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Signal Signature and Transcriptome Changes of *Arabidopsis* During Pathogen and Insect Attack

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Plant defenses against pathogens and insects are regulated differentially by cross-communicating signaling pathways in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play key roles. To understand how plants integrate pathogen- and insect-induced signals into specific defense responses, we monitored the dynamics of SA, JA, and ET signaling in *Arabidopsis* after attack by a set of microbial pathogens and herbivorous insects with different modes of attack. *Arabidopsis* plants were exposed to a pathogenic leaf bacterium (*Pseudomonas syringae* pv. *tomato*), a pathogenic leaf fungus (*Alternaria brassicicola*), tissue-chewing caterpillars (*Pieris rapae*), cell-content-feeding thrips (*Frankliniella occidentalis*), or phloem-feeding aphids (*Myzus persicae*). Monitoring the signal signature in each plant-attacker combination showed that the kinetics of SA, JA, and ET production varies greatly in both quantity and timing. Analysis of global gene expression profiles demonstrated that the signal signature characteristic of each *Arabidopsis*-attacker combination is orchestrated into a surprisingly complex set of transcriptional alterations in which, in all cases, stress-related genes are overrepresented. Comparison of the transcript profiles revealed that consistent changes induced by pathogens and insects with very different modes of attack can show considerable overlap. Of all consistent changes induced by *A. brassicicola*, *Pieris rapae*, and *F. occidentalis*, more than 50% also were induced consistently by *P. syringae.* Notably, although these four attackers all stimulated JA biosynthesis, the majority of the changes in JA-responsive gene expression were attacker specific. All together, our study shows that SA, JA, and ET play a primary role in the orchestration of the plant's defense response, but other regulatory mechanisms, such as pathway cross-talk or additional attacker-induced signals, eventually shape the highly complex attacker-specific defense response.

Additional keywords: innate immunity, microarray, plant defense responses.

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The data sets of all ATH1 hybridizations are deposited at NASCarrays of the Nottingham Arabidopsis Stock Centre.

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Plants are abundantly present on earth and are at the basis of almost all food webs. Each of the approximately 300,000 plant species is attacked by a multitude of other organisms, such as insects and pathogens. The number of insect species is estimated to be in the order of 6 million, 50% of which are herbivorous (Schoonhoven et al. 1998). The biodiversity of pathogenic microorganisms is less well characterized but it is general knowledge that plant pathogens are a common threat to plants. To effectively combat invasion by microbial pathogens and herbivorous insects, plants have evolved sophisticated defensive strategies to "perceive" attack by pathogens and insects, and to translate this "perception" into an appropriate defensive response (Dangl and Jones 2001; Dicke and Hilker 2003; Pieterse and Van Loon 2004). These induced defense responses are regulated by a network of interconnecting signal transduction pathways in which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play key roles (Dicke and Van Poecke 2002; Glazebrook 2001; Pieterse and Van Loon 1999; Reymond and Farmer 1998; Thomma et al. 2001). SA, JA, and ET accumulate in response to pathogen infection or damage caused by insect feeding, resulting in the activation of distinct sets of defense-related genes (Glazebrook et al. 2003; Reymond et al. 2004; Schenk et al. 2000). Compelling evidence for the significance of SA, JA, and ET in plant defense came from studies using mutant and transgenic plants affected in either SA, JA, or ET signaling (Pieterse et al. 2001; Pozo et al. 2005). For instance, SA-defective signaling mutants and transgenics are often more susceptible to pathogen infection than wild-type plants (Delaney et al. 1994; Nawrath and Métraux 1999; Wildermuth et al. 2001). Blocking the response to JA generally renders plants more susceptible to herbivorous insects (Howe et al. 1996; Kessler et al. 2004; McConn et al. 1997), although enhanced susceptibility toward necrotrophic pathogens has been

reported as well (Staswick et al. 1998; Thomma et al. 1998). Furthermore, analysis of mutants affected in ET signaling demonstrated that ET plays a modulating role in many plant defense responses (Hoffman et al. 1999; Knoester et al. 1998; Lund et al. 1998).

Although the importance of SA, JA, and ET in induced plant defense is clear, evidence is accumulating that their signaling pathways cross-communicate (Dicke and Van Poecke 2002; Felton and Korth 2000; Feys and Parker 2000; Kunkel and Brooks 2002; Pieterse and Van Loon 1999; Reymond and Farmer 1998; Rojo et al. 2003). For instance, activation of SA-dependent systemic acquired resistance (SAR) has been shown to suppress JA signaling in plants, thereby prioritizing SA-dependent resistance to microbial pathogens over JA-dependent defense that is, in general, more effective against insect herbivory (Felton and Korth 2000; Stout et al. 1999; Thaler et al. 2002b; Thaler et al. 1999). Pharmacological and genetic experiments have indicated that SA-mediated suppression of JA-inducible gene expression plays an important role in this process (Glazebrook et al. 2003; Peña-Cortés et al. 1993; Van Wees et al. 1999), and sometimes can work in both directions (Glazebrook et al. 2003; Niki et al. 1998). The antagonistic effect of SA on JA signaling recently was shown to be controlled by a novel function of the defense regulatory protein NPR1 in the cytosol (Pieterse and Van Loon 2004; Spoel et al. 2003). Cross-talk between defense signaling pathways is thought to provide the plant with a powerful regulatory potential, which helps the plant to "decide" which defensive strategy to follow, depending on the type of attacker it is encountering. Yet, it also may allow attackers to manipulate plants to their own benefit by shutting down induced defense through influences on the signaling network (Kahl et al. 2000).

