Transcriptional Profiling of Sorghum Induced by Methyl Jasmonate, Salicylic Acid, and Aminocyclopropane Carboxylic Acid Reveals Cooperative Regulation and Novel Gene Responses[1][w]

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We have conducted a large-scale study of gene expression in the C4 monocot sorghum (*Sorghum bicolor*) L. Moench cv BTx623 in response to the signaling compounds salicylic acid (SA), methyl jasmonate (MeJA), and the ethylene precursor aminocyclopropane carboxylic acid. Expression profiles were generated from seedling root and shoot tissue at 3 and 27 h, using a microarray containing 12,982 nonredundant elements. Data from 102 slides and quantitative reverse transcription-PCR data on mRNA abundance from 171 genes were collected and analyzed and are here made publicly available. Numerous gene clusters were identified in which expression was correlated with particular signaling compound and tissue combinations. Many genes previously implicated in defense responded to the treatments, including numerous pathogenesis-related genes and most members of the phenylpropanoid pathway, and several other genes that may represent novel activities or pathways. Genes of the octadecanoic acid pathway of jasmonic acid (JA) synthesis were induced by SA as well as by MeJA. The resulting hypothesis that increased SA could lead to increased endogenous JA production was confirmed by measurement of JA content. Comparison of responses to SA, MeJA, and combined SA+MeJA revealed patterns of one-way and mutual antagonisms, as well as synergistic effects on regulation of some genes. These experiments thus help further define the transcriptional results of cross talk between the SA and JA pathways and suggest that a subset of genes coregulated by SA and JA may comprise a uniquely evolved sector of plant signaling responsive cascades.

Plant defense systems against invading pathogens are being elucidated in numerous plant species (Ekengren et al., 2003; Nimchuk et al., 2003). Studies of gene expression in wild-type and mutant Arabidopsis (*Arabidopsis thaliana*) genotypes in response to signaling compounds and pathogens have established the existence of interacting signaling pathways regulated by salicylic acid (SA), jasmonic acid (JA), and ethylene (E), and changes in mRNA abundance of many gene classes (Maleck et al., 2000; Schenk et al., 2000; Brodersen et al., 2002; Scheideler et al., 2002; Tao et al., 2003). SA is well known to regulate both local and systemic resistance to many pathogens (Ryals et al., 1996; Durner et al., 1997). Genes both upstream (*EDS1* and *4*, *PAD 4*, and *SID2/EDS16*; Falk et al., 1999; Jirage et al., 1999; Nawrath and Metraux, 1999) and downstream (*NPR1/NIM1*; Cao et al., 1997; Ryals et al., 1997; Shah et al., 1999) of SA synthesis are known to mediate the SA response. Arabidopsis plants overexpressing salicylate hydroxylase (*NahG*) in order to degrade endogenous SA have allowed assessment of the role of SA in many additional responses (Ryals et al., 1996). The pathways of E biosynthesis and action have been well characterized (Bleecker 1999; Alonso et al., 2003). E produced during wounding has been suggested to activate JA biosynthesis (Laudert and Weiler, 1998), and E is likely to play a role in antimicrobial defense by acting with JA to induce a number of pathogenesis-related (PR; Xu et al., 1994; Penninckx et al., 1998; Kunkel and Brooks, 2002) and other defense genes. JA is known to uniquely regulate expression of the plant defensin *PDF1.2*, thionin, and several other antimicrobial proteins (Reymond and Farmer, 1998) and to compose a defense pathway distinct from, but interacting with, the SA pathway.

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JA also mediates plant defense against insects, and appears to mobilize antimicrobial defenses predominantly effective against necrotrophic pathogens, while the SA-mediated defense response is effective against biotrophic fungi, bacteria, and viruses (Thomma et al., 2001; Murphy and Carr, 2002).

Lipids are connected to the plant defense response through their potential action as signaling molecules (Laxalt and Munnik, 2002; Maldonado et al., 2002) and through the fatty acid linolenic acid (LA) acting as the precursor for 12-oxophytodienoic acid (OPDA) and JA synthesis via the octadecanoid (ODA) pathway (Creelman and Mullet, 1997). Membrane breakdown by lipases after wounding is known to generate LA and to promote JA production. The first three steps in the ODA pathway occur in the chloroplast. LA is oxygenated to 13-hydroxylinolenic acid by lipoxygenases (LOX), followed by dehydration to epoxyoctadecatrienoic acid by the Cyt P450 allene oxide synthase (AOS). The resulting unstable intermediate allene oxide is then cyclized by allene oxide cyclase (AOC) to form OPDA, the precursor of JA. OPDA then undergoes conversion to JA by 12-oxo-phytodienoate reductase (OPR3). This appears to require export of OPDA to the peroxisome, since OPR3 is targeted there (Strassner et al., 2003). β-Oxidation of the resulting oxo-cyclopentane heptanoic acid by a number of possible enzymes then occurs in the peroxisomes and glyoxisomes to form JA.

Cross talk between the SA and JA antimicrobial defense pathways in plants is currently of great interest (Glazebrook et al., 2003); however, studies on several representative genes in these pathways have revealed that the interactions between them are complex. SA has been shown to block JA induction of several defense related genes, including proteinase inhibitors (Doares et al., 1995), *PDF1.2*, *LOX2*, and *VSP* (Spoel et al., 2003), and several basic PR genes (Niki et al., 1998). As a result of this type of repression, SA and JA pathways have been considered antagonistic. Based largely on evidence from *NahG* overexpressing Arabidopsis, SA is currently proposed to block JA synthesis (Heck et al., 2003; Spoel et al., 2003), although the specific mechanism of such an inhibition is not known. SA had previously been suggested to inhibit JA synthesis by preventing the conversion of 13-hydroperoxylinolenic acid to OPDA in tomato (*Lycopersicon esculentum*; Pena-Cortez et al., 1993). However, more recent evidence has indicated this is not the case in Arabidopsis (Laudert and Weiler, 1998). Although acetylsalicylic acid has been shown to block JA synthesis by acetylation of AOS (Pan et al., 1998), SA lacks the acetyl group and does not have this ability (Harms et al., 1998). Rather, SA is known to increase the activity of the two enzymes (AOS and AOC) required for this conversion and to result in increased production of OPDA (Laudert and Weiler, 1998). Recent evidence suggests that SA blocks JA induction of at least some genes by an unknown mechanism occurring in the cytoplasm (Spoel et al., 2003). Conversely to SA antagonism of the JA defense pathway, evidence also exists that JA antagonizes SA regulation of certain PR genes (Niki et al., 1998; Gupta et al., 2000). However, SA and JA are also known to commonly induce a large number of genes in Arabidopsis (Schenk et al., 2000), and coapplication of SA and JA synergistically induced *PR1b* in tobacco (Xu et al., 1994).

Genome-scale studies of gene expression in response to defense signaling compounds have not been reported in grasses. Therefore, we initiated work on these pathways in the C4 monocot sorghum (*Sorghum bicolor*), and have made the resulting data sets publicly accessible. Sorghum is known to synthesize numerous unique cyanogenic glycosides, flavonoids, and other compounds as part of its broad defense response arsenal (Nicholson et al., 1987; Hipskind et al., 1990; Lo et al., 1999), reflective of its significant genetic diversity (Menz et al., 2004) and wide adaptive capabilities. The three-gene pathway for synthesis of the defensive compound dhurrin was recently transferred from sorghum into Arabidopsis, resulting in insect resistant plants (Tattersall et al., 2001). This demonstrates the potential of sorghum as a resource for gene discovery and supplementation of our current understanding of plant defense. We have produced microarrays based on a collection of 12,982 unique cDNAs and have here utilized these arrays to define the response of sorghum to the signaling compounds SA, methyl jasmonate (MeJA), and the immediate ethylene precursor aminocyclopropane carboxylic acid (ACC).

RESULTS

Experimental Design and Assessment of Reproducibility

To minimize experimental variability and ensure accurate representation of changes in mRNA abundance, a standardized regime for plant growth, signaling compound treatments, and replication was used for all experiments (Fig. 1). Full MIAME-compliant descriptions will be available at http://fungen.org/sorghum.htm. Seedlings of BTx623, a standard inbred sorghum line, were grown in hydroponics for 8 d prior to treatment. Three biological replicates and matched controls were collected from each of two different experiments for each compound analyzed. At each time point, 10 to 12 seedlings were harvested from each biological replicate for RNA extraction and production of cDNA, microarray probes, and quantitative reverse transcription (RT)-PCR (qRT-PCR). A minimum of six microarray slides were hybridized for each of the 12 compound $\times$ time $\times$ tissue conditions, and a total of 102 slides were included in the primary determination of significantly regulated genes. TIF files of the raw data from all slides will be available at http://fungen.org/sorghum.htm. Less than 2.6% of the elements did not show detectable expression in any of the treatments, with detectability defined as a mean fluorescence intensity greater than double the

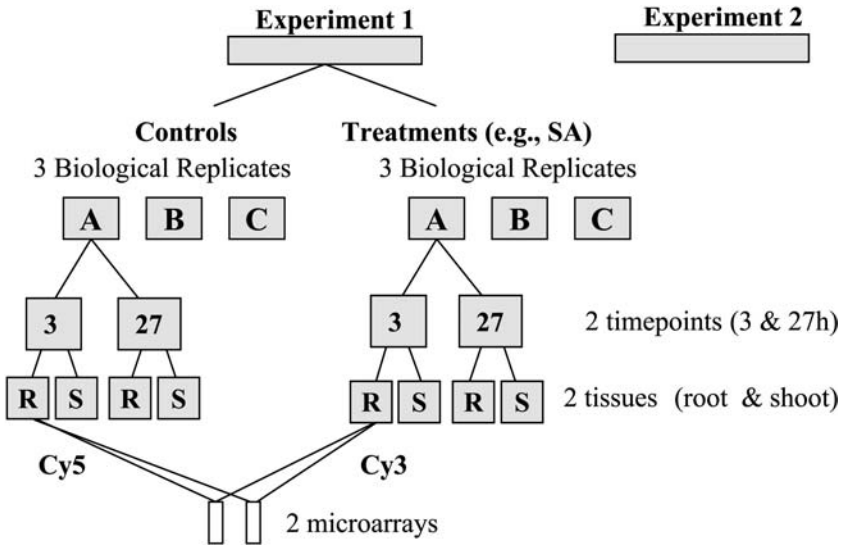

Figure 1. Experimental design and replication. An abbreviated flow diagram of the design used for plant growth and treatment, tissue collection, and microarray replication. The full pathway culminating in assay of two microarray slides with probes from control versus SA-treated 3-h root tissue is shown. Two complete runs of the treatment experiment with each compound were conducted, with each run containing three biological replicates in separate hydroponic growth buckets. As used in this report, the term ''condition'' would refer (in this example) to 3-h SA-treated root tissue and would be represented by a total of 12 microarray slides.

background mean fluorescence intensity, and a mean signal of at least 200 fluorescence units (Galbraith, 2003). The microarray sensitivity and false positive rate were determined by separate self-versus-self hybridization experiments (data not shown). Based on the error model developed from these, we adopted a 1.5-fold response cutoff, which gave a 1.5% false-positive rate based on six slide replicates (the lowest no. of replicates for any condition in the primary microarray analysis). In addition, the regulation data were further filtered for significance to retain genes passing the 1.5-fold response cutoff at $P \leq 0.05$ (''Materials and Methods''). The mRNA abundance of 171 genes found significantly regulated by the compounds on microarrays was also evaluated by qRT-PCR.

Overall Gene Regulation in Response to SA, MeJA, and ACC

Of the 12,982 cDNAs present on the microarray, a total of 6,438 were significantly regulated (more than 1.5-fold induced or suppressed; $P \leq 0.05$) in at least one of the 12 conditions. Among the three treatments, SA and MeJA altered mRNA levels of substantially more genes than ACC (Fig. 2), similar to what has been observed in Arabidopsis (Schenk et al., 2000). Among the 6,438 sorghum genes significantly induced by the treatments, 1,704 were unique to SA, 1,487 to MeJA, and 669 to ACC. Among genes significantly suppressed, 245 were unique to SA, 665 to MeJA, and 166 to ACC. Many genes were coregulated by SA, MeJA, and ACC. As has also been documented in Arabidopsis (Schenk et al., 2000), the largest individual group of genes coinduced in sorghum was between SA and MeJA (1,031 genes), and the largest corepressed group was also between SA and MeJA (579 genes). In addition, 284 genes were commonly induced by all three compounds, and 443 were commonly suppressed (Fig. 2).

