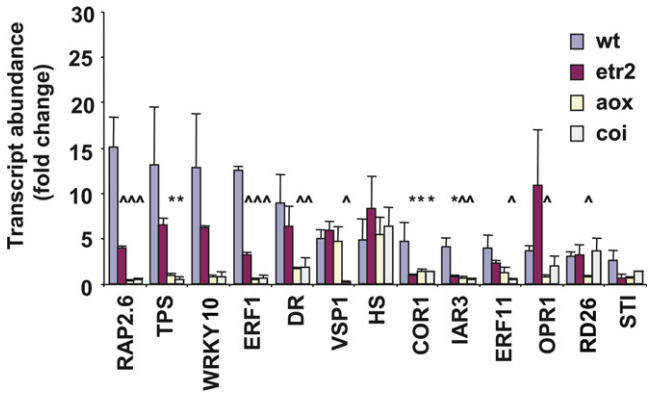

Fig. 9. Change of transcript abundance in wt, *etr2*, *aoc*, or *coi* plants treated for 2 h with ocimene. Mean fold-change of transcript-abundance in treated plants over non-treated plants with standard error ($n = 3$) detected by RT-PCR. Symbols (∗, ∧) indicate change of transcript levels in mutants as significantly different from wt plants at 90% (∗) or 95% (∧) confidence intervals. *RAP2.6*:At1g43160; *TPS04*:At1-g61120; *WRKY40*:At1g80840; *ERF1*:At3g23240;*DR*:At2g34930; *VSP1*:At5g24780; *HS*:At2g29500; *COR1*:At1g19670; *IAR3*:At1g51760; *ERF11*:At1g28370; *OPR1*:At1-g76680; *RD26*:At4g27410; *STI*:At1g62740.

et al., 2008), and also has substantial overlap with the transcriptome of *A. thaliana* and other species affected by herbivory as recorded on other array platforms, specifically with regard to up-regulation of octadecanoid signaling (e.g. De Vos et al., 2005; Kempema et al., 2007; Ralph et al., 2006a,b; Reymond et al., 2004). Our findings of monoterpene-induced up-regulation of octadecanoid pathway transcripts along with the monoterpene-induced increase of MeJA and the monoterpene-induced increase of JAZ proteins, as well as the altered responses in the *aoc* and *coi* mutants, warrant future work to test if responses to monoterpenes are mediated through octadecanoid signaling processes that also function in wound- or insect-induced plant stress responses. The apparent lack of JA accumulation, which contrasts with induced formation of MeJA, could be due to possible metabolism of JA, including formation of the MeJA and JA-conjugates. It is important to note that previous work by Kishimoto et al. (2005, 2006a) showed that *jar1-1* dependent jasmonate signaling is not required for the full *allo*-ocimene-induced resistance response of *A. thaliana* against the fungal pathogen *B. cinerea*.

4. Conclusions

In conclusion, our results from a series of carefully replicated experiments provide new information about the large-scale response of *A. thaliana* to monoterpenes. The results described here will guide future experiments to test mechanisms of monoterpene-induced plant responses with the inclusion of the available genetic and genomic tools for the *Arabidopsis* model system. The present study provides an unprecedented plethora of new molecular targets for functional dissection of plant-to-plant or within-plant volatile signaling.

5. Experimental

5.1. Plant and insect materials

A. thaliana (Col-0) seeds were provided by Dr. Ljerka Kunst (University of British Columbia, Vancouver, Canada). Stable (T3) plants containing pinII-*GUS* were generated by floral dip transformation (Clough and Bent, 1998) with *Agrobacterium tumefaciens* containing a pinII::GUS construct in pCAMBIA1300 vector. The potato pinII promoter was provided by Dr. Robert Thornburg (Iowa State University, USA). Fourth generation mutant lines (*aoc*, *coi1*, *etr2*) were from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc/). Seeds of wild-type, mutant, and transformed plants were germinated on 50% MS media, individual seedlings transferred to $6 \times 4 \times 4$ cm plastic pots with soil mix (Sunshine Mix 5; SunGro, Canada), and grown for approximately two weeks at 22 °C, 16/8 h light/dark, 70 μmoles/m^2/s of light intensity to approximately 3–4 cm height.

5.2. Plant treatment

For exposure to monoterpene or MeJA volatiles, potted plants were placed in a fume hood on a clean, aluminum foil-covered surface under a 1-L glass beaker together with a cotton ball of 1–2 cm diameter containing a defined concentration of the volatile compound (0.4–4 μL for GUS assays; 4 μL for microarray and RT-PCR gene expression studies). Compounds were used individually without solvents added. The following pure (90–99%) monoterpenes were from Sigma–Aldrich (Oakville, Canada): (+)-α-pinene (99%), (−)-β-pinene (99%), (+)-β-pinene (99%); myrcene (90%); racemic linalool (95%), racemic limonene (96%). A 95% pure mixture of 70% (*E*)-β-ocimene, 10% (*Z*)-β-ocimene, and 15% *allo*-ocimene was from International Flavours and Fragrance (New York, USA). MeJA (95%) was from Sigma–Aldrich. Control plants were treated in the same fashion without any compound added to the cotton ball. Plants were exposed to volatiles released from the cotton ball for 0.5, 2, 6 or 24 h. Above-ground tissues were harvested, frozen in liq. N_2, and stored at −80 °C prior to RNA or metabolite extraction. GUS staining assays (Jefferson, 1987) were done with leaves detached from treated plants (at 2 h).