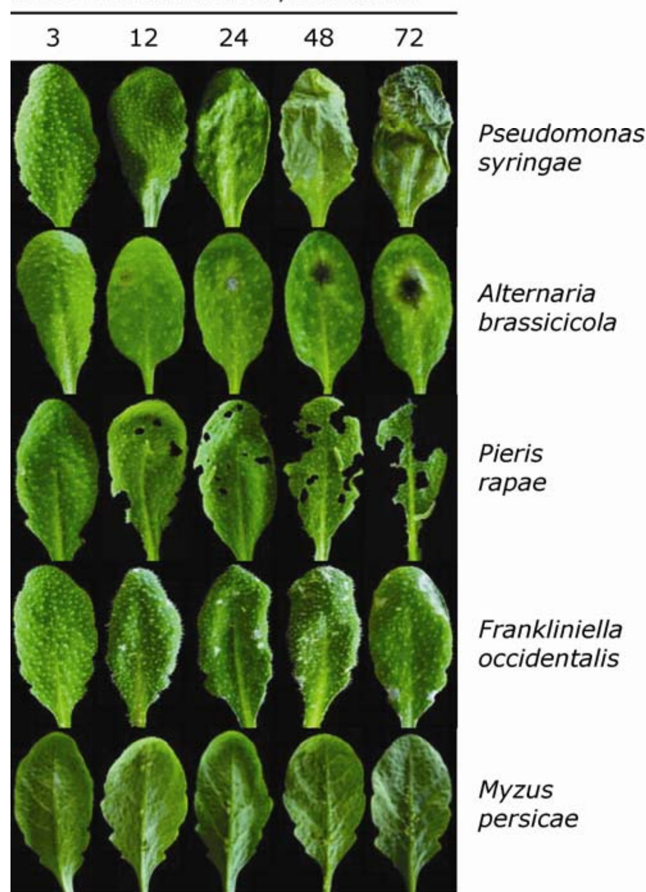

Fig. 1. Symptom development in *Arabidopsis* upon pathogen and insect attack. Symptom development on *Arabidopsis* leaves at different time points after inoculation or infestation with the necrotizing bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000(*avrRpt2*), the necrotrophic fungal leaf pathogen *Alternaria brassicicola*, tissue-chewing caterpillars of the cabbage white butterfly (*Pieris rapae*), cell-content-feeding larvae of the Western flower thrips (*Frankliniella occidentalis*), or phloem-sucking green peach aphids (*Myzus persicae*).

In order to study the role of pathway cross-talk in plant innate immunity, it is important to have insight into the dynamics of SA, JA, and ET signaling during different plant-attacker combinations. The role of SA, JA, and ET in plant defense has been studied for several plant-microbe and plant-insect interactions (Dicke and Van Poecke 2002; Glazebrook 2001; Pieterse et al. 2001). However, most of these studies have been performed in different plant species, often using single plant-microbe or plant-insect combinations. Moreover, the large variation in experimental conditions in these studies makes it difficult to integrate the results and draw overall conclusions. Therefore, we monitored the dynamics of SA, JA, and ET signaling in a single plant species (*Arabidopsis thaliana*) in response to attack by a range of microbial pathogens and herbivorous insects with very different modes of action. To relate our findings to those by others, we investigated the response of *Arabidopsis* to the well-characterized microbial pathogens *Pseudomonas syringae* pv. *tomato* and *Alternaria brassicicola* and the herbivorous insects *Pieris rapae, Myzus persicae,* and *Frankliniella occidentalis*. The production of SA, JA, and ET was monitored during these five *Arabidopsis*-attacker interactions, and related to global gene expression profiles using Affymetrix ATH1 whole-genome GeneChips.

RESULTS

Arabidopsis pathogens and insects.

Arabidopsis has been proven to be an excellent model for studying a wide variety of plant-pathogen and plant-insect interactions (Kunkel 1996; Van Poecke and Dicke 2004). To study the dynamics of the response of *Arabidopsis* to different microbial pathogens and herbivorous insects simultaneously, we chose two well-characterized *Arabidopsis*-pathogen interactions and three *Arabidopsis*-insect interactions in which the attackers deploy very different modes of attack.

P. syringae is a bacterial leaf pathogen that causes extensive chlorosis and necrotic spots (Whalen et al. 1991). Analyses of *Arabidopsis* signaling mutants have shown that basal resistance to this pathogen is predominantly dependent on SA (Delaney et al. 1994; Nawrath and Métraux 1999; Wildermuth et al. 2001), although components of the JA and ET signaling pathways have been demonstrated to contribute to resistance against this pathogen as well (Ellis et al. 2002; Pieterse et al. 1998). The transcriptome of *Arabidopsis* in response to *P. syringae* pv. *maculicola* infection has been well-studied (Glazebrook et al. 2003). Recently, Tao and associates (2003) provided evidence that a large part of the differences in transcriptional changes between the compatible and incompatible interactions is quantitative. Therefore, to induce a strong response in the plant, we chose to use avirulent *P. syringae* pv. *tomato* DC3000, carrying the avirulence gene *avrRpt2*. Pressure infiltration of whole *Arabidopsis* leaves with *P. syringae* pv. *tomato* DC3000(*avrRpt2*) resulted in collapse of the leaf tissue within the first 48 h after inoculation, which is typical for this incompatible interaction (Fig. 1).

A. brassicicola is a necrotrophic fungal pathogen that provokes spreading necrotic lesions on leaves. In contrast to basal resistance against *P. syringae*, SA is not required for defense against this pathogen, because *Arabidopsis* genotypes impaired in SA accumulation retain the strong level of resistance that is

characteristic for the wild-type Col-0 plants (Thomma et al. 1998; Van Wees et al. 2003). Basal resistance against *A. brassicicola* is compromised in the phytoalexin-deficient mutant *pad3* and the JA-response mutant *coi1*, indicating that the *Arabidopsis* phytoalexin camalexin and JA signaling are required for defense against *A. brassicicola* (Thomma et al. 1998, 1999). In our comparative study, we used the *pad3* mutant as the susceptible host for studying a compatible *Arabidopsis–A. brassicicola* interaction. After inoculation with *A. brassicicola*, necrotic lesions developed gradually to a size that spanned half the width of the leaf 3 days after inoculation (Fig. 1).