Clustering of Expression Data by Compound × Tissue Interaction

To begin the process of identifying groups of coregulated genes that respond to JA, SA, and/or ACC, we performed two-way ANOVA testing on expression data from the 6,438 significantly regulated ($P < 0.05$) genes. Data from 14 slides in each of the six compound × tissue groups (84 slides total) were analyzed using the Bonferroni multiple testing correction with a 0.3 cutoff. Under these conditions, 0.3 genes of the 6,438 were expected to be selected by chance. This resulted in a list of 400 genes with regulation patterns displaying statistically significant interaction between compound and tissue. Annotations of the expressed sequence tags (ESTs) representing induced genes in the ANOVA experiment were based on sequence similarity with genes of other organisms, applying cutoffs of blast score 50 and e-value of e^{-10} (see Supplemental Table I). For convenience, we refer to them by their potential function on this basis, although functionality of the sorghum genes has yet to be determined. ESTs without homology to named genes or specific cDNAs are designated ''no homology.'' A heatmap was constructed using the GeneSpring program (Agilent Technologies, Redwood City, CA) in two steps: first, clustering the genes by relatedness of expression pattern (vertical dimension, Fig. 3) and, second, clustering the compound, tissue, and time parameters by relatedness across the expression data (horizontal dimension, Fig. 3). The tree was built using an Average-Linkage algorithm (Shi et al., 2000), and standard correlation on log-transformed ratios (parametric test, assuming equal variances). Many clusters corresponding to specific compound × tissue × time regulation patterns were visible. The three experimental parameters (compound, tissue, and time) at the base of the main clustergram show that the data clustered into three undivided blocks corresponding

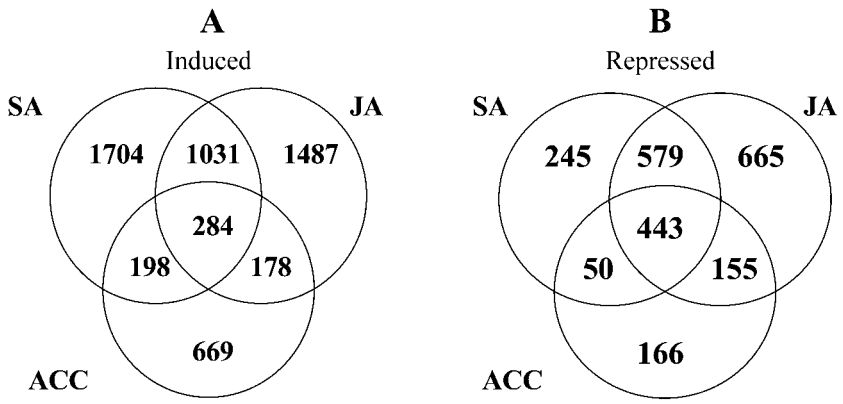

Figure 2. Overall regulatory relationships of 6,438 genes significantly induced or repressed by treatments with SA, MeJA, and ACC. Represented are genes that were (A) induced or (B) repressed more than 1.5-fold, respectively, relative to untreated controls and for which this regulation was statistically significant at $P \leq 0.05$ (Benjamini and Hochberg F.D.R.). Data were derived from a total of 102 microarray slides representing 12 compound × tissue × time conditions. Regulation fold values used for each gene were means of data from no fewer than six microarray slides for any one condition.

to the three compounds used. Within each compound block, two undivided blocks emerged corresponding to root and shoot, and within each compound × tissue block, two unbroken time blocks (3 and 27 h) formed in all cases. This indicated that overall expression patterns characteristic of each condition were very consistent across the 84 slides included in the total ANOVA dataset. Correlation coefficients of data from replicate slides within each condition are given in Supplemental Table II. Most conditions gave regulation data with good consistency, although several of the ACC conditions gave lower correlation coefficients, presumably due to the lower dynamic range of regulation in those conditions.

Sorghum homologs of genes with putative roles in cell wall synthesis, pathogen resistance, calcium and metal binding, photosynthesis, regulation of oxidative state, and modulation of plant signaling compound synthesis and response showed distinctive patterns of regulation (Fig. 3). Notable were inductions in shoot of putative defense-related and cell wall metabolism-related genes by MeJA (cluster 1) and by both MeJA and SA (cluster 2). Coinduced by MeJA and ACC were genes potentially implicated in defense and oxidative regulation (cluster 3), while putative histones and auxin-responsive factors were suppressed by all three compounds (cluster 4). ACC uniquely induced (cluster 5) several genes with possible involvement in defense, signaling, drought-response, and calcium regulation, and others in some of these classes were coregulated by all three compounds (cluster 6). Clusters 7 and 8 included sorghum genes with potential roles in defense, heat shock proteins, and several other classes that were induced by most of the compounds, while cluster 9 contained a number of putative photosynthesis-related genes that were suppressed in shoots, particularly by MeJA.

Specific Functional Gene Groups Were Regulated by SA, MeJA, and/or ACC

Separately from the ANOVA analysis, individual microarray gene regulation data files from the 6,438 significantly regulated genes under the 12 conditions were sorted into biological categories using a custom gene ontology filter with Excel (Microsoft, Redmond, WA). Due to space limitations, the resulting 24 biologically clustered data files are available as supplemental tables (Supplemental Tables I–XXIV). From these data, 171 ESTs representing major defense-related biological categories and the three compounds were chosen for further analysis of gene expression by qRT-PCR. A portion of these ESTs was also selected from outside the major biological categories based upon their particularly strong regulation by one or more compounds as determined by microarray analysis. A subset of 97 of these ESTs and their fold regulation by microarray and qRT-PCR analyses are shown in Table I (data for all 171 ESTs are presented in Supplemental Table III). Many of the individual ESTs shown represent common regulation patterns of many isologs that were observed. A total of 505 comparisons between qRT-PCR and microarray fold-inductions among these 97 ESTs gave a 92% overall agreement in direction of regulation, while the rate among the entire 171 ESTs analyzed (757 comparisons) was 81.6%. Discrepancies between the results from the two methods are widely recognized and are often attributed to cross hybridization of gene family members on microarrays, differences in hybridization on surfaces versus solution hybridization, and/or better quantitation of low abundance transcripts by RT-PCR. Potential functionality of the genes described below is ascribed based on homology to genes of known function from other organisms. Function in sorghum has yet to be determined.

Cell Wall-Related Genes

A putative peroxidase (CF772269) was moderately to strongly induced across most tissues and times by all three treatments, as determined both by microarray and by qRT-PCR (Table I, Oxidative). A putative cinnamyl alcohol dehydrogenase gene (BG049125) was strongly induced by SA and MeJA across most tissue-time combinations (Table I, Phenylpropanoid/

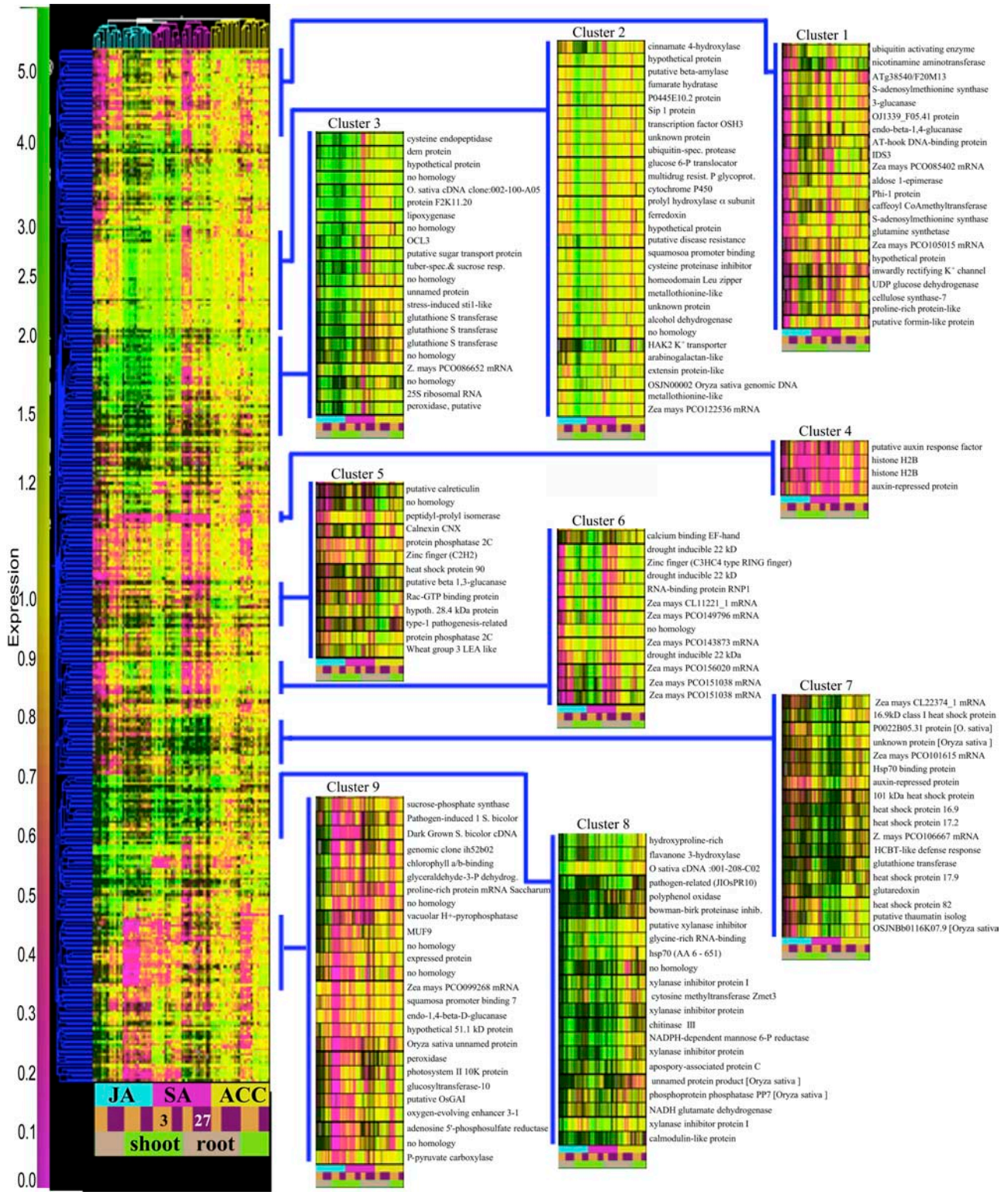

Figure 3. Regulatory patterns of 400 genes displaying significant interaction between defense compound and tissue. Clustergram shows results of two-way ANOVA analysis for significance of interaction between defense compound and tissue. Data were drawn from a total of 84 microarray slides, with 28 slides per defense compound, 14 slides per tissue for each compound. Statistical significance was determined with the Bonferroni correction at 0.3 (0.3/6,438 genes selected by chance) using Genespring 6.1 (Agilent Technologies). Tree at left of main heatmap shows clustering of expression profiles among the genes, tree at top of heatmap shows relatedness of expression data by compound, tissue, and time. Color bar at base of heatmap indicates vertically oriented zones among the data corresponding to compound × tissue × time conditions. Fold expression data for each individual gene across the conditions are indicated by colored horizontal bars across the main map; green, induced; yellow, unchanged; and magenta, suppressed. Magnified sections of the main map corresponding to visual clusters are shown at right, with putative gene functions identified for genes in the magnified clusters (blast scores and e-values available in Supplemental Table I). Smallest-order vertical stripes within each horizontal gene color bar represent individual feature data from a single slide.

Table I. Induction folds of selected genes from biological groups assessed by microarray and qRT-PCR.

ns= microarray results were non-significant **XXX** = between 2 and 5 fold induced ***XXX*** = 5 fold or more induced XXX = 4 fold or more suppressed n.a. = qRT-PCR unsucessful