5.3. Microarray gene expression analysis

RNA was isolated using Trizol reagent (Invitrogen; Burlington, Canada) following manufacturer's protocol with a second $CHCl_3$

extraction. RNA was precipitated using 0.5 volumes of *i*-PrOH and 0.5 volumes of 0.8 M Na_3-citrate. RNA quality was assessed by agarose gel separation and spectrophotometrically. Reverse transcription of RNA, cDNA labeling and microarray hybridizations were performed as described in Ehlting et al. (2005). Details of the 70-mer microarray platform have previously been described (Ehlting et al., 2005). In the present study we used the same platform with an extended AROS V3.0 oligo set (Operon Biotechnologies, Huntsville, USA; www.operon.com/arrays/oligosets_arabidopsis.php) of 29,110 Tm-optimized 70-mers representing 26,173 protein-coding genes, 28,964 protein-coding gene transcripts, and 87 microRNA genes from *A. thaliana*. Oligo design was based on the ATH1 release 5.0 of the TIGR arabidopsis genome annotation database (www.tigr.org) and the 4.0 release of the miRNA Registry at the Sanger Institute (www.sanger.ac.uk). Microarray hybridizations were done with equal numbers of Cy3- and Cy5-labeled treatment and control RNA samples to eliminate fluorescent dye bias. Initial hybridizations were performed with two independent biological and two technical replicates for the 2 h ocimene treatment to confirm technical reproducibility. All subsequent array hybridizations included four independent biological replicates. Microarray slides were scanned on a Packard Bioscience BioChip Technologies Model ASCEX00 Scan Array (Montreal, Canada). Laser power was adjusted to optimal signal/background levels for each experiment with background fluorescence not exceeding 250 pixels. Spots were quantified using the Imagene software (BioDiscovery, Marina Del Rey, USA). Grids were manually placed and spot finding was performed using the "auto adjust" function three times. Median pixel intensity was used for all analyses. To limit user bias flagging was avoided. Each channel was background corrected using the lowest 10% of foreground intensities. The two channels of each array were then normalized to each other using *vsn* thereby generating the delta h measure for each array. Using all slides, a linear model was used to estimate all effects and their standard errors. Student's *t*-test was used to compute a *p*-value for each effect. *Q*-values were computed to adjust for the false discovery rate. Additional data processing was done using customized scripts for R and Bioconductor (The R Development Core Team, http://www.r-project.org). Only spots showing reproducible expression at $p \leqslant 0.05$ were assessed; spots with $\geqslant$2-fold differential expression were submitted to the TAIR GO annotation database search engine (http://www.arabidopsis.org/tools/bulk/go/index.jsp), and placed into functional categories. Genome representation of GO categories were from the TAIR GO annotation database using whole genome characterization. Significant differential representation between genomic abundance and transcript abundance was calculated using a test for equality of proportions. Microarray data were obtained according to MIAME standards and are available in the European Bioinformatics Institute (EBI) database under the accession numbers E-MEXP-899 and E-MEXP-901.

5.4. Quantitative RT-PCR analysis

For cDNA synthesis, 2.5 μg of RNA was treated with 1 μL of DNAse I (Invitrogen) in a final volume of 10 μL at room temperature for 15 min, followed by addition of 1 μL of 25 mM EDTA and incubation for 10 min at 65 °C, and further addition of 1 μL oligo dT, 1 μL 10 mM dNTPs and 1 μL water and additional incubation for 5 min at 65 °C. Reactions were placed on ice and spun down at 3000 rpm. 5X RT buffer, 2 μL 0.1 M DTT and 1 μL SUPERSCRIPT II RT® were added to a final 20 μL volume. The reaction was incubated for 1 h at 42 °C. The resulting cDNA was diluted 10-fold with water. RT-PCR was conducted using Qiagen QuantiTect SYBR Green Mastermix (Mississauga, Canada) in final reaction volumes of 20 μL with PCR reactions done as described in Ehlting et al. (2005). Actin was used to normalize expression data between samples. In all RT-PCR experiments, transcript levels were measured with three independent biological replicates, and each biological replicate was analyzed in two or three technical replicates. Primers used for RT-PCR are listed in (Supplementary Table 2).