Tissue-chewing caterpillars of the cabbage white butterfly (*Pieris rapae*) are specialists on cruciferous plant species (Van Loon et al. 2000). Defense against caterpillar feeding in plants has been suggested to be mainly regulated by JA-dependent defense responses (Kessler and Baldwin 2002; Van Poecke and Dicke 2002). In *Arabidopsis*, *Pieris rapae* feeding has been shown to induce expression of JA-responsive genes (Reymond et al. 2000, 2004) and to induce direct and indirect defenses that involve SA, JA, and ET (Reymond et al. 2004; Stotz et al. 2000, 2002; Van Poecke and Dicke 2004; Van Poecke et al. 2001). Moreover, tomato plants affected in JA production or perception are more susceptible to caterpillar feeding than wild-type plants (Howe et al. 1996; Thaler et al. 2002a). In this study, first-instar larvae of *Pieris rapae* immediately started to feed when they were placed onto the leaf tissue. Caterpillar feeding caused a severe, progressing damage to the leaf tissues (Fig. 1).

Western flower thrips (*F. occidentalis*) cause extensive damage on many plant species, including *Arabidopsis* (Yudin et al. 1986). Thrips are cell-content-feeding insects that penetrate single cells with a stylet to suck out the contents (Kindt et al. 2003). JA plays an important role in defense against cell-content-feeding herbivores. Tomato mutant *def1*, compromised in JA signaling, shows enhanced susceptibility to thrips feeding. Moreover, overexpression of JA-inducible prosystemin, a signal peptide involved in the wound-induced expression of protease inhibitors (PIs), resulted in plants highly resistant to thrips damage (Li et al. 2002). *Arabidopsis* leaves infested with *F. occidentalis* displayed white chlorotic spots, so-called silver scars, which were located mainly at the leaf edges. During the course of the experiments, the symptoms became more severe (Fig. 1).

Green peach aphids (*M. persicae*) are generalists that feed on the plant's phloem sap using a sucking mode of action. The aphids carefully maneuver their stylets around the epidermal and mesophyl cells before inserting them into the phloem, thereby inflicting minimal wounding to the plant (Tjallingii and Hogen Esch 1993). *M. persicae* feeding has been shown to induce the expression of both SA- and JA-responsive genes (Moran and Thompson 2001), suggesting a role for both signals in defense against aphid feeding. Ellis and associates (2002) demonstrated that *M. persicae* population development is reduced on *Arabidopsis* mutant *cev1,* which constitutively expresses JA-responsive genes. Moreover, aphid population development was much faster on the JA-insensitive mutant *coi1*, indicating that JA plays an important role in defense against *M. persicae* (Ellis et al. 2002; Moran and Thompson 2001). In our study, *M. persicae* was allowed to feed for 72 h. During this 72-h time course, the aphids fed predominantly on the main vein at the abaxial side of the *Arabidopsis* leaves without causing any visible symptoms (Fig. 1).

Signal signature.

To investigate the dynamics of SA, JA, and ET production during the different *Arabidopsis*-attacker combinations, we monitored the production of these signals after pathogen and insect attack. Because the progress of disease or damage caused by the pathogens and the insects differed among the *Arabidopsis*-attacker combinations (Fig. 1), the time points for tissue harvest were selected from early to late stages of infection or infestation and, thus, are not always identical for each *Arabidopsis*-attacker combination. For SA and JA measurements, leaf tissue from 20 plants per plant-attacker combination and untreated controls were harvested at each time point and immediately frozen in liquid nitrogen. For ET determinations, 10 plants per plant-attacker combination were placed in gas-tight vials immediately after pathogen inoculation or insect infestation. The production of SA, JA, and ET during the first 72 h after pathogen or insect attack is shown in Figure 2. *P. syringae* infection induced a strong increase in the production of all three signal molecules. JA production was detectable as early as 3 h after inoculation, whereas SA and ET levels were increased significantly from 12 h onward. Similar to the *Arabidopsis–P. syringae* interaction, inoculation of *Arabidopsis* with *A. brassicicola* resulted in a strong increase in JA and ET production. Enhanced JA levels were detectable at 3 h after inoculation, whereas ET levels started to increase between 12 and 24 h postinoculation. *A. brassicicola* did not induce an increase in SA levels.

None of the insects induced a detectable increase in SA accumulation (Fig. 2). Moreover, the magnitude of JA and ET production was much lower in response to insect infestation than during pathogen attack. However, this may be due to the fact that the number of cells contributing to the defense response upon pathogen infection is higher than that upon insect infestation. Feeding by tissue-chewing caterpillars of *Pieris rapae* induced a modest, but significant increase in ET production and a clear increase in JA production. Cell-content-feeding larvae of the Western flower thrips *F. occidentalis* also induced an increase in JA biosynthesis, whereas ET levels remained unchanged. No changes in the production of JA or ET were detectable in response to infestation of *Arabidopsis* with phloem-sucking *M. persicae* aphids.

Together, these results demonstrate that the accumulation patterns of SA, JA, and ET differ highly in composition, magnitude, and timing during the different plant-pathogen and plant-insect combinations. The combined patterns of SA, JA, and ET production subsequently will be referred to as the signal signature.

Attacker-induced marker gene expression.

To investigate in how far the specific patterns of defense signal production during each plant-attacker combination correspond with a coordinate activation of SA-, JA-, or ET-responsive genes, we first analyzed the expression of the well-characterized marker genes *PR-1* (SA responsive), *VSP2* (JA responsive), *PDF1.2* (JA and ET responsive), and *HEL* (ET responsive). To be able to correlate the signal signatures with the gene expression patterns, RNA was isolated from the same leaf samples as those used for the SA and JA determinations. *P. syringae* induced the expression of all the SA-, JA-, and ET-responsive marker genes, whereas *A. brassicicola* triggered only the JA- and ET-responsive marker genes *PDF1.2* and *HEL* (Fig. 3) Furthermore, *Pieris rapae* and *F. occidentalis* induced the JA-responsive marker genes *VSP2* and *PDF1.2*, respectively. No clear accumulation of any marker gene transcripts could be detected in *M. persicae*-infested plants.