		SA								ACC								JA							
3' GenBank accession #	3' Abbreviated Clone name	3 h root		3 h shoot		27 h root		27 h shoot		3 h root		3 h shoot		27 h root		27 h shoot		3 h root		3 h shoot		27 h root		27 h shoot		
		qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	**Putative identification**
	Defense signaling																									
CF771133	DSBF1_14_D07	**2.02**	ns	***27***	**2.9**	***5.8***	**3.37**	***47***	ns	0.88	ns	1.19	ns	1.46	ns	1.27	**3.1**	1.635	ns	0.41	ns	***20.5***	***12***	0.8	ns	serine/threonine-specific protein kinase homolog T5F17.120 [A. thaliana]
BM330823	PIC1_57_A08	1.54	ns	**2.14**	1.95	**2.35**	ns	**4.33**	**3.32**	1.13	ns	1.09	ns	0.91	ns	1.27	ns	1.82	ns	0.62	ns	**3.596**	ns	**2.39**	ns	probable serine/threonine kinase (EC 2.7.1.-) SNFL1 [Sorghum bicolor]
BE592414	WS1_94_D12	0.9	ns	***5***	**2.3**	1.51	ns	1.51	1.8	1.03	ns	0.61	ns	1.14	ns	1.01	ns	0.771	ns	1.27	ns	0.92	ns	0.97	ns	Pto-like serine/threonine kinase (Fragment) [Musa acuminata]
BE593730	WS1_101_E03	***7.8***	**2.07**	***39***	**4.09**	***5***	1.84	**3.48**	ns	1.35	ns	1.47	ns	1.28	ns	***7.75***	ns	0.43	ns	***5.8***	ns	0.806	ns	1.11	ns	Similar to PTK2 protein tyrosine kinase 2 [Homo sapiens]
BE638195	WS1_96_E09	1.28	ns	**2.88**	***12***	1.56	ns	1.69	***7.7***	0.78	0.65	0.54	ns	0.57	0.65	1.08	ns	**3.792**	ns	**3.33**	ns	0.804	ns	**4.08**	***5.6***	protein kinase, lysine deficient 4 [Rattus norvegicus]
BI139762	IP1_48_H07	1.41	**2.19**	1.62	ns	1.69	ns	0.37	ns	0.57	ns	0.92	ns	1.05	ns	0.9	ns	**4.136**	**2.64**	***6.3***	***8.7***	**4.974**	**3.27**	**4.41**	**2.34**	Putative choline kinase [Oryza sativa]
BG933609	OV1_32_D07	***14***	ns	***30***	ns	***17***	ns	**3.15**	ns	1.1	ns	1.64	ns	**3.07**	ns	1.02	ns	***12.6***	**4.95**	***33***	**3.23**	***15.7***	***6.5***	***24***	**2.49**	NPK1-related protein kinase-like protein [Oryza sativa]
BE598412	PI1_82_H03	1.61	ns	0.69	ns	**3**	ns	1.11	0.55	1.53	ns	1.38	ns	0.08	ns	***21.4***	0.54	0.708	ns	0.44	0.45	1.362	**2.44**	1.14	0.15	mitogen-activated protein kinase 4 [Petroselinum crispum]
BM330410	PIC1_51_F08	0.2	0.64	0.43	0.68	**2.31**	ns	**2.04**	0.65	0.64	ns	1.23	ns	0.03	ns	***141***	ns	0.173	0.37	0.14	ns	0.545	0.23	0.51	0.54	serine/threonine kinase-like protein [Oryza sativa]
BG050463	FM1_69_F01	0.54	ns	0.53	ns	0.2	ns	1.49	ns	1.25	ns	1.25	ns	***9.1***	ns	0.18	ns	0.463	0.56	0.44	ns	0.093	0.26	0.31	ns	Similar to mitogen-activated protein kinases [Oryza sativa]
BM331204	PIC1_71_A03	1.64	ns	**2.4**	**3.67**	**4.57**	**3.95**	**3.25**	**3.96**	0.86	ns	1.18	ns	1.16	ns	1.04	ns	**2.641**	1.82	**3.67**	1.93	***5.47***	ns	***6.8***	***6.3***	Protein phosphatase 2C-like protein [Oryza sativa]
CF757073	DSAF1_13_D04	0.08	0.55	1.15	ns	1.57	ns	0.3	ns	1.1	ns	1.51	ns	1.53	0.62	1.11	ns	***1116***	***7***	**2.2**	***6.3***	***3621***	***16***	***11***	***17***	Putative acid phosphatase (Fragment) [Hordeum vulgare]
BG356045	EM1_19_F06	0.96	ns	1.47	ns	***30***	ns	**4.04**	ns	0.12	ns	0.83	ns	***15***	ns	0.77	ns	1.014	ns	0.75	ns	***336***	***5.2***	1.16	ns	leucine-rich repeat protein LRP [Sorghum bicolor]
BG739281	EM1_81_B02	**4.11**	**4.03**	***5.2***	**4.95**	**3.89**	ns	1.04	ns	1.24	ns	1.1	ns	1.32	ns	1.18	ns	0.899	ns	1.17	ns	1.407	ns	1.27	ns	Putative zinc finger protein [Arabidopsis thaliana]
BM328251	PIC1_22_H04	1.43	1.62	***5***	**3.18**	**2.13**	**3.19**	1.64	ns	1.19	ns	1.01	ns	0.67	ns	1.23	ns	1.473	ns	**2.68**	ns	1.693	ns	**4.89**	***5.2***	Probable WRKY transcription factor 3 [A. thaliana]
BG103121	RHIZ2_18_D08	0.69	ns	0.41	ns	0.58	ns	0.52	ns	**3.89**	**2.88**	**2.89**	ns	**4.28**	ns	**3**	ns	0.27	0.64	0.62	ns	0.243	ns	**2.19**	ns	Putative EREBP-type transcription factor [Oryza sativa]
AW284841	LG1_278_G01	**4.91**	ns	***8.3***	1.72	***8***	**2.81**	**3.79**	ns	1.69	ns	1.39	ns	**2.49**	ns	0.9	ns	0.469	ns	0.42	ns	1.729	ns	1.31	ns	Ethylene responsive element binding factor 4-like [Thellungiella halophila]
CF772823	DSBF1_39_G12	***13***	ns	***29***	ns	***20***	1.8	***7.8***	ns	1.57	ns	1.28	ns	***8.7***	ns	1.86	ns	***6.21***	**2.13**	**4.79**	ns	***37.9***	**4.44**	***29***	0.61	WRKY-like protein (WRKY transcription factor 75) [Arabidopsis thaliana]
BG557968	EM1_39_H01	0.58	ns	0.27	0.62	0.48	ns	1.18	1.72	***5***	ns	**2.96**	**2.49**	***5.8***	**3.93**	**3.41**	**2.8**	0.207	ns	0.61	ns	0.146	ns	1.57	ns	Putative EREBP-type transcription factor [Oryza sativa]
BM331200	PIC1_70_H11	0.92	ns	**3.2**	**4.02**	0.95	ns	**2.1**	**2.47**	0.99	ns	1.33	ns	1.22	ns	1.49	ns	0.537	ns	1.08	ns	0.96	ns	1.14	**3.31**	nitrilase-like protein [imported] [Oryza sativa]
AW677238	DG1_6_G11	1.94	ns	***5.3***	ns	1.61	ns	1.9	ns	1.2	ns	1.55	ns	1.8	ns	0.36	ns	**2.009**	ns	0.65	ns	***8.37***	0.49	***11***	ns	Putative AP2 domain transcriptional regulator [Oryza sativa]
	Phenylpropanoid/secondary compounds																									
BG273296	OV2_25_F03	***32***	***12***	***33***	***24***	***54***	***69***	***7***	**3.92**	1.06	ns	1.48	ns	1.91	ns	1.22	ns	***6.13***	**3.28**	0.66	**3.44**	***9.41***	***8.2***	1.75	ns	Putative flavonol glucosyltransferase [Oryza sativa]
BG049125	OV1_23_F04	***32***	***5.6***	***25***	***6.5***	***26***	**3.44**	**4.52**	1.91	1.26	ns	1.31	ns	1.64	ns	1.3	ns	***11.1***	**4.37**	1.73	**4.35**	***22.9***	***10***	***21***	***5.1***	putative cinnamyl-alcohol dehydrogenase [Oryza sativa]
AW680925	WS1_8_F09	1.6	**2.09**	**2.35**	**2.9**	1.05	**2.09**	1.29	**2.9**	1.33	ns	0.98	ns	**2.53**	ns	1.15	ns	1.193	1.59	**3.02**	**3.6**	**3.845**	**4.82**	***5.1***	**4.45**	Similarity to chalcone-flavonone isomerase [Arabidopsis thaliana]
BE366509	PI1_34_B02	0.9	1.97	**4.61**	**2.15**	0.8	**2.85**	**4.59**	**3.22**	**2.18**	1.87	0.89	ns	***7.4***	ns	**2.58**	ns	**2.64**	**3.06**	**4.42**	ns	**3.414**	***37***	***23***	**2.09**	Putative flavanone 3-hydroxylase (Hypothetical protein) [Oryza sativa]
BM330867	PIC1_57_E11	1.18	ns	**3.14**	**2.17**	1.33	ns	**2.05**	**2.17**	0.67	ns	1.2	ns	0.65	ns	1.19	ns	**2.826**	**4.91**	0.88	**3.58**	***6.94***	***6.3***	**2.04**	***9.3***	Isoflavone reductase homolog (EC 1.3.1.-) [Lupinus albus]
BG273207	OV2_28_B10	***8***	ns	***571***	0.59	1.79	ns	0.87	0.46	***20***	ns	n.a.	ns	n.a.	ns	0.51	ns	0.167	ns	***69***	ns	0.49	0.28	***9***	0.24	pectin methylesterase-like protein [Oryza sativa]
BG102626	RHIZ2_35_C12	0.04	ns	1.19	ns	1.18	ns	0.34	ns	1.27	ns	1.28	ns	1.2	0.62	0.75	ns	0.333	ns	1.79	1.87	***5.67***	ns	***6.8***	**4.96**	Cyanogenic beta-glucosidase dhurrinase-2 [Sorghum bicolor]
BG158602	RHIZ2_42_F06	0.16	1.75	1.68	ns	0.41	ns	***6.4***	ns	1.58	ns	0.98	ns	***18***	ns	0.07	ns	***22.5***	***8***	***87***	***45***	0.541	***7.7***	0.13	***17***	Putative 0-deacetylbaccatin III-10-O-acetyl transferase [Oryza sativa]
AW747472	WS1_68_C01	0.07	0.37	0.12	0.49	0.03	ns	1.56	ns	0.91	ns	1.52	ns	**3.58**	ns	0.46	ns	0.014	0.26	0.04	0.41	0.046	0.54	0	0.54	Thiazole biosynthetic enzyme 1-2, chloroplast precursor [Zea mays]
	Pathogenesis-related																									
AW284118	LG1_269_H05	1.76	ns	***11***	***6.5***	**3.29**	**2.56**	**4.81**	**2.79**	1.82	1.66	0.8	ns	**2.38**	ns	1.4	ns	0.966	ns	0.61	ns	**3.775**	ns	1.6	ns	Putative pathogenesis related protein [Oryza sativa]
AW676734	DG1_13_H03	***7.3***	**3.56**	**2.94**	ns	***11***	***6.5***	***5.8***	***8.5***	**3.15**	**2.54**	1.22	ns	***37***	ns	1.29	ns	***6.21***	**3.11**	1.03	ns	***156***	***82***	***5.6***	**3.41**	pathogenesis-related protein PR10 [Sorghum bicolor]
BG053390	RHIZ2_8_C04	1.46	**2.61**	1.93	**3.03**	**4.74**	**2.66**	**2.25**	ns	1.68	ns	1.33	ns	1.58	ns	1.24	ns	***10.3***	ns	***20***	**2.07**	***33.2***	ns	***39***	ns	Putative thaumatin isolog [Oryza sativa]
BM318308	PI1_77_A09	0.07	ns	0.12	0.44	0.02	ns	0.26	0.32	**2.57**	**2.24**	0.75	ns	***14***	ns	1.85	ns	3E-05	ns	0.63	ns	0.003	ns	0.62	ns	probable beta-1,3-glucanase [imported] [Arabidopsis thaliana]
AW746272	WS1_40_E04	**2.97**	**4.19**	***16***	***16***	***9.3***	***6.7***	***40***	***19***	1.13	ns	1.34	ns	1.68	ns	1.43	ns	***7.78***	***5.4***	**3.44**	**2.23**	***20***	***27***	***15***	***7.9***	chitinase (EC 3.2.1.14) III C10701 [Oryza sativa]
AW679749	WS1_31_F02	0.63	ns	***21***	ns	***7.4***	ns	**4.86**	ns	1.09	ns	***5.7***	ns	0.9	ns	1.7	ns	0.388	**3.7**	**2.36**	**2.86**	***11.9***	**4.01**	***5.1***	**4.04**	probable wound-induced protein T9A4.6 [Arabidopsis thaliana]
	SA/Ethylene/JA specific																									
CF772606	DSBF1_37_B04	0.13	ns	1.02	ns	***5.1***	ns	***102***	***42***	0.94	ns	1.11	ns	***14***	ns	1.85	ns	***133***	1.91	***11***	***7.8***	***99.3***	1.94	***49***	***79***	benzothiadiazole-induced protein (clone WCI-3) [Triticum aestivum]
BI245631	IP1_68_B04	**2.92**	***7.5***	***12***	**2.56**	***5.1***	ns	***35***	1.92	**1**	ns	**2.61**	ns	***5.8***	ns	1.85	ns	**3.227**	ns	**4.55**	ns	***15.7***	ns	***43***	ns	jasmonic acid regulatory protein [Oryza sativa]
BF705195	RHIZ2_2_F12	**2.35**	**2.8**	1.86	***5.5***	**3.27**	**3.29**	***5.9***	***5.5***	1.06	ns	1.63	ns	0.75	ns	1.96	ns	0.475	ns	1.12	***13***	**3.131**	**3.72**	**4.77**	***5.3***	probable 1-aminocyclopropane-1-carboxylate oxidase [O. sativa]
BE360455	DG1_63_G10	0.44	ns	0.3	ns	0.13	ns	1.49	ns	0.4	ns	1.08	ns	0.39	ns	1.99	ns	***5.92***	**3.48**	***13***	1.65	**2.085**	1.95	***1710***	**3.53**	Putative ethylene-forming enzyme [Oryza sativa]
	Protease/inhibitors																									
CF758097	DSAF1_27_C06	**2.65**	ns	**2.42**	**2.68**	**3.69**	ns	***104***	***62***	1.08	ns	0.7	ns	**4.21**	ns	0.94	ns	***317***	***5.7***	***36***	***11***	***811***	***15***	***58***	***48***	Bromelain-like thiol protaease (Putative bromelain FB1035) [O. sativa]
AW747582	WS1_73_G06	***20***	**4.68**	***166***	***51***	***21***	***5.1***	***7.9***	**2.35**	0.99	ns	1.23	ns	1.98	ns	1.48	ns	1.068	ns	***6.5***	**2.02**	***6.43***	ns	***21***	***5.1***	ATP-dependent Clp protease ATP-binding subunit precursor [O. sativa]
AW680689	WS1_6_H02	***33***	**4.8**	**2.62**	**2.17**	***281***	***27***	***54***	***8.5***	**3.17**	ns	1.6	ns	***13***	ns	1.49	ns	***30.5***	***49***	**2.18**	**2.55**	***3714***	***170***	***40***	***10***	Similar to Zea mays bowman-birk proteinase inhibitors WIP1) [O. sativa]
AW679450	WS1_24_D01	0.43	ns	**4.44**	**3.79**	1.08	ns	1.93	**3.79**	0.99	ns	1.73	ns	**2.45**	ns	1.11	ns	***99.2***	***19***	***25***	***17***	***352***	***150***	***33***	***12***	Bowman-Birk type trypsin inhibitor (WTI) [Triticum aestivum]
AI723849	RHIZ1_28_B11	0.67	1.7	**3.66**	**3.12**	1.39	ns	**3**	1.86	1.2	ns	1.66	ns	0.03	ns	na	ns	***104***	***9.4***	***19***	***19***	***357***	***20***	***28***	***14***	putative Bowman-Birk serine proteinase inhibitor
CF758792	DSAF1_36_F07	0.48	ns	1.9	1.76	0.36	ns	**2.03**	1.76	0.9	ns	1.59	ns	0.5	0.62	**2.18**	ns	***6.44***	***7.2***	***11***	***15***	***5.5***	***9.2***	***17***	***21***	subtilisin/chymotrypsin inhibitor [Zea mays]
AW747623	WS1_74_C11	**3.18**	**3.39**	***100***	***5.6***	***10***	***5.3***	***70***	***7***	1.07	ns	1	ns	1.69	ns	1.97	ns	***7.19***	***6.9***	**3.53**	***9.5***	***26.2***	***31***	***33***	***8.5***	Putative xylanase inhibitor protein [Oryza sativa]
CF758771	DSAF1_36_D09	**3.25**	**4.33**	***25***	***30***	***7.1***	**4.33**	***43***	***30***	1.15	ns	1.45	ns	1.82	ns	1.53	ns	***5.24***	***8***	**2.72**	**4.36**	***21.3***	***28***	***16***	***10***	Putative xylanase inhibitor protein [Oryza sativa]
BE353141	DG1_4_A01	**2.65**	**2.64**	***22***	***6.4***	**4.97**	**2.64**	***30***	***6.4***	0.64	ns	1.05	1.52	1.67	1.75	1.37	1.97	**4.816**	***6.4***	**2.06**	***5***	***19.1***	***56***	**4.1**	***9.5***	Xylanase inhibitor protein I precursor [Triticum aestivum]
AW680920	WS1_8_F04	***5.7***	**3.97**	***9.1***	***6.6***	***9.4***	***5.6***	1.1	ns	1.3	ns	1.13	ns	**2.08**	ns	0.91	ns	0.688	ns	**2.35**	ns	1.61	ns	**3.69**	ns	Bax inhibitor-1 (BI-1) (OsBI-1) [Oryza sativa]