5.5. Metabolite analysis

Tissue extraction for MeJA analysis was done as described by Meyers et al. (2003). Tissues were ground to a fine powder with a mortar and pestle under liquid nitrogen. Extraction buffer (0.1% HCL in MeOH) was added (2 mL/g tissue fresh weight) and extracts transferred to 1.5 mL microcentrifuge tubes, shaken for 5 min, and centrifuged at 3000 rpm for 1 min. Extraction of tissue pellets was repeated four times. Supernatants from four extractions per sample were pooled, transferred into dark glass vials and concentrated under N_2 to 300 μL. Residual solids were removed by centrifugation and the MeOH-extract analysed on an Agilent (Canada) 1100 LC-MSD-Trap XCT plus system equipped with a Rapid Resolution HT Zorbax SB-C18 4.6 × 50 mm, 1.8 μm column. Instrument settings and analytical conditions were: positive ion electrospray with dry temperature set at 350 °C; nebulizer pressure at 65psi; dry gas flow at 12 L/min; column flow at 1.5 mL/min; column temperature at 50 °C; mobile phase was H_2O with 0.2% HCO_2H (A) and CH_3CN with 0.2% HCO_2H (B) with a gradient program of 10% B at 0 min to 80% B at 6 min; injection vol: 30 μL in triplicates. MeJA was identified by comparison of mass spectra and retention times with those of authentic standard (Sigma–Aldrich). For quantitative MeJA analysis, standard curves were generated. Seven pure standards (1.4–89.6 ng) of MeJA were combined with 16.5 ng of d_3-MeJA (Sigma–Aldrich), then injected into the LC–MS. Analyte standard curves were calculated using Bruker Daltonik LC/MSD Trap Software 5.2 build 382. d_3-MeJA was also used as internal standard to assess recovery (consistently ~50%) from plant extracts.

JA was analyzed by HLPC-MS/MS at the Plant Biotechnology Institute of the National Research Council of Canada. JA was analyzed by HLPC-MS/MS at the Plant Biotechnology Institute of the National Research Council of Canada. Fresh plant material was frozen in N_2 and ground to a fine powder. Samples of 300 mg were extracted with 3 mL of MeOH:H_2O:glacial AcOH (90:9:1; v/v/v) and addition of the internal standard [50 ng 2,2-d_2-jasmonic acid (Galka et al., 2005) dissolved in 15% CH_3CN in H_2O + 0.1% HCO_2H] by sonication (5 min) followed and shaking (4 °C, 5 min). Samples were centrifuged to pellet debris. Supernatant was transferred to a clean tube and pellets re-suspended in 2 mL of extraction solution. Extraction was repeated three times and extracts combined. MeOH was then evaporated under a constant N_2 stream. A solution of 2 mL of 0.1 M NaOH was added to the residual water phase and neutral components were removed by extraction with CH_2Cl_2 (3 mL). The H_2O layer was transferred to a clean tube. The dichloromethane layer was re-extracted with a fresh portion (2 mL) of 0.1 M NaOH. Both aqueous layers were combined and acidified with 5% aq. HCl (on ice) followed by the partitioning with 1 mL of EtoAC:cyclohexane (1:1, v/v) solvent mixture. The organic phase was collected and the water phase was extracted a second time with the EtoAc:cyclohexane (0.5 mL) mixture. The organic fractions were pooled and the solvent was evaporated under constant N_2 stream. The samples were reconstituted in 200 μL of 15% CH_3CN in 0.1% HCO_2H. Analysis of JA was carried out by HPLC/ES–MS/MS utilizing an HP1100 series binary solvent pump and autosampler (Hewlett-Packard) coupled to a Quattro LC™ quadrupole tandem mass spectrometer *via* a Z-spray™ interface (Micromass, Manchester, UK). A 100 × 2.1 mm C18 ACE® HPLC column (Advanced Chromatography Technologies, Aberdeen, Scotland) was used. Mobile phase A comprised 1% HCO_2H in HPLC-grade water; mobile phase B comprised HPLC-grade CH_3CN. Sample volumes of 5 μL were injected onto the column at a flow rate of 0.2 mL/min under initial

conditions of 5% B, which was maintained for 3 min, then increased to 50% at 20 min. B was again increased to 60% at 30 min and to 80% at 35 min. The mobile phase composition was returned to initial conditions by 40 min and the column allowed to equilibrate for 20 min before the next injection. The analytes were ionized by negative-ion electrospray using the following conditions: capillary potential 2.5 kV; cone voltage 30 V; desolvation gas flow 505 L/h; source and desolvation gas temperatures, 120 °C and 350 °C, respectively. The MS function employed to quantify the endogenous analyte d_0JA and its internal standard d_2JA was Multiple Reaction Monitoring (MRM) in which the first quadrupole mass filter was set to the m/z of the deprotonated molecular ion (208.8 and 210.8, respectively) and the second mass filter is set to the m/z of a fragment product (59.3 and 61.3, respectively). Collision energy was 12 eV and collision gas pressure was 2×10^{-3} mbar. Analytical procedures analogous to those reported in Ross et al. (2004) were employed to determine the quantities of JA in the plant extracts. The calculated average recovery of JA and 2,2-d_2-jasmonic acid during the extraction of plant tissue samples was 78%.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.02.011.

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