Because aphids damage only a small number of cells while probing for feeding sites, we made use of the transgenic *Arabidopsis* Col-0 lines *PDF1.2:GUS* and *PR-1:GUS* to examine local aphid-induced marker gene expression in more detail. The *PDF1.2:GUS* and *PR-1:GUS* lines contain a translational fusion

of the *uidA* reporter gene with the JA- and ET-responsive promoter of the *PDF1.2* gene, and the SA-responsive promoter of the *PR-1* gene, respectively. No β-glucuronidase (GUS) activity was detected in *PDF1.2:GUS* plants in response to *M. persicae* feeding. In contrast, aphid feeding strongly induced expression of the SA-responsive *PR-1* promoter in the cells surrounding the feeding sites on the main vein (Fig. 4).

To similarly investigate local effects of thrips and caterpillar feeding on *PR-1* and *PDF1.2* marker gene expression, GUS activity also was assessed in *F. occidentalis*- and *Pieris rapae*-infested *PR-1:GUS* and *PDF1.2:GUS* plants. Thrips feeding locally activated the *PR-1* promoter to a moderate level (Fig. 4), which apparently was too low to be detected in the RNA isolated from whole rosettes (Fig. 3). Damage caused by caterpillar feeding had no effect on GUS activity in *PR-1:GUS* plants. Both *F. occidentalis* and *Pieris rapae* induced the expression of the *PDF1.2* promoter around the feeding site. The latter was not detected in the RNA from whole rosettes of *Pieris rapae*-infested plants (Fig. 3).

These results indicate that the expression patterns of the marker genes correlate only to a limited extent with the accumulation patterns of the signaling compounds themselves. For instance, JA production in *P. syringae*-infected plants was detectable earlier and to a fivefold higher level than in *Pieris*