Table 1, continued

ns= microarray results were non-significant **XXX** = between 2 and 5 fold induced ***XXX*** = 5 fold or more induced XXX = 4 fold or more suppressed n.a. = qRT-PCR unsucessful

		SA								ACC								JA								
3' GenBank	3' Abbreviated	3 h root		3 h shoot		27 h root		27 h shoot		3 h root		3 h shoot		27 h root		27 h shoot		3 h root		3 h shoot		27 h root		27 h shoot		
accession #	Clone name	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	qPCR	µarray	Putative identification
	Oxidative																									
AW677268	DG1_6_C05	***505***	***12***	***145***	***173***	***143***	***38***	***26***	***17***	**2.05**	ns	1.27	ns	1.81	ns	1.81	ns	***239***	***21***	***8.8***	***10***	***571***	ns	***11***	ns	Glutathione S-transferase GST 34 (EC 2.5.1.18) [Zea mays]
AW747673	WS1_75_A09	***8.6***	***13***	***56***	***44***	***9.2***	***12***	***5***	**3.66**	0.81	ns	1.31	ns	1.56	ns	1.39	ns	***11.9***	***24***	***24***	***13***	***12.5***	ns	***30***	ns	Putative cytochrome P450 [Oryza sativa]
AW745838	WS1_37_G04	0.57	***9.5***	***12***	***15***	***7.2***	***10***	**2.35**	**3.13**	0.96	ns	1.02	ns	1.41	ns	1.41	ns	1.029	ns	1.59	ns	**2.332**	ns	1.22	ns	Thioredoxin H-type (TRX-H) (Phloem sap 13 kDa protein-1) [Oryza sativa]
BG559720	RHIZ2_72_G09	***21***	**4.61**	***63***	***23***	***96***	***28***	***6***	**3.79**	1.87	ns	1.25	ns	**2.36**	ns	1	ns	***10.7***	***8.7***	**4.88**	***11***	***13.4***	ns	**4.15**	***11***	alternative oxidase AOX2 precursor [Zea mays]
BE357624	DG1_21_A04	0.48	ns	0.89	1.86	0.29	ns	1	1.86	0.31	ns	1.17	1.55	1.04	ns	1.3	ns	1.429	**3.67**	0.98	***20***	0.64	1.51	***7.1***	***5.5***	Putative NADPH oxidase [Oryza sativa]
AW922393	DG1_18_G12	***25***	ns	***180***	ns	***22***	ns	***10***	ns	0.95	ns	1.37	ns	0.55	ns	1.03	ns	0.721	ns	0.86	**2.42**	**3.64**	ns	***6.2***	**2.97**	alternative oxidase AOX3 precursor [Zea mays]
CF772269	DSBF1_31_E01	**2.31**	**3.44**	**3.87**	**3.29**	**3.15**	**4.3**	**4.82**	**3.23**	***6.5***	***5.3***	**4.14**	**2.07**	***15***	ns	**4.08**	ns	1.935	ns	***12***	**2.15**	***14.4***	***11***	***9***	ns	putative peroxidase [Oryza sativa]
	Fatty acid metabolism/JA synthesis																									
BG464526	EM1_70_G12	1.38	1.82	**4.44**	**2.83**	1.58	**2.24**	**2.29**	**2.2**	1.04	ns	1.25	ns	1.26	ns	1.45	ns	**2.052**	1.82	**2.04**	**4.36**	**3.3**	**3.3**	**3.35**	***6.7***	Acyl-CoA oxidase ACX3 [Arabidopsis thaliana]
BE592413	WS1_94_D11	***5.4***	**2.91**	***76***	***9.2***	**3.9**	ns	**3.96**	1.86	0.89	ns	1.26	ns	0.86	ns	0.82	ns	**2.429**	ns	***20***	**3.55**	**4.072**	1.93	***19***	**3.76**	Putative acyl-CoA synthetase [Capsicum annuum]
BF480926	FM1_15_B03	1.91	**2.16**	**3.41**	1.75	1.72	**2.42**	1.91	1.76	0.57	ns	0.85	ns	1.18	ns	1.08	ns	0.434	ns	0.44	ns	0.126	ns	0.85	ns	probable lipase [Zea mays]
BG048471	OV1_13_H09	0.39	ns	0.6	ns	0.24	ns	0.85	ns	0.8	ns	1.44	ns	0.86	ns	1.17	ns	***14.5***	**3.78**	***22***	***8.2***	***28***	***8.7***	***38***	***14***	w-3 fatty acid desaturase [Oryza sativa]
AW747671	WS1_75_A04	**4.59**	**2.65**	1.09	ns	**3.63**	ns	1.75	ns	1.41	ns	0.87	ns	0.76	ns	**2.87**	ns	1.603	ns	0.46	ns	1.135	ns	**4.76**	ns	Putative lysophospholipase [Arabidopsis thaliana]
BF586333	FM1_27_E12	0.32	ns	**3.94**	**2.57**	0.37	ns	***11***	**2.57**	1.53	ns	**4.36**	1.85	**2.43**	ns	**3.23**	ns	***16.9***	***7.9***	***78***	***27***	**4.648**	**3.28**	***136***	***37***	Lipoxygenase (EC 1.13.11.12) [Zea mays]
BG559771	RHIZ2_73_D11	0.77	**2.25**	**3.62**	**3.63**	0.71	**2.25**	1.66	**3.63**	1.21	ns	1.34	ns	1.89	ns	1.3	ns	***10.9***	**3.27**	***35***	1.92	**4.728**	ns	***34***	1.85	Allene oxide synthase (EC 4.2.1.92) [Hordeum vulgare]
AW745827	WS1_37_H03	0.6	**2.26**	**2.07**	1.63	0.69	1.98	1.32	ns	1.26	ns	1.58	ns	1.38	ns	1.26	ns	***5.42***	ns	***15***	ns	***5.18***	ns	***16***	ns	Allene oxide cyclase (EC 5.3.99.6) [Oryza sativa]
BE358964	DG1_37_B12	***5.4***	**2.02**	1.86	1.91	**4.23**	**2.09**	**3.42**	**2.12**	0.97	1.65	1.26	ns	1.88	ns	1.6	ns	1.904	**2.64**	1.34	ns	**2.155**	***23***	**3.73**	1.84	12-oxophytodienoate reductase (OPR1) [Arabidopsis thaliana]
BE358737	DG1_31_B03	0.84	ns	***7.7***	**2.4**	0.78	ns	1.76	**2.4**	1.08	ns	1.22	ns	1.64	1.59	1.5	1.76	**3.057**	**3.79**	***6.1***	***14***	**4.316**	**4.41**	***16***	***13***	12-oxophytodienoate reductase 3 [Lycopersicon esculentum]
CF772670	DSBF1_37_H12	**2.23**	***6.8***	***10***	**3.12**	***7.7***	***7.3***	n.a.	**2.57**	0.74	ns	n.a.	ns	**3.19**	ns	1.11	ns	***5.28***	**2.33**	n.a.	1.68	1.356	**3.57**	***13***	ns	AIM1 protein [Arabidopsis thaliana]
	Metal binding/related																									
BG047941	OV1_25_F12	0.14	ns	***5.9***	1.71	0.08	ns	***40***	***5.5***	1.1	ns	0.72	ns	1.72	ns	1.05	ns	0.009	ns	**3.74**	ns	8E-04	ns	**2.15**	ns	Putative calcium binding EF-hand protein [Hordeum vulgare]
BF656370	FM1_49_H04	**4.54**	**2.79**	***14***	***12***	**4.94**	ns	**3.98**	**3.66**	0.81	ns	1.44	ns	0.89	ns	**2.46**	ns	1.323	**2.06**	**3.62**	**2.58**	***31.8***	**3.3**	**2.78**	**3.04**	Putative selenium binding protein [Oryza sativa]
	HSP/Chaperones																									
CF770431	DSBF1_7_E11	***738***	***27***	***38***	***12***	***136***	***14***	***6.1***	**4.52**	**2.76**	ns	1.05	ns	0.66	ns	1.37	ns	0.154	ns	0.75	ns	***30.2***	ns	0.82	ns	17.5 kDa class II heat shock protein [Zea mays]
AW283680	LG1_222_B11	**3.3**	**3.99**	***16***	**3.88**	***6.1***	**2.73**	0.88	ns	1.47	ns	0.75	ns	**2.29**	ns	0.98	0.48	0.713	ns	**2.48**	ns	1.402	2	1.14	ns	Heat shock cognate 70 kDa protein 2 [Lycopersicon esculentum]
AW746348	WS1_40_A06	***87***	***25***	***105***	***8.7***	***134***	***23***	***59***	**3.53**	0.96	ns	1.05	ns	0.97	ns	1.11	ns	0.419	ns	1.26	ns	***10.7***	**2.08**	**3.04**	1.81	heat shock protein 16.9 [Pennisetum glaucum]
AW747351	WS1_67_D11	***101***	***7.1***	***15***	1.76	***111***	**4.18**	**3.68**	ns	0.62	ns	0.99	ns	0.53	ns	0.82	ns	0.185	ns	0.82	ns	0.779	0.61	1.02	0.59	101 kDa heat shock protein (Fragment) [Zea mays]
	Membrane/Transport																									
AW678176	WS1_13_G07	***8.6***	**3.07**	***36***	1.87	***7.2***	**4.76**	***6.2***	**2.01**	0.46	ns	0.44	ns	**2.48**	ns	0	ns	1.45	**4.7**	***6.1***	ns	**4.636**	***23***	**4.52**	0.57	PDR-like ABC transporter [Oryza sativa]
CF772054	DSBF1_28_E02	0.66	ns	1.81	**4.32**	**2.64**	**4.68**	1.65	ns	1.24	ns	1.2	ns	**2.05**	ns	1.48	ns	0.886	ns	1.45	**2.32**	1.007	***9.1***	1.43	**3.61**	High-affinity potassium uptake transporter [Debaryomyces occidentalis]
CF757790	DSAF1_22_C12	0.78	ns	1.02	ns	**2.37**	ns	**2.4**	***5.9***	1.37	ns	0.97	ns	0.98	ns	1.03	ns	1.075	ns	***40***	***21***	***5.46***	**2.31**	***49***	***16***	ABC transporter-like protein [Oryza sativa]
	Photosynthesis/chloroplast																									
AW285440	LG1_251_E07	0.04	ns	0.68	ns	1.52	ns	0.85	0.66	1.49	ns	1.42	ns	0.02	ns	***99.2***	0.5	0.187	ns	0.3	0.39	***25.5***	ns	0.15	0.11	Phosphoenolpyruvate carboxylase 3 [Sorghum bicolor]
BM327935	PIC1_19_C04	0.3	ns	0.94	ns	0.39	ns	0.7	0.64	1.78	ns	1.26	ns	0.02	ns	***58.7***	ns	0.117	ns	0.11	ns	0.125	ns	0.02	ns	Signal recognition particle 54 kDa protein [A thaliana]
BM318011	PI1_36_F08	0.07	ns	0.5	ns	1.21	**2.47**	0.62	0.47	1.18	ns	1.44	ns	0.01	ns	***154***	0.57	0.095	ns	0.26	ns	n.a.	**2.18**	0.08	0.15	PsbP domain protein, putative [Arabidopsis thaliana]
AW672324	LG1_358_G11	0.05	ns	0.24	0.62	***14***	1.68	0.24	0.24	1.19	ns	1.37	ns	0.01	ns	***107***	0.42	0.005	ns	0.12	0.36	n.a.	ns	0	0.05	Chlorophyll a/b-binding protein WCAB precursor [Triticum aestivum]
	Miscellaneous other function																									
BM327868	PIC1_18_E03	***5.1***	***5.1***	***8.9***	***12***	**3.35**	**3.24**	1.39	1.76	1.14	ns	1.04	ns	1.2	ns	1.97	ns	**2.769**	**2.5**	**3.89**	**3.69**	**4.157**	**4.01**	***7.4***	**4.14**	Riboflavin biosynthesis protein ribA [A. thaliana]
BM317859	OV1_16_D08	0.64	ns	0.39	ns	***7.1***	ns	**4.79**	**3.66**	***63***	ns	0.58	ns	0.96	ns	1.45	ns	0.186	ns	***17***	***6.2***	0.155	0.37	***40***	***10***	S-like RNase [Triticum aestivum]
CF756248	DSAF1_4_A06	0.78	ns	0.71	ns	***8.4***	1.77	***6.7***	**3.84**	1.35	ns	1.1	ns	1.01	ns	1.57	ns	0.904	ns	***9.1***	***7.2***	n.a.	1.7	**3.78**	**3.81**	ribonuclease S homolog [Zea mays]
BG412449	OV2_34_C11	**4.84**	**4.39**	***42***	***32***	***9.6***	***6***	***12***	***7.3***	1.54	ns	1.13	0.62	**3.49**	ns	1.71	ns	**2.621**	**2.18**	1.8	**2.19**	***9.58***	***6.3***	**3.28**	**3.1**	Beta-glucosidase (Fragment) [Musa acuminata]
AW679941	WS1_33_H03	***10***	**3.55**	**4.56**	***5.9***	**4.46**	***6.4***	1.77	1.9	0.85	ns	1.1	ns	1.44	ns	1.54	ns	**2.947**	1.75	1.09	1.9	***5.14***	**2.12**	1.12	1.54	Putative ripening regulated protein [Oryza sativa]
AW672441	LG1_359_C07	0.39	ns	0.65	ns	**2.54**	**4.81**	0.76	0.63	***7.6***	**2.56**	1.53	ns	**3.86**	ns	1.15	ns	0.49	**2.12**	1.54	ns	**2.64**	***14***	0.42	**2.33**	Putative NAM (no apical meristem) NAC-domain protein [Oryza sativa]
AW679110	WS1_22_G08	0.98	**2.66**	**2.19**	***6.1***	1.51	**3.3**	**2.25**	ns	0.98	ns	**4.67**	ns	0.96	ns	**2.82**	ns	0.944	ns	0.24	ns	1.596	ns	1.16	ns	Putative ubiquitin-conjugating protein [Oryza sativa]
BI211613	IP1_59_G01	0.22	0.5	0.13	0.38	0.03	0.28	1.05	ns	0.42	0.5	0.93	ns	0.47	0.51	0.87	0.56	0.041	ns	0.58	ns	0.001	0.18	0.41	0.55	Phosphoethanolamine methyltransferase [Triticum aestivum]
BE592378	WS1_94_A05	0.29	ns	0.26	0.31	0.24	0.23	0.38	0.53	0.71	ns	0.8	0.65	0.89	ns	0.94	0.44	0.356	0.59	0.55	0.28	0.217	0.21	0.24	0.26	Histone H2B [Triticum aestivum]
AW679114	WS1_22_G03	1.54	ns	***34***	***7.7***	0.11	ns	***54***	***15***	0.55	0.61	0.79	ns	0.43	0.63	1.1	ns	0.273	ns	0.6	**2.12**	0.004	0.32	0.3	ns	Rab 17 dehydrin-like
CF758054	DSAF1_26_F12	0.07	ns	0.87	ns	***6.7***	ns	0.62	ns	1.91	ns	1.37	ns	n.a.	ns	1.06	0.64	***2359***	***15***	***16***	***8.2***	n.a.	***14***	***5.7***	***6.9***	Hydroxynitrile lyase (EC 4.1.2.11) [Sorghum bicolor]
BF585835	FM1_25_B01	0.79	ns	0.97	ns	0.78	ns	0.63	ns	1.1	ns	1.53	ns	0.76	ns	1.35	ns	**2.265**	***28***	1.5	**2.36**	**2.649**	***7***	1.37	***9.2***	probable glucose regulated repressor protein [Arabidopsis thaliana]
CF771144	DSBF1_14_E10	0.37	ns	0.96	ns	1.39	ns	0.42	ns	0.67	ns	0.59	0.5	0.81	0.58	0.63	ns	35537	**4.5**	***5.7***	***5.3***	***366***	**2.55**	**2.42**	**4.82**	O-methyltransferase ZRP4 (EC 2.1.1.-) (OMT) [Zea mays]
BG158601	RHIZ2_42_F05	1.22	1.76	**4.45**	ns	0.47	ns	**2.16**	ns	0.94	ns	1.4	ns	***24***	ns	0.04	ns	***14.8***	***11***	***314***	***19***	***21.7***	***7.4***	***483***	***11***	Patatin-like protein [Sorghum bicolor]
BI099490	IP1_42_H07	**2.86**	**3.64**	***8.3***	***5.9***	**3.82**	**4.51**	**4.08**	**2.13**	1.84	ns	1.23	ns	***6.4***	ns	0.3	ns	**4.389**	***5.8***	**4.65**	***5.8***	***9.32***	***13***	**3.62**	***5.9***	putative cytidine deaminase [Zea mays]
AW285221	LG1_235_H09	1.4	ns	1.82	ns	0.2	ns	0.55	0.54	0.69	0.6	1.08	ns	0.78	0.21	0.11	ns	0.367	0.46	0.12	ns	0.056	0.17	0.1	0.53	herbicide safener binding protein 1 [Zea mays]

Microarray fold numbers (µarray) for each gene indicate global mean fold inductions (relative to untreated controls) from all slides in each compound x tissue x time condition. Fold inductions for qRT-PCR data (qPCR) indicate 2^ (deltaCT control/delta CT treatment). Gene suppressions are expressed as decimals (1/fold). cDNA template for the qRT-PCR reactions were made from the same RNA samples used to generate the microarray cDNA probes. GenBank accession numbers refer to 3' sequences of the ESTs, and these sequences were used for design of primers for qRT-PCR. 3' abbreviated clone names refer to clone designations in the sorghum database at http://fungen.org.

Blast scores and expect values are available by using supplementary table SIV, or online at http://fungen.org/sorghum.htm., accessed by clone name.

secondary compounds), representative of a number of sorghum homologs of this gene. Also implicated in lignin production, several caffeoyl CoA *O*-methyltransferases were induced in shoots by SA and MeJA (Supplemental Tables I–XXIV). Also presumably involved in cell wall fortification, four cellulose synthase homologs were induced in shoots by MeJA on microarrays. Further, a pectin methylesterase-like gene was up-regulated by SA, as were a cell wall-associated hydrolase (BE593926), two putative dermal glycoproteins (AW746846 and AW680228), and a putative Hyp-rich glycoprotein (BI211604; Supplemental Tables I–XXIV).

Genes Involved in Lipid Metabolism and the Oxylipin Pathway/Jasmonate Synthesis

We observed induction of several putative phospholipases, including a phospholipase A2, by SA as well as by MeJA and ACC (Fig. 4). Patatin also exhibits phospholipase A2 functionality (Dhondt et al., 2000; Holk et al., 2002), and expression of a number of sorghum patatin homologs was induced sharply by MeJA and also moderately by SA (Fig. 4). Sorghum homologs of most genes in the ODA pathway, including LOX, AOS, AOC, and 12-oxophytodienoate reductase (*OPR1* and *OPR3*-like), were markedly

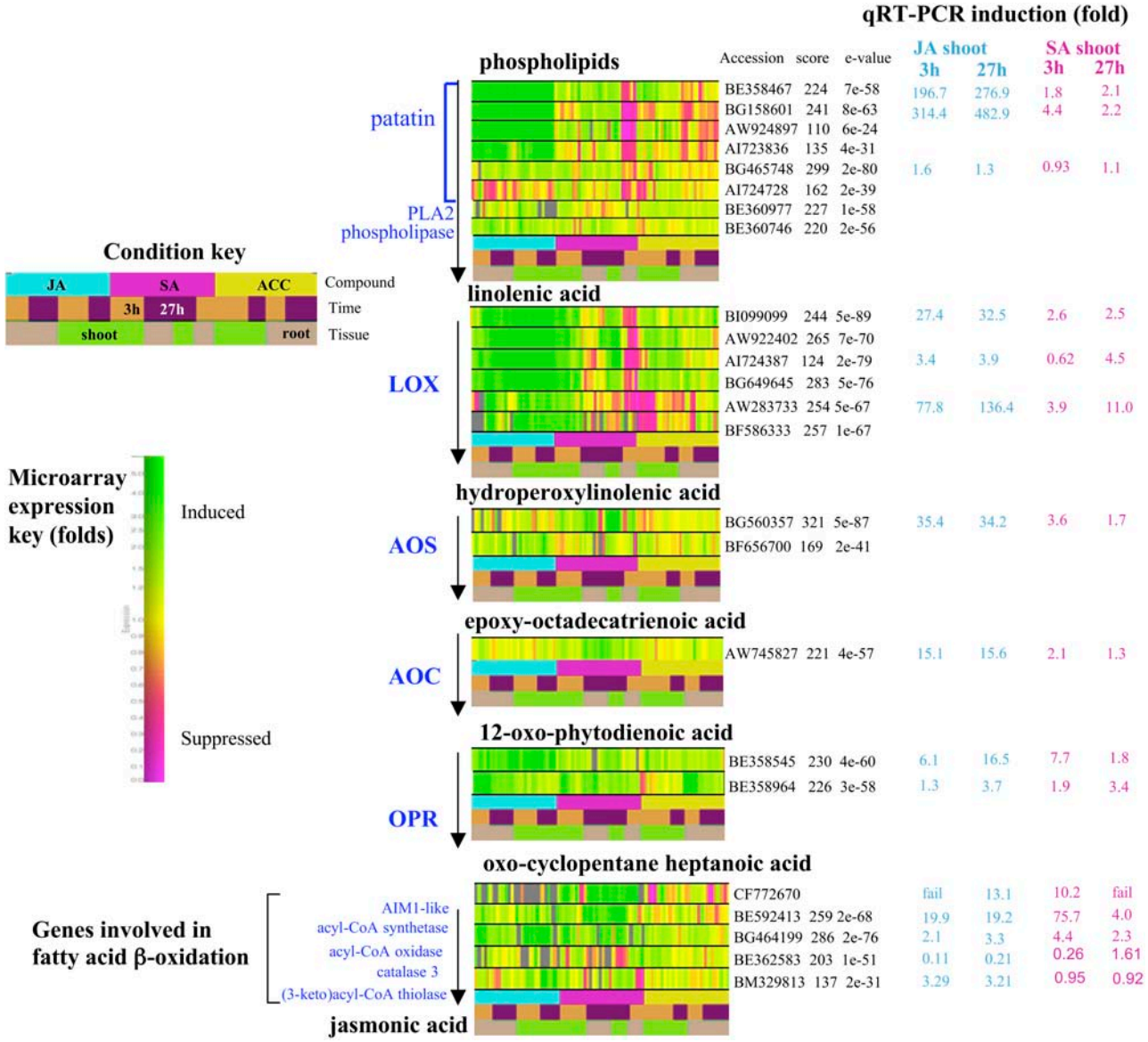

Figure 4. Gene regulation of ODA pathway members by SA, MeJA, and ACC. Shown is the regulation of sorghum genes putatively encoding ODA pathway enzymes and of genes potentially associated with the pathway. The ODA pathway is indicated by arrows, and intermediate products are shown in black. Names of pathway enzymes and associated genes are shown at left in blue: phospholipase A2 (PLA2), LOX, AOS, AOC, and OPR. Mean microarray expression data are shown in heatmap format, as described in Figure 3. Accession numbers and blast scores of sorghum clones putatively in the pathway are shown to right of the color bars. Data from qRT-PCR are shown to the right of the accession number representing each gene.

induced by MeJA and by SA (Fig. 4; Table I, Fatty acid metabolism/JA synthesis), although the clones assayed by qRT-PCR were in some cases more induced by MeJA. Four sorghum LOX homologs were induced by both compounds in shoots, but interestingly, the induction was shoot specific by SA but not MeJA (Fig. 4). Two clones potentially encoding AOS were induced both by MeJA and SA, but one appeared more induced by SA, while the other was more induced by MeJA. A sorghum AOC homolog appeared slightly more induced by SA than by MeJA. Two genes putatively encoding OPRs were each induced by SA and MeJA, though with different kinetics. qRT-PCR showed a homolog of *OPR1* from Arabidopsis was generally more induced by SA than by MeJA, while the reverse was true for a homolog of *OPR3* from tomato, except in 3-h shoots (Table I, Fatty acid metabolism/JA synthesis). Downstream of 12-oxophytodienoic acid reduction, the ODA pathway includes three rounds of β-oxidation. A sorghum homolog of the *AIM1* gene from Arabidopsis, encoding a fatty acid oxidase (Richmond and Bleecker, 1999), an acyl-CoA synthetase, and an acyl-CoA oxidase, were also confirmed by qRT-PCR to be induced by both SA and MeJA. Both of these classes of genes are known to function in fatty acid β-oxidation (Schenk et al., 2003). Microarray results also showed induction by SA and MeJA of a possible catalase 3 and a (3-keto) acyl-CoA thiolase, both of which also carry out fatty acid oxidation. Consistent with the observed induction of most members of the ODA pathway by SA, we also found that increased JA accumulation resulted from the SA treatment, particularly at the 3-h time point (Fig. 5). Also potentially related to oxylipin signaling, a sorghum gene (BI245631) with homology to a JA-regulatory protein from Arabidopsis was strongly upregulated by both SA and MeJA at the 27-h time point, while MeJA specifically, strongly, and rapidly induced transcription of an apparent w-3 fatty acid desaturase homolog (BG048471; Table I, Fatty acid metabolism/JA synthesis), a lipid modification enzyme previously implicated in response to fungal elicitation (Kirsch et al., 1997).