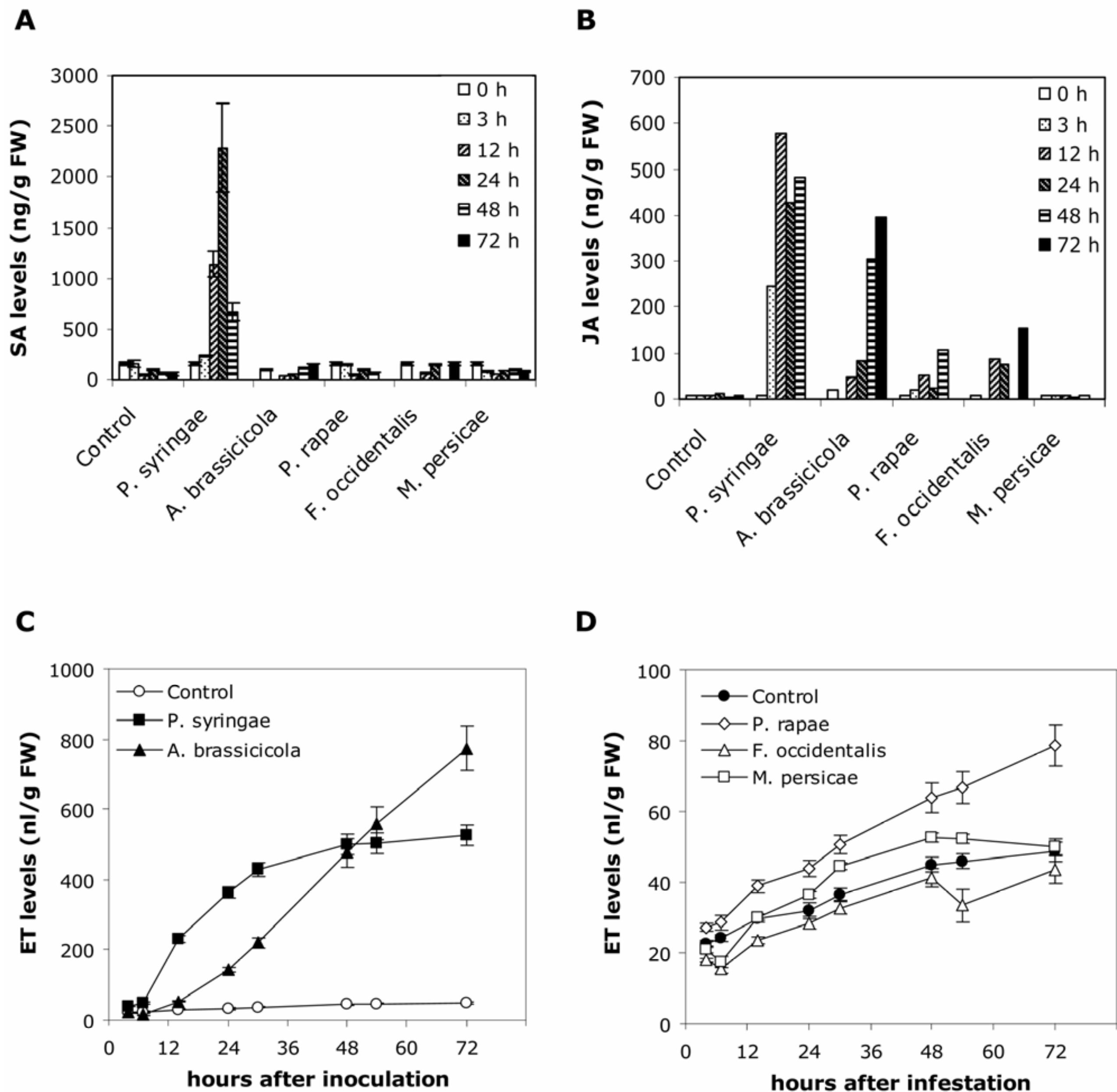

Fig. 2. Signal signature of *Arabidopsis* upon pathogen and insect attack. **A,** Endogenous levels of free salicylic acid (SA) in *Arabidopsis* plants at different time points after inoculation or infestation with *Pseudomonas syringae* pv. *tomato* DC3000(*avrRpt2*), *Alternaria brassicicola*, *Pieris rapae, Frankliniella occidentalis,* or *Myzus persicae*. Values presented are means (±standard error [SE]) of five samples, each consisting of four rosettes that received the same treatment. **B,** Jasmonic acid (JA) levels in *Arabidopsis* plants at different time points after pathogen inoculation or insect infestation. The values presented are from 20 pooled rosettes that received the same treatment. Cumulative ethylene production over a 72-h period in leaves of *Arabidopsis* after inoculation with **C,** *P. syringae* pv. *tomato* DC3000(*avrRpt2*) or *A. brassicicola* or **D,** after infestation with *Pieris rapae, F. occidentalis*, or *M. persicae*. The represented values are means (±SE) for 10 plants that received the same treatment. Inoculations with *A. brassicicola* were performed on the Col-0 mutant *pad3-1*, which is a susceptible host for this pathogen. All other inoculations or infestations were carried out with Col-0 plants. Depending on the progress of the symptoms inflicted by the respective pathogens and insects, harvesting of leaf tissue for SA and JA determinations were omitted at some time points (missing bars in **A** and **B**). FW = fresh weight.

rapae-infested plants. Nevertheless, *VSP2* transcript levels accumulated faster and to a higher level after caterpillar feeding. Furthermore, the timing and magnitude of JA biosynthesis during *Pieris rapae* and *F. occidentalis* feeding was comparable. However, the expression patterns of JA-responsive genes *PDF1.2* and *VSP2* were clearly different.

Global expression profiles of *Arabidopsis* upon pathogen and insect attack.

To explore the complexity of the transcriptional changes of *Arabidopsis* in response to pathogen or insect attack, we analyzed the transcriptome of *Arabidopsis* at two time points after pathogen infection or insect infestation using Affymetrix ATH1 whole-genome GeneChips. Because a detailed qualitative analysis of the transcript profiles of each *Arabidopsis*-attacker combination is beyond the scope of this study, we will focus on the comparison of the transcript profiles between the different *Arabidopsis*-attacker combinations. The time points used for the microarray analysis were selected on the basis of the signal signature (Fig. 2) and the marker-gene expression (Fig. 3), and are listed in Table 1. To be able to relate gene expression to relative SA, JA, and ET levels, RNA was prepared from the same plant material as was used for the determination of the signal signature (Fig. 2). RNA was prepared from four biological replicates, each consisting of five plants. These replicates were pooled to reduce noise arising from biological variation. The transcript profile of each pool was obtained by hybridization of an Affymetrix ATH1 GeneChip representing approximately 23,750 *Arabidopsis* genes (Redman et al. 2004). After hybridization, expressed genes were identified using GeneChip Operating Software (GCOS), which uses statistical criteria to generate a “present” or “absent” call for genes represented by each probe set on the array. The average number of detectable genes (with present call) was 13,729 (60,2%), which is in good agreement with the 60% previously reported by Redman and associates (2004).

Expression values from each pooled sample were normalized globally using GCOS. To validate the global normalization, the fold change in expression level of a set of nine genes previously identified as representative, constitutively expressed controls (Kreps et al. 2002) was calculated. As expected, the fold-change ratio in attacker- over mock-treated leaves was close to 1 for most of these genes for all interactions and time points tested (Table 1).

To identify attacker-responsive genes, the transcript profile of each selected time point of each *Arabidopsis*-attacker combination was compared with the transcript profile of their respective mock-treated control plants that were grown under identical conditions and were harvested at the same two time points as the attacker-induced plants. To identify a robust set of pathogen- and insect-responsive genes, we chose an experimental set-up in which we selected for genes of which changes in expression level were evident during the whole time frame monitored for each of the *Arabidopsis*-attacker combinations. The following conservative selection criteria were applied. First, per *Arabidopsis*-attacker combination, the expression level had to be detectable (P-flag generated by GCOS) and the hybridization intensity had to be >40 units in at least two of four data sets. Second, the change in expression level in attacker-treated leaves compared with that in mock-treated control leaves had to be at least two-fold. To avoid false positives, we required the changes to occur at both time points and to be in the same direction. Only those probe sets were selected that met these stringent selection criteria at both time points tested.

Validation of microarray data.

To validate the GeneChip results, we compared the relative expression values of the marker genes *PR-1*, *PDF1.2*, and *HEL* with the relative mRNA levels on the Northern blots. *VSP2* was left out of this analysis because it is not represented

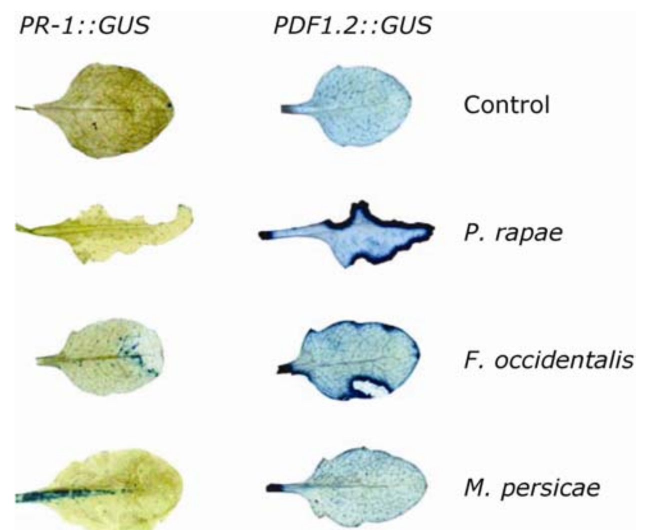

Fig. 4. Histochemical staining of β-glucuronidase (GUS) activity in leaves of transgenic *Arabidopsis PR-1:GUS* and *PDF1.2:GUS* lines after insect feeding. Photographs were taken from representative leaves that were fed on for 24 h by *Pieris rapae* or for 72 h by *Frankliniella occidentalis* or *Myzus persicae*. Silver scars inflicted by *F. occidentalis* feeding appear as a clear white zone at the edge of the leaf.

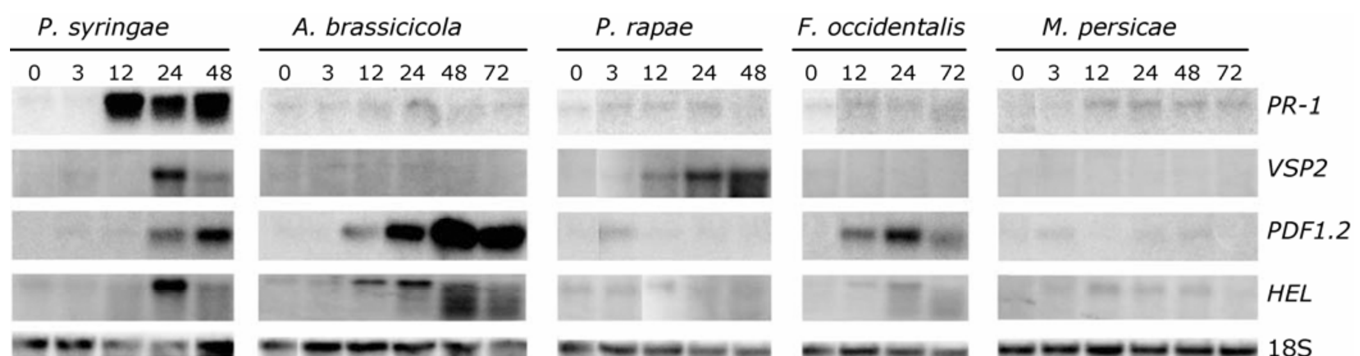

Fig. 3. Northern blot analysis of salicylic acid (SA)-, jasmonic acid (JA)-, and ethylene (ET)-responsive marker genes in *Arabidopsis* upon pathogen and insect attack. Transcript levels of SA-responsive (*PR-1*), JA-responsive (*VSP2* and *PDF1.2*), and ET-responsive (*PDF1.2* and *HEL*) marker genes in *Arabidopsis* leaves at different time points after inoculation or infestation with *Pseudomonas syringae* pv. *tomato* DC3000(*avrRpt2*), *Alternaria brassicicola*, *Pieris rapae, Frankliniella occidentalis*, or *Myzus persicae*. Equal loading of RNA samples was checked using a probe for 18S rRNA.

on the ATH1 GeneChip. Hybridization signals on the northern blots were quantified using a Phosphor Imager and the fold-change relative to the respective controls calculated. Of 30 combinations tested (three marker genes × five *Arabidopsis*-attacker combinations × two timepoints), 29 matched with the microarray data, indicating that the relative expression levels of the marker genes correlated well between GeneChip and Northern blot hybridization (Table 2). In addition, we determined the transcript levels of five attacker-specific genes (At1g30700, At4g26150, At4g15210, At1g72260, and At5g62360) in each of the five *Arabidopsis*-attacker combinations and their respective mock-treated controls, using quantitative real-time polymerase chain reaction (Q-RT-PCR). Figure 5 shows the fold-change induction of the selected genes in the different *Arabidopsis*-attacker combinations as determined by microarray analysis (left panel) and Q-RT-PCR (right panel). Although fold induction in gene expression, especially for low abundant mRNAs, has been shown to differ between the two methods (Czechowski et al. 2004), the relative expression patterns of the five attacker-specific genes were highly similar, indicating that the relative expression levels of the genes tested correlated well between GeneChip and Q-RT-PCR analysis.

To further validate the GeneChip data obtained, we compared the selected pathogen- and insect-responsive genes with those identified in other transcript profiling studies in which the same or similar *Arabidopsis*-attacker combinations were used (Glazebrook et al. 2003; Moran et al. 2002; Reymond et al. 2000, 2004; Tao et al. 2003; Van Wees et al. 2003; Verhagen et al. 2004). Although the experimental set-up, such as age of the plant material upon harvest, time points after inoculation, and type of microarray used, often differed in these studies, a large number of genes behaved similarly (data not shown). For instance, 65% of all the *P. syringae*-responsive genes identified in our study that also are represented on the *Arabidopsis* Genome 8K array of Affymetrix also were identified as being *P. syringae* responsive by Tao and associates (2003). Moreover, 79% of all the *A. brassicicola*-responsive genes identified in our study that also are present on the *Arabidopsis* Genome 8K array, also were identified as being *A. brassicicola* responsive by Van Wees and associates (2003). All together, these results indicate that our experimental set-up and stringent selection criteria resulted in the selection of a robust set of pathogen- and insect-responsive genes.

Functional analysis of differentially expressed genes.

All differentially expressed genes identified in the five *Arabidopsis*-attacker combinations were classified according to their functional categories derived from the Gene Ontology tool at The *Arabidopsis* Information Resource (TAIR) (Rhee et al. 2003). The distribution of the identified probe sets over the

Table 1. Fold-change ratio of representative constitutively expressed control genes in the different *Arabidopsis*-attacker combinations compared with mock-treated *Arabidopsis* plants