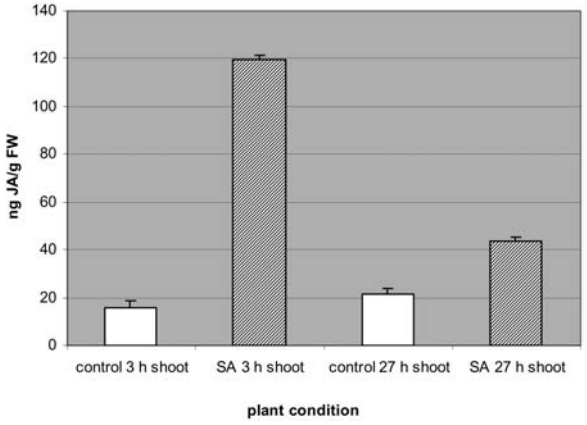

Figure 5. SA induces transient accumulation of JA in sorghum. Endogenous JA levels were measured in shoots of control and 1 mM SA-treated seedlings, at 3- and 27-h time points, respectively. Samples were assayed by gas chromatography-mass spectrometry and relative abundance quantitated using a ^{13}C-JA internal standard. Shown are mean levels from three biological replicates of the experiment. Error bars represent SD.

Secondary Metabolic Pathways Leading to Compounds Involved in Defense

Microarray analysis showed that genes encoding many enzymes in the general phenylpropanoid pathways of phytoalexin, anthocyanin, and lignin synthesis were coordinately induced in response to SA and MeJA (Supplemental Tables I–XXIV). These included Phe ammonia lyase, cinnamate 4-hydroxylase, cinnamyl alcohol dehydrogenase, cinnamoyl-CoA reductase, chalcone synthase, chalcone-flavanone isomerase, flavanone 3-hydroxylase, dihydroflavonal-4-reductase, isoflavone reductase, and leucoanthocyanidin dioxygenase. In many cases, genes apparently encoding multiple isoforms were coregulated. Inductions of a subset of these were confirmed by qRT-PCR (Table I, Phenylpropanoid/secondary compounds). Also regulated were genes potentially involved in terpenoid and alkaloid production. A putative 0-deacetylbaccatin III-10-*O*-acetyl transferase gene (BG158602) potentially involved in taxol synthesis was substantially induced primarily by MeJA and also by SA in 27-h shoot tissue (Table I, Phenylpropanoid/secondary compounds). A putative thiazole biosynthetic enzyme transcript (AW747472) was one of the genes most strongly suppressed in the entire collection by MeJA as well as SA but was essentially unchanged by ACC, as assessed by qRT-PCR (Table I, Phenylpropanoid/secondary compounds). Sorghum is well known to synthesize the cyanogenic glycoside dhurrin in response to insect attack. SA strongly but transiently suppressed several dhurrinase transcripts in 3-h roots, while transcription in shoots was essentially unchanged (Table I, Phenylpropanoid/secondary compounds). By 27 h, root transcript levels were similar to controls, while levels in shoots were about 3-fold suppressed. This contrasted with a 5- to 7-fold induction of dhurrinase transcripts by MeJA within 27 h as assessed by microarray and qRT-PCR.

Genes Potentially Implicated in Defense Signaling/Activation

Regulation of a number of genes encoding putative Leu-rich repeat (LRR) proteins was observed. Particularly interesting were a sorghum LRR, LRP (BG356045; Table I, Defense signaling), which was strongly induced by all three treatments, specifically in 27-h root tissue and a homolog of a nucleotide-binding site-LRR type resistance gene product from rice (*Oryza sativa*; AW680410) that was strongly suppressed by SA and MeJA but moderately induced by ACC, as assessed by qRT-PCR. In total, more than 183 kinases were signif-

icantly regulated by one or more of the three compounds based on microarray analysis, and a subset of these were assayed by qRT-PCR. These included genes with homology to a number of kinases implicated in plant defense, as well as to many kinases with no such known role. A possible homolog of rice *NPK1* (BG933609) was sharply induced by SA as well as MeJA. However, potential homologs of a PTK2 protein Tyr kinase (BE593730) involved in Ca^{2+}-induced regulation of ion channel and mitogen-activated protein kinase functions in humans, and a Pto-like Ser/Thr kinase (BE592414) well known to function as an R-gene in the Pseudomonas/tomato pathosystem were induced most commonly by SA (Table I, Defense signaling), as was a Ser/Thr-specific protein kinase homolog T5F17.120 from Arabidopsis. Two sorghum genes with homology to (respectively) a mitogen-activated protein kinase from parsley (BE598412) and a Ser/Thr kinase-like protein from rice (BM330410) were sharply and quite specifically induced in 27-h shoot tissues by ACC (Table I). A putative choline kinase (BI139762) was primarily induced by MeJA. Genes with homology to several other kinases were primarily suppressed by the treatments (Table I, Defense signaling). A possible homolog (AW677238) of an APETALA2 domain transcriptional regulator centrally involved in defense gene activation was induced by MeJA as well as by SA (Table I, Defense signaling), as were several putative members of the WRKY group of defense-related transcription factors. As assayed by qRT-PCR, a WRKY factor-like gene (CF772823) was robustly induced by both SA and MeJA, while another WRKY-like homolog (BM328251) was more modestly induced with the same pattern. Contrasting this, two EREBP-type transcription factor-like homologs (BG103121 and BG557968) were consistently induced specifically by ACC and were mildly suppressed by SA and MeJA (Table I, Defense signaling). SA-induced increases of a nitrilase-like transcript (BM33120) potentially involved in PR gene regulation (Xu et al., 1994) were observed primarily in leaf by qRT-PCR (Table I, Defense signaling).

Direct-Acting Antimicrobial Effectors

As expected, strong regulation by the compounds was observed among sorghum homologs of members of the major PR gene families, such as PR1, glucanases (PR2), chitinases (PR3), thaumatin-like (PR5), and PR10 genes, as well as a number of genes annotated in GenBank as "pathogenesis-related" but not possessing homology to known PR genes (Table I, Pathogenesis-related). A majority of the pathogenesis-related genes on the array were induced by both SA and MeJA, with many also induced by ACC. Interestingly, however, several glucanases appeared specifically induced by ACC. Analysis by qRT-PCR confirmed this for one of them (BM318308), also verifying a moderate to strong suppression by SA and MeJA treatments (Table I, Defense signaling). A large number of genes putatively encoding protease inhibitors (Bowman-Birk, subtilisin-chymotrypsin, and Cys proteases) were up-regulated on microarrays in response to both SA and MeJA (Table I, Protease/inhibitors; Supplemental Tables I–XXIV). Transcripts with homology to certain proteases were also induced by both SA and MeJA, notably bromelain-like thiol- and an ATP-dependent Clp type (Table I, Protease/inhibitors). Other protease-like genes induced by SA and generally by MeJA included CAAX prenyl proteases, a nucellin-like aspartic protease, a putative Ser protease, chloroplast FtsH protease, and a ubiquitin-specific protease. Numerous genes with homology to the wheat endoxylanase inhibitor XIP1 were substantially up-regulated in response to both SA and MeJA treatments in nearly all tissues and time points, as assessed by both microarray and by qRT-PCR (Table I, Protease/inhibitors). However, specific cDNAs appeared consistently among the most highly (AW747623) or least highly induced (BE353141), respectively. Six sorghum XI-like genes formed a cluster in the two-way ANOVA analysis, demonstrating significantly similar regulation of these genes across the three compound treatments and two tissues (Fig. 3). Among other pathogenesis-related genes present in this cluster were homologs of PR10, chitinase III, and flavanone 3-hydroxylase.

Genes Involved in Regulation of Cellular Redox State

Many genes potentially related to regulation of cellular oxidative status were strongly regulated, as measured by both microarrays and qRT-PCR, under the SA and MeJA treatments. However, the ACC treatment induced relatively few of these, and generally to much lower levels (Table I, Oxidative). One exception to this pattern was a putative peroxidase (CF772269), which was moderately to strongly induced by all three treatments (Table I, Oxidative). The induction of many additional putative peroxidase genes was demonstrated by microarray analysis (Supplemental Tables I–XXIV). We also observed several transcripts homologous to oxalate oxidases and germin, known to promote oxidative defense responses, up-regulated by SA and MeJA on microarrays, although these were not assayed by qRT-PCR. NADPH oxidase is a major known producer of hydrogen peroxide contributing to the oxidative burst (Alvarez et al., 1998). As assayed by qRT-PCR, a putative NADPH oxidase (BE357624) transcript was modestly suppressed by SA in roots but essentially unchanged by any of the compounds in shoots, with the exception of a 7-fold induction in 27-h MeJA shoots. Among other genes involved in regulation of oxidative state, many glutathione *S*-transferase-like transcripts were induced by SA and MeJA within 3 h in both root and shoot tissues, and mRNA levels remained elevated through 27 h. The alternative oxidase AOX2 is a mitochondrial electron transport protein responsible for oxidative respiration and has also been connected with cellular redox status during the hypersensitive response (Ordog et al., 2002). We observed strong in-

duction of a putative AOX2 precursor (BG559720) by SA as well as by MeJA across the tissue time point combinations (Table I, Oxidative).

Genes Not Previously Associated with Major Plant Pathogenesis Defense Pathways

We observed 5- to 9-fold induction of two putative S-like RNase transcripts (BM317859 and CF756248) by microarray analysis as well as by qRT-PCR following SA treatment in the 27-h tissues (Table I, Miscellaneous other function). Interestingly, the same transcripts were induced by MeJA, but induction was specific to the shoot rather than specific to the 27-h time point as in the SA treatment. Microarray and qRT-PCR analyses showed two putative cytidine deaminases were up-regulated by JA and one of them (BI099490) also by SA (Table I, Miscellaneous other function). A sorghum homolog (BM327868) of the Arabidopsis riboflavin biosynthesis enzyme RibA was up-regulated by both SA and MeJA. This induction occurred in both root and shoot tissues and was rapid and transient in response to SA, compared to a slower but sustained increase in response to MeJA (Table I, Miscellaneous other function).

In addition, genes putatively encoding homologs of patatin, several acid phosphatases (one VSP homolog), CG31048 protein from *Drosophila melanogaster*, a selenium-binding protein, hydroxynitrile lyase, putative reverse-transcriptase, cytidine deaminase, and an *O*-methyltransferase ZRP4 were induced by MeJA. On the other hand, MeJA suppressed homologs of a β-glucosidase, putative retroelement, herbicide safener-binding protein, and two hypothetical proteins from yeast and Arabidopsis, respectively (Table I, Miscellaneous other function). Genes outside major groups induced by SA included transcripts putatively encoding β-glucosidase, cytidine deaminase, selenium-binding protein, and dehydrin-like and ripening-related proteins, respectively, while those repressed included phosphoethanolamine (PE) methyltransferase, herbicide safener-binding protein, histone 2B, and the same two hypothetical proteins also repressed by MeJA (Table I, Miscellaneous other function). Genes outside major groups notably induced by ACC encoded a putative No Apical Meristem (NAM) protein and a phosphoglycerate dehydrogenase, while the only gene consistently suppressed by ACC was the apparent PE methyltransferase also suppressed by SA and MeJA (Table I, Miscellaneous other function).

Gene Regulation under SA+MeJA Simultaneous Signaling

qRT-PCR was used to assess the effect of simultaneous SA+MeJA on regulation of genes from three categories: (1) genes induced primarily by MeJA, (2) genes primarily induced by SA, and (3) genes induced by both SA and MeJA individually, and results are presented in Figure 6.

Effect of SA+MeJA on 18 MeJA-Induced Genes

Induction folds of nearly all MeJA-induced genes assessed in shoot tissue at both the 3- and 27-h time points were reduced more than 25% by SA+MeJA. Exceptions to this SA antagonism of JA were genes encoding a putative chalcone flavanone isomerase and a thaumatin isolog at 3-h posttreatment and an S-RNase like protein at 27 h (Fig. 6A). In these four cases, SA+MeJA caused higher expression than MeJA alone, and except for the chalcone-flavanone isomerase, the increases were synergistic (greater than the sum of individual inductions).