		Fold change[a]										
		Pseudomonas syringae		*Alternaria brassicicola*		*Pieris rapae*		*Frankliniella occidentalis*		*Myzus persicae*		
Annotation	**AGI No.**	***t* = 12**	***t* = 24**	***t* = 24**	***t* = 48**	***t* = 12**	***t* = 24**	***t* = 12**	***t* = 24**	***t* = 48**	***t* = 72**	**AVG ± SD**
Polyubiquitin, UBQ10	At4g05320	1.06	0.91	0.74	0.83	1.06	0.75	1.02	1.05	1.35	1.67	1.04 ± 0.28
Eucaryotic init. Fact. elF-4A1	At3g13920	1.06	1.36	1.07	1.25	0.88	0.81	0.86	1.00	0.87	1.18	1.03 ± 0.19
Aquaporin, PIP-1B	At2g45960	0.36	0.75	1.43	1.08	0.96	1.07	0.85	0.92	0.48	0.22	0.81 ± 0.37
40S ribosomal protein S16	At2g09990	0.80	1.09	1.10	1.09	0.79	0.95	0.89	1.04	0.75	1.01	0.95 ± 0.14
Actin 2	At3g18780	0.65	0.56	1.23	0.86	0.85	0.77	0.79	0.87	0.84	1.55	0.90 ± 0.29
Pl. membr. H^+-ATPase, AHA1	At2g18960	0.74	1.58	1.03	0.86	1.18	1.13	0.89	1.11	1.15	1.11	1.08 ± 0.23
Tubulin, β-4	At5g44340	0.85	0.62	0.88	0.99	0.88	0.79	0.82	0.75	1.20	1.83	0.96 ± 0.34
Calmodulin-1	At5g37780	1.81	0.79	0.75	1.02	1.02	0.81	1.10	1.02	2.28	3.16	1.38 ± 0.79
Ca-dep. protein kinase, CPK3	At4g23650	1.10	1.27	0.98	1.36	0.91	0.87	0.95	0.95	1.37	1.22	1.10 ± 0.19
AVG ± SD	…	0.94 ± 0.40	0.99 ± 0.35	1.02 ± 0.22	1.04 ± 0.18	0.95 ± 0.12	0.88 ± 0.14	0.91 ± 0.10	0.97 ± 0.11	1.14 ± 0.52	1.44 ± 0.80	…

[a] Fold-change ratios (attacker/mock) are based on gene expression profiles of leaves of Col-0 plants at indicated time points (*t* = 12, 24, 48, or 72 h) after inoculation or infestation; AVG = average and SD = standard deviation.

Table 2. Comparison of microarray and Northern blot data of the marker genes *PR-1*, *PDF1.2*, and *HEL* in different *Arabidopsis*-attacker combinations

		Fold-change					
		PR-1		*PDF1.2*		*HEL*	
Attacker	**Time**	**Mic[a]**	**Nor[b]**	**Mic**	**Nor**	**Mic**	**Nor**
Pseudomonas syringae	12 h	60.4	50.5	–1.7	1.1	1.7	1.1
	24 h	38.3	25.5	7.7	15.7	2.7	8.8
Alternaria brassicicola	24 h	–1.5	<1	5.0	50.2	2.7	1.4
	48 h	2.5	1.4	126.7	77.5	12.5	2.2
Pieris rapae	12 h	–1.2	<1	–1.4	<1	-2.2	<1
	24 h	3.1	1.4	–1.4	1.4	-1.1	<1
Frankliniella occidentalis	12 h	–1.2	1.2	6.6	6.3	2.8	1.5
	24 h	2.6	1.3	11.4	27.0	3.3	3.2
Myzus persicae	48 h	2.8	1.7	12.4	<1	4.0	2.1
	72 h	5.1	1.3	–2.2	<1	4.0	1.3

[a] Fold changes are marked in solid red when the corresponding genes were selected as responsive to the attacker indicated (consistently showed a greater than twofold change in the same direction (up or down) in the microarray data sets (Mic)). Fold-changes are marked in solid green when the corresponding genes did not consistently show a greater than twofold change in the same direction (up or down) in the microarray data sets.

[b] Signal intensities on the Northern blots were quantified using a Phosphor Imager and compared with the untreated control. The calculated fold changes are given in the same color as the corresponding fold changes in the microarray analysis when they were comparable. Light red or light green are given when the fold change on the Northern blot was in the same direction but, in contrast to the microarray analysis, was below twofold.

different functional categories is shown in Figure 6A. To evaluate the importance of a given functional category, the percentage of differentially expressed genes belonging to each functional category was compared with the degree of representation of the respective functional category in the genome. The results of this comparison for the up- and downregulated genes is shown in Figure 6B. The predominant functional category that is overrepresented in the upregulated gene sets of four of five *Arabidopsis*-attacker combinations represent genes involved in the response to abiotic and biotic stress. In the *Arabidopsis-M. persicae* interaction, genes from this category are overrepresented as well, although the predominant overrepresented category represents genes involved in so-far-unspecified biological processes ("other biological processes").

Of the differentially expressed genes that are downregulated during the *Arabidopsis–A. brassicicola* interaction, genes involved in the response to abiotic and biotic stress clearly are overrepresented. This indicates that, in addition to differential activation, repression of stress-related genes also occurs during the response of *Arabidopsis* to this pathogen. In the *Arabidopsis–Pieris rapae* and *Arabidopsis–F. occidentalis* interactions, genes involved in other biological processes clearly are overrepresented in the downregulated gene sets. However, the specific biological gene functions are diverse, impeding any speculation as to their biological relevance. In the interactions of *Arabidopsis* with *P. syringae* and *M. persicae,* none of the functional categories are clearly overrepresented among the downregulated genes.

Comparison of transcriptome changes induced by pathogen and insect attack.

The number of genes that are consistently up- or downregulated in the different *Arabidopsis*-attacker combinations is shown in Table 3. Of all the attackers investigated, *M. persicae* induced the largest number of changes (2,181). This is remarkable because aphid feeding caused virtually no visual symptoms compared with the extensive damage caused by the other attackers. *P. syringae* infection resulted in a similar number of consistent changes (2,034), whereas the number of consistent changes in the other *Arabidopsis*-attacker combinations was much lower (151 to 199). It must be noted that, in all *Arabidopsis*-attacker combinations, many more genes showed a more than twofold change in expression at a single point in time. Because these changes are not as robust as the consistent changes, they were not analyzed further.