Effect of SA+MeJA on 11 SA-Induced Genes

Treatment with SA+MeJA reduced induction of three genes, encoding putative alternative oxidase AOX3 and two small heat shock proteins, after 3 h compared to inductions by SA alone (Fig. 6B). In contrast, five genes were more induced by SA+MeJA than by SA alone, including a putative dehydrin, *PR1*, *EREBP4*, Ser/Thr kinase, and a 101-kD heat shock protein (HSP). Interestingly, at the 27-h time point, all but one of the genes were reduced in induction by SA+MeJA compared to their induction by SA alone. The only exception was the dehydrin-like protein, in which SA+MeJA continued to give increased induction.

Effect of SA+MeJA on 22 Genes Induced by SA as Well as MeJA

The most striking increases in induction by SA+MeJA at the 3-h time point were in genes with homology to a leucoanthocyanidin dioxygenase, Bowman-Birk proteinase inhibitor, and a PR10 (Fig. 6C). At the 27-h time point, genes encoding sorghum homologs of a leucoanthocyanidin dioxygenase, chitinase, protein phosphatase 2C, *NPK1*-related protein kinase, JA-regulatory protein, and a putative xylanase inhibitor were also more induced by SA+MeJA than by either compound alone. On the other hand, SA+MeJA substantially reduced induction levels for seven genes at the 27-h time point compared to induction caused by either compound alone. These putatively encoded sorghum homologs of a glutathione *S*-transferase, alternative oxidase AOX2, cytidine deaminase, PR10, 1-aminocyclopropane-1-carboxylate oxidase, flavanone 3-hydroxylase, and a 12-oxophytodienoate reductase (OPR1).

DISCUSSION

Large-scale transcriptional responses to pathogens and defense compounds have been documented in Arabidopsis (Maleck et al., 2000; Schenk et al., 2000; Van Zhong and Burns, 2003). However, such studies in grasses have not been reported. Further, previous

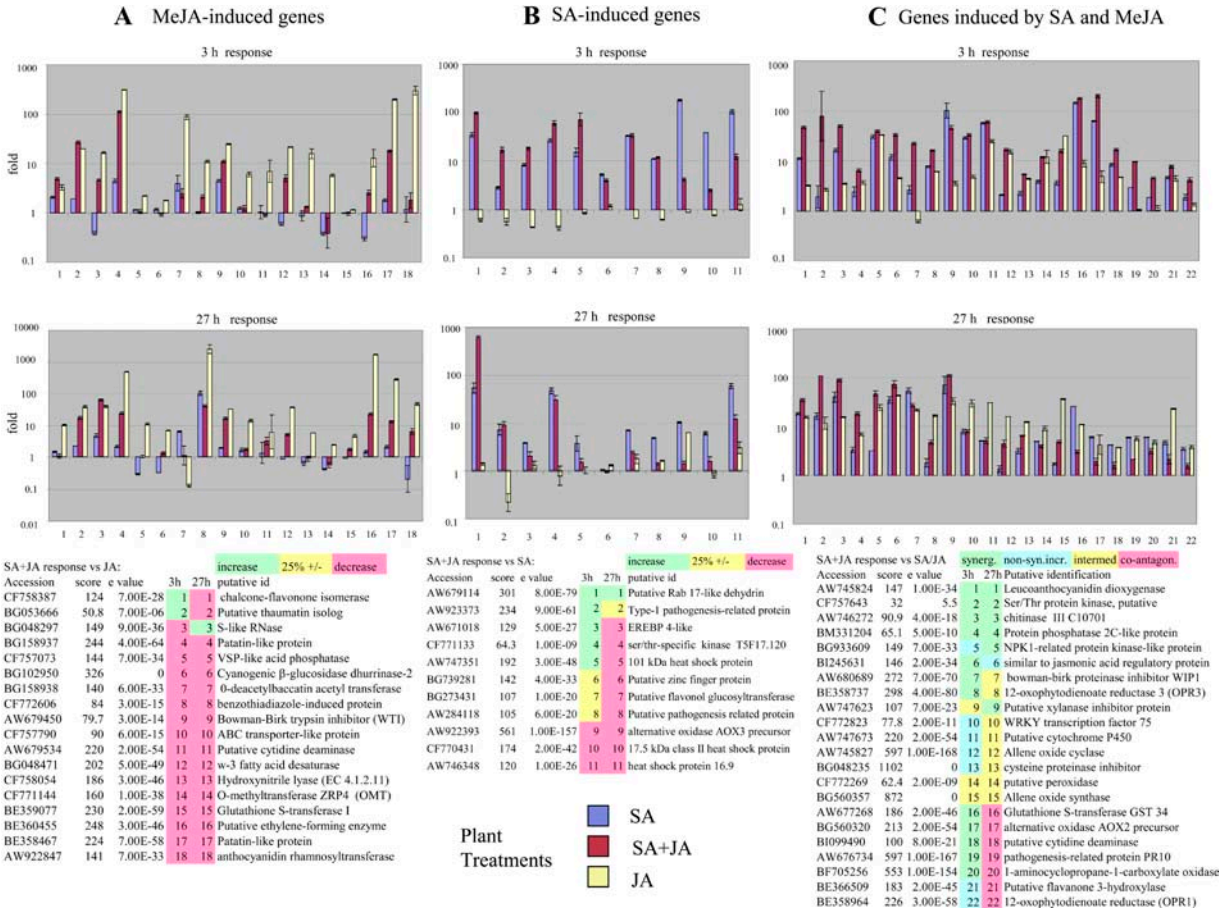

Figure 6. Regulation by simultaneous SA+MeJA of genes primarily induced by MeJA, SA, and by both SA and MeJA individually. Three gene groups were composed representing genes: (1) primarily MeJA induced, (2) primarily SA induced, and (3) induced individually by both MeJA and SA. Pooled cDNAs from three biological replicates of the SA+MeJA experiment were used as template for qRT-PCR determination of mRNA abundance. Shoot tissues were assayed from the 3-h posttreatment (top graph of each section) and 27-h posttreatment (bottom graph of each section) time points. Gene identification numbers used in graphs are shown at the bottom of each section, as are blast scores and e-values. Colored zones beneath each number show gene regulation relative to individual compounds, as indicated above each gene list. Graphs show induction by SA+MeJA of each numbered gene relative to control tissue. Y-axes indicate fold induction (log scale). Error bars represent SD of the inductions, derived as described in ''Materials and Methods.''

studies generally utilized a smaller set of cDNAs than the 12,982 unigenes used here. Thus, it was of interest to create a foundational, publicly accessible resource on sorghum gene expression in response to exogenous SA, MeJA, and ACC. While pathogen infection may be more biologically significant, unraveling the complexities of specific pathogen-induced pathways in sorghum requires knowledge of baseline gene expression patterns from the pure signaling compounds. The responses of sorghum to pathogen infection will be covered in a separate report. Defense genes are well represented in the unigene set, via inclusion of cDNAs from two libraries of pathogen-infected plants, and 12 other cDNA libraries (http://fungen.org/Projects/Sorghum/SorghumUnigeneSet.htm). Due to the ample replication depth of the microarray experiments, a large number of genes showed statistically significant responses to one or more of the treatments, including some potentially representing novel defense functionalities. We focus discussion here upon the response of genes likely involved in synthesis of known defense signals.

SA has been thought to block JA synthesis (Pena-Cortez et al., 1993) and/or action, based on evidence that SA blocked JA induction of proteinase inhibitor genes (Doares et al., 1995) and that JA production and JA-responsive gene regulation after *Pseudomonas syringae* infection was greater in *NahG* expressing Arabidopsis plants than in wild-type plants (Spoel et al., 2003). In contrast to this paradigm, we observed induction of putative homologs of genes composing the entire ODA pathway for JA synthesis by SA as well as MeJA, although inductions by SA were considerably lower than by MeJA at some steps (Fig. 4; Table I, Fatty acid metabolism/JA synthesis). Patatins are lipid acyl hydrolases with high phospholipase A2 activity (Dhondt et al., 2000) and are thus potential producers of lipid signals and/or precursors of OPDA and

initiators of jasmonate synthesis. SA induced several putative patatin and phospholipase homologs by 3 h, consistent with an SA-responsive release of LA that could then promote OPDA and JA synthesis. However, only small inputs of LA are required to generate significant amounts of JA, suggesting that the 200- to 500-fold induction of patatin homologs could also play a more direct role in defense. Beyond lipases, genes potentially involved in all steps of the ODA pathway were also induced by SA as well as by MeJA (Fig. 4). Only OPR3 is efficient for JA synthesis (Schaller et al., 2000). The sorghum putative OPR3 homolog was preferentially induced by SA at the 3-h time point; thus, it appeared that SA stimulation of JA production might be greater at this early point. Measurement of JA content by GC-MS confirmed this (Fig. 5). This also agrees with the early increase in JA observed after infection of Arabidopsis with *P. syringae* Pst/avrRpt2 (Heck et al., 2003), which is known to generate endogenous SA. JA production was found to be higher in SA-degrading *NahG* plants than in wild-type plants 2 d postinfection, and this was taken as evidence that SA suppressed JA production. Nevertheless, increases in JA were observed in wild-type plants after the infection (Spoel et al., 2003), contrary to the idea that SA produced during this interaction should suppress JA synthesis. Further, JA levels were higher in wild type than in the *NahG* line within the first 24 h after infection (Heck et al., 2003), suggesting that the presence of SA provided an early stimulation of JA production. These findings may be reconcilable if SA induction of modest increases in JA is a mechanism to activate a broader range of genes than SA alone, but that continued presence of SA then refines the selection of genes activated by suppressing a subset of JA-induced genes not effective in the particular challenge. For example, three putative proteinase inhibitors assayed by qRT-PCR were all strongly induced by MeJA, while SA induction was similarly high for one, but relatively mild for the other two (Table I, Protease/inhibitors). The isoform induced by both SA and MeJA alone was also synergistically induced by SA+ MeJA at 3 h, while a primarily MeJA-induced isoform was reduced in expression by SA+MeJA (Fig. 6A). Previous studies as well as this work have found large groups of genes coinduced by SA and MeJA (Schenk et al., 2000, 2003). Recent work also suggests that SA/JA coregulation conditions resistance to some pathogens (van Wees et al., 2000; Berrocal-Lobo et al., 2002). The JA precursor OPDA is a potent inducer of defense responses, which are in some cases different than those induced by JA (Fleigmann et al., 2003). The finding that SA greatly increased OPDA production (Laudert and Weiler, 1998) implies that OPDA may be an important component in, or branch of, the SA defense pathway. This is further supported by systemic increases in OPDA seen after SAR induction by *P. syringae* (Landgraf et al., 2002). Thus, a substantial amount of SA-induced ODA pathway activity may exit the pathway as OPDA rather than as JA.

An interesting relationship was also apparent between genes putatively involved in E and JA signaling. A putative JA-regulatory protein gene was found by qRT-PCR to be up-regulated by ACC and SA, while a putative E-forming enzyme cDNA was specifically induced by MeJA, and a homolog of ACC oxidase was induced by MeJA as well as SA (Table I, SA/E/JA specific). The finding that E can induce a putative JA-regulatory gene and vice-versa could bear upon the observed similarity in pathways of downstream gene induction between MeJA and E (Kunkel and Brooks, 2002). JA and E have been suggested to stimulate each other's synthesis through AOS (Laudert and Weiler, 1998).

SA/MeJA Antagonism

For the great majority of the 18 mainly MeJA-induced genes examined here, SA+MeJA gave lower induction than did MeJA alone, as has been reported for other JA-induced genes. However, degrees of antagonism by SA+MeJA varied considerably (Fig. 6A). SA antagonism of gene induction by MeJA operates in the cytoplasm and requires NPR1, a key regulator of PR-gene expression in Arabidopsis (Spoel et al., 2003). Conversely, to SA antagonism of JA responses, MeJA antagonized induction by SA in three of 11 genes at 3 h, and 10 of 11 genes at 27 h, with varying degrees of reduction (Fig. 6). Such antagonisms have been seen as a mechanism evolved to conserve resources by limiting defense responses to genes effective against microbial pathogens versus insects (Zhu-Salzman et al., 2004), or to fine-tune antimicrobial defenses to counter SAR-inducing biotrophic versus JA-inducing necrotrophic pathogens (Thomma et al., 2001). Pathogens may also have evolved the ability to turn SA/JA antagonisms to their own advantage. *P. syringae* uses the JA mimic coronatine to induce the JA pathway, resulting in a reduced PR protein accumulation and increased pathogen susceptibility (Zhao et al., 2003).

SA/MeJA Cooperative Regulation

Synergistic gene regulation by SA and MeJA has previously been reported for PR1b (Xu et al., 1994). We observed such synergism for genes from several groups including PR proteins, enzymes of the ODA and phenylpropanoid pathways, oxidative regulation, other defense-associated proteins, and genes with diverse functions (Fig. 6). Overall, synergistic induction was the most common response to SA+MeJA among the 22 genes examined here that were induced by both SA and MeJA alone. This may indicate the copresence of elements uniquely responsive to the SA and JA pathways (respectively) on the promoters of these genes, providing additional transcriptional capacity through the second compound. Alternatively, certain elements may be activated by both pathways (Spoel et al., 2003), and the presence of SA+MeJA may

have heightened transcription. Enhanced resistance to pathogens is a logical outcome of cooperative induction by SA and JA, and evidence of this has been observed (van Wees et al., 2000; Berrocal-Lobo et al., 2002). A further, novel SA/MeJA coantagonism was demonstrated here, indicating bidirectional suppressions between SA and MeJA may operate simultaneously on certain genes. This suggests two or more independent molecular switches control flux through the SA and JA pathways. Apparently, both pathways can be switched off (coantagonism) or on (synergism) simultaneously. In Arabidopsis, *ssi1* appears to define one of these switches between the SA and JA pathways (Shah et al., 1999), and within the E/JA pathway AtMYC2 (*JAI1/JIN1*) and ERF may constitute two others (Lorenzo et al., 2004).

Beyond broadening the number and functional categories of genes displaying antagonisms and synergisms by SA and MeJA, our results also showed a substantial difference in regulation patterns between 3 and 27 h. This may reflect a period of adjustment to the presence of both signals. The finding that markedly more antagonisms were present at the later point may indicate that the implementation of gene repression itself requires gene expression or time to otherwise activate the process. The differential responses to SA+MeJA also provide information that could be important for further work in identifying promoter elements responsive to these defense compounds.

MATERIALS AND METHODS

Full MIAME compliant descriptions of methods connected with production of sample material, sample tracking, and microarray experiments are available at http://fungen.org/sorghum.htm.

Hydroponic Plant Growth and Treatments

Sorghum (*Sorghum bicolor*) L. Moench cv BTx623 seeds were surface sterilized with 30% bleach, rinsed three times with excess distilled water, and germinated in moist paper for 3 d, then transferred to aerated hydroponic growth buckets (35–40 seedlings per bucket) containing 0.5× Hoagland solution. Seedlings were grown in growth chambers under 12-h photoperiod with incandescent and fluorescent light (750 μmol s^{-1} m^{-2}) at 32°C (day)/22°C (night) and constant 50% humidity. Eight days after imbibition, three buckets of seedlings were treated with each compound, and three separate control buckets were similarly manipulated but without addition of the compound. Treatments with SA (Sigma, St. Louis), MeJA (Bedoukian Research, Danbury, CT), and the E precursor ACC (ACPC, Sigma) were carried out as follows. Stock solutions of either SA, MeJA, ACC, or SA+MeJA were added to nutrient solutions of hydroponically grown, 8-d-old sorghum seedlings, resulting in final concentrations of 1 mM SA, 100 μM MeJA, 0.5 mM ACC, and 1 mM SA/100 μM MeJA. ACC was used rather than gaseous E to avoid the potential collateral effects of sealing plants in a closed vessel. ACC and MeJA treatments of seedlings were each conducted in separate growth chambers from the respective control or from other treatments to avoid potential effects of volatile E or MeJA on other plants. For all treatments, the nutrient solution was refreshed once, 3 d prior to addition of the compounds. Plants were grown under conditions described above, before, during, and after the treatment. Roots and shoots of control and treated plants were harvested at 3 and 27 h, respectively. Tissues were frozen in liquid nitrogen upon harvest.

Experimental Design and Replication

Microarrays were hybridized with probes from a total of 12 plant conditions, comprised of three defense compounds × two harvest time points × two tissue types (Fig. 1). Each of these individual conditions was represented by four to six biological replicates (two separately conducted experiments × two–three hydroponic buckets in each experiment). Microarray probes were prepared from control and treated RNAs extracted from each of these four to six replicates and were each used to query two individual microarray slides. This resulted in the following numbers of slides assayed: for SA, 3-h shoot, 10; 27-h shoot, 9; 3-h root, 9; 27-h root, 6; for ACC, 3-h shoot, 10; 27-h shoot, 10; 3-h root, 9; 27-h root, 8; and for JA, 3-h shoot, 9; 27-h shoot, 8; 3-h root, 8; 27-h root, 6.

RNA Extraction and Microarray Probe Preparation

From each individual condition, frozen tissues from 10 to 12 seedlings were ground under liquid nitrogen with a mortar and pestle. RNA was extracted from 200 mg of tissue powder per sample, using a Trizol-based RNA extraction method (Molecular Research Center, Cincinnati). cDNAs were prepared from 40 μg of total RNA per sample using Superscript II reverse transcriptase (Promega, Madison, WI), and microarray probes labeled with Cy3 and Cy5 were prepared from the cDNA using the Genisphere 3DNA microarray labeling system according to manufacturer's instructions (Genisphere, Montvale, NJ). The cDNA equivalent of 8 μg input RNA each from control and treated plants (respectively) were used for each slide hybridization. Slides were hybridized as described previously (Zhu-Salzman et al., 2004) and scanned with a Scanarray 5000 (Packard Bioscience, Billerica, MA) using the line scan function to balance signals.

Preparation of Microarrays

A total of 117,682 sequenced sorghum ESTs were clustered into 16,801 contigs based on 3′ sequence, referred to as Milestone version 1 (M.-M. Cordonnier-Pratt, personal communication). From the 16,801 contigs, 12,982 cDNAs were successfully amplified by PCR as judged by agarose gel electrophoresis (data not shown). Of 768 randomly chosen clones, all were confirmed accurate by resequencing. Thus, each feature on the microarrays used here represents a singleton or cluster of two or more members, and the resulting microarray is expected to represent approximately 25% of the sorghum gene based on the prediction of approximately 50,000 genes in diploid grass genomes such as rice and sorghum (Goff et al., 2002). The results of this clustering can be explored at http://fungen.org/sorghum.htm using MAGIC Gene Discovery Viewer, which is a Java program that can be downloaded from that page (Cordonnier-Pratt et al., 2004). Clones were cherry picked into 384-well plates from the plasmid preparations from which EST sequences were obtained using a Biomek 2000 and the associated Hit Picking wizard (Beckman-Coulter, Fullerton, CA). cDNA inserts were amplified in 96-well format with universal PCR primers in 150-μL reactions using chimney plates (USA Scientific, Ocala, FL). PCR products were purified with Montage PCR filter plates (Millipore, Billerica, MA) recovered in 60 μL of water and reassembled into 384-well plates. All liquid transfers from 384- to 96-well format and back were done with a Hydra96 (Maxtrix Technologies, Hudson, NH). Samples were lyophilized, redissolved in 30 μL of water, and divided into two equal aliquots. One was lyophilized and saved for future use. To the other, 15 μL of ArrayIt Spotting Buffer (Telechem, Sunnyvale, CA) was added. These prepared samples were spotted onto UltraGAPS microarray slides with bar codes (Corning, Corning, NY) using ArrayIt Stealth SMP3 microarray pins (Telechem, Sunnyvale, CA) and an OmniGrid 100 (Genomic Solutions, Ann Arbor, MI). All information pertaining to sample selection and tracking was parsed into the MAGIC Database (Cordonnier-Pratt et al., 2004), which was in turn used to identify features on the arrays. In addition, after recovering samples from the PCR filter plates, they were evaluated by agarose gel electrophoresis, with all observations entered into the same database.

Data Analysis

Scanned images were analyzed using the Digital Genome program (MolecularWare, Cambridge, MA) to locate spots and quantitate signal and background levels. After applying a low signal cutoff (if mean signal intensity <2× mean background intensity, then mean signal intensity made equal to mean background intensity), mean signal intensity and mean background

intensity data were input into Genespring 6.1 (Agilent Technologies, Redwood City, CA). Microarray data from all biological and slide replicates within each of the conditions were pooled and normalized using the Genespring Lowess algorithm. Results for each condition were then filtered by expression to eliminate genes with mean fold changes of less than 1.5-fold up or down and further filtered by confidence (one sample Student's *t* test *P*-value, using Benjamini and Hochberg F.D.R. multiple testing correction), to retain only genes in which expression changes versus the untreated control were significant at $P \leq 0.05$. The resulting 12 individual condition gene lists were then compiled into a single nonredundant gene list of 6,438 genes. This gene list was then queried with each condition experiment to generate the 12 microarray base expression datasets. To develop lists of candidate genes for qRT-PCR validation, these base datasets for each condition were sorted by predicted biological function (based on the top BLASTX hit with a described function) using a custom ontology filter set to develop functional clusters and then the clusters sorted based on fold changes in expression. Genes remaining unclassified were also sorted by fold changes in expression. Clones were selected for qRT-PCR validation from these lists based on high regulation, likely biological relevance, and low SDs. These base datasets as well as all the raw expression data are included in Supplemental Tables I to XXIV. Correlation coefficients were generated across slide replicates within each condition using the Genespring program (Agilent Technologies) by 1-distance value from the hierarchical condition clustering tree. TIF images from the microarrays, as well as MIAME-compliant uniform experimental data are available from the University of Georgia at http://fungen.org/sorghum.htm.

Quantitative RT-PCR

RNAs from the same extracts used for microarray experiments were pooled for each of the 12 (compound × tissue × time) conditions in proportion to their representation among the microarray experiments. RNA pools for each of the 12 conditions were converted to cDNA template for qRT-PCR using random hexamer primers and Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA). All qRT-PCR reactions representing a given condition used template from the same original large-scale cDNA synthesis reaction. qRT PCR was performed in duplicate 10-μL reactions for each clone of interest using Sybr Green mastermix (Applied Biosystems) for the main reactions and TAQMAN Universal PCR mastermix (Applied Biosystems) with VIC probe labeling for ribosomal control reactions. No-template control reactions using untranscribed RNA controls confirmed that no interfering products derived from genomic DNA were present. Primers for amplifying genes of interest were designed using Primer Express (Applied Biosystems). Amplification specificity was determined by dissociation curve analysis. Mean induction folds were calculated as $2^{\wedge}$ ($\Delta\Delta$CT), and SD range of replicate reactions was calculated by: upper error bar = $2^{\wedge}$ ($\Delta\Delta$CT + s), lower error bar = $2^{\wedge}$ ($\Delta\Delta$CT − s), where:

$$\Delta\Delta CT = (\Delta CT_{\text{control cDNA}}) - (\Delta CT_{\text{treatment cDNA}})$$

$$\Delta CT = (\text{mean CT cDNA}_{\text{test primers}}) - (\text{mean CT cDNA}_{\text{ribosomal primers}})$$

$$S = \sqrt{((\text{SD of CT}_{\text{test primers}}{}^2) + (\text{SD of CT}_{\text{ribosomal primers}}{}^2))}.$$

Annotation of ESTs

Microarray features cited are identified by the GenBank 3′ accession number. Full annotation information including 3′ and 5′sequences, database clone names, BLASTX identifications and match quality, 3′ and 5′expect values, and match lengths are viewable at http://fungen.org/sorghum.htm, accessed by clone name. The total number of element positions on the microarray was 17,733. Of these, 12,983 elements were confirmed by gel electrophoresis to contain a single PCR band. In Supplemental Table IV are annotation data for the full 17,733 element positions on the microarray, including the following information for both 3′ and (when available) 5′ sequences: clone name, PIRnref ID, GenBank ID, BLASTX identifications, expect values, and BLASTX scores. Provisional electronic annotation of ESTs was obtained by BLASTX (Altschul et al., 1990, 1997) against full-coding-length entries from the PIR-NREF database (Wu et al., 2002). Blast scores and e-values of genes given functional consideration in this paper are shown in the figures and tables. Only genes with blast scores of >60 and e-values of $<e^{-}10$ are considered potential homologs of genes with known function for discussion purposes.

Determination of JA Content

JA was measured using isotope dilution selective ion monitoring-gas chromatography-mass spectrometry. Samples (100 mg) of powdered shoot tissues from control and 1 mM SA-treated plants (one sample each from three biological replicates) were added to 0.8 mL of MeOH with the inclusion of 50 ng 1,3-[^{13}C]-JA internal standard (Creelman and Mullet, 1995) per sample. Samples were extracted by shaking at 250 rpm, 50°C for 30 m, centrifuged 5 m, and supernatants removed to a collection tube. The tissues were then sequentially reextracted with 0.8 mL of 1:1 (v:v) MeOH:acetone and with 0.8 mL of acetone, respectively. The pooled extracts were dried and soluble components redissolved in 240 μL of 30% MeOH:70% 0.1 N acetic acid by vortex mixing. The resulting extracts were filtered through 0.2-μM syringe filters and 180 μL was applied to a Phenomenex μBondapak C-18 column (300 × 3 mm; Phenomenex, Torrance, CA). Constituents were separated on a linear gradient from 30% to 100% MeOH in 0.1 N acetic acid over 13.5 m at 1.6 mL per minute. Fractions were collected based on elution times previously determined with authentic JA standard. Eluted fractions were dried and methylated with 40 μL of ethereal diazomethane twice, dried, and resuspended in ethyl acetate. Samples were chromatographed on a HP5890 GC with a 15-m × 0.32-mm DB-5 column (J&W Scientific, Folsom, CA) with a linear ramp from 60°C to 240°C over 6 m. Ions 193 and 224 (endogenous) and 195 and 226 (internal standard) were quantified with a HP5970B mass spectrometer in selected ion monitoring mode. The endogenous level of JA was calculated based on the ratio of the peak areas of ions 224 and 226.

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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