To evaluate the complexity of the transcriptional changes induced during the five different *Arabidopsis*-attacker combinations, we made a pairwise comparison of the overlap between the selected probe sets. In the majority of the comparisons, the overlap is relatively small (Table 3), indicating that most of the differentially expressed genes are specific for the respective *Arabidopsis*-attacker combinations. However, more than 50% of all consistent changes elicited by *A. brassicicola* (68%), *Pieris rapae* (52%), and *F. occidentalis* (72%) also are triggered consistently by *P. syringae*, suggesting that these genes are commonly activated or repressed during these *Arabidopsis*-attacker interactions. Interestingly, these four attackers all induced a considerable increase in JA levels (Fig. 2), suggesting that JA may be the common regulator of the overlapping gene sets.

To investigate the role of JA in the regulation of the overlapping gene sets, we identified probe sets representing JA-responsive genes among the selected attacker-responsive genes. To this end, 5-week-old Col-0 plants were treated with 0.05 mM methyl jasmonate (MeJA) and harvested 0, 1, 3, and 6 h later. RNA from these plants was used to prepare probes for the hybridization of Affymetrix ATH1 GeneChips. Probe sets showing a more than twofold change (up or down) on at least two of the time points tested were selected as described above. The resulting 2,209 probe sets were considered to represent JA-responsive genes. Comparison of these JA-responsive genes among the selected attacker-responsive probe sets revealed that 32% of the *P. syringae*-responsive genes are responsive to MeJA (Table 4). The percentages of JA-responsive genes among the *A. brassicicola*-, *Pieris rapae*-, and *F. occidentalis*-induced changes were even higher (44, 55, and 69%, respectively), indicating that JA plays a dominant role in the transcriptional reprogramming of *Arabidopsis* in response to these attackers. Pairwise comparisons of the overlap between JA-responsive genes in the four *Arabidopsis*-attacker combinations revealed that, of all JA-responsive, *Pieris rapae*-induced

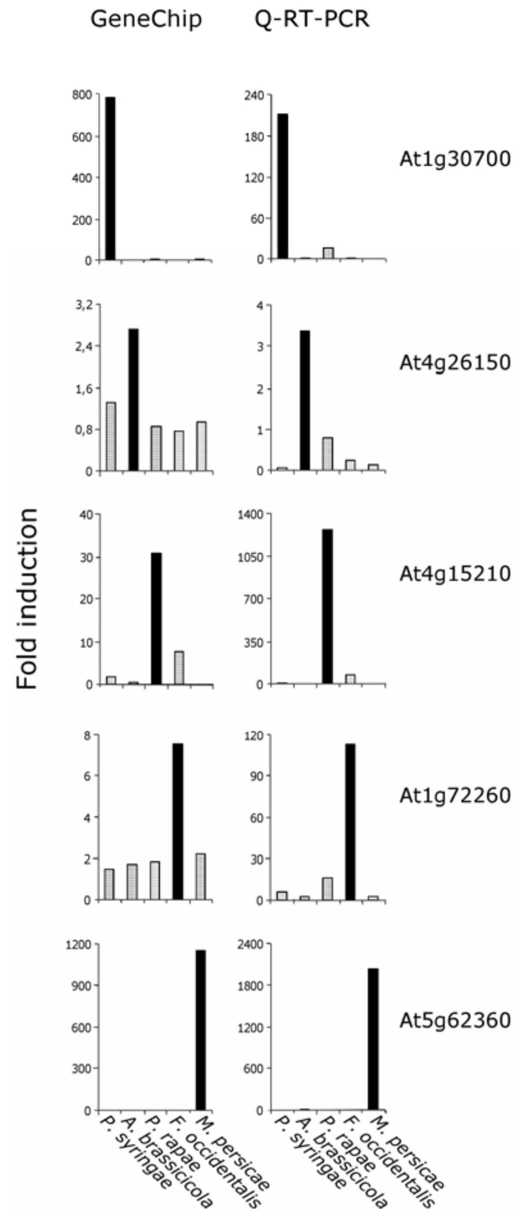

Fig. 5. Comparison of microarray and quantitative real-time polymerase chain reaction (Q-RT-PCR) analysis of five attacker-specific genes in the different *Arabidopsis*-attacker combinations. Fold induction of five attacker-specific genes (At1g30700, At4g26150, At4g15210, At1g72260, and At5g62360) after infection or infestation of *Arabidopsis* by *Pseudomonas syringae*, *Alternaria brassicicola*, *Pieris rapae*, *Frankliniella occidentalis*, or *Myzus persicae.* On the left, the fold-change patterns from the microarray analysis. On the right, the fold-change patterns from the Q-RT-PCR analysis.

changes, 66% also are induced by *P. syringae* (Table 4). In the *Arabidopsis*-*F. occidentalis* and the *Arabidopsis*-*A. brassicicola* interactions, this percentage is even higher (80 and 85%, respectively), indicating that the JA-induced defense responses triggered by these attackers show considerable overlap. However, this does not hold for all *Arabidopsis*-attacker combinations. For instance, when the JA-responsive genes among the *Pieris rapae*- and *F. occidentalis*-induced changes were compared with the JA-responsive genes among the *A. brassicicola*-induced ones, the overlap was relatively low (6 to 17%). These results indicate that although, attackers with very different modes of action (e.g., *F. occidentalis* and *P. syringae*) may induce similar sets of JA-responsive genes, the majority of the JA-responsive genes are affected in an attacker-specific manner, indicating that other factors besides JA shape the final outcome of the defense response.

DISCUSSION

Plants require a broad range of defense mechanisms to effectively combat invasion by microbial pathogens or attack by herbivorous insects. These mechanisms include pre-existing physical and chemical barriers, as well as inducible defense responses that become activated upon pathogen infection or insect herbivory. A concerted action of these defensive activities helps the plant to minimize damage caused by the attacker. The signal molecules SA, JA, and ET have been implicated in many plant-pathogen and plant-insect interactions (Dicke and Hilker 2003; Pieterse and Van Loon 1999). Despite the evident overlap in signaling that is triggered upon pathogen or insect attack, the plant response is highly dependent on the plant-attacker combination. Little is known about how plants coordinate attacker-induced signals into specific defense responses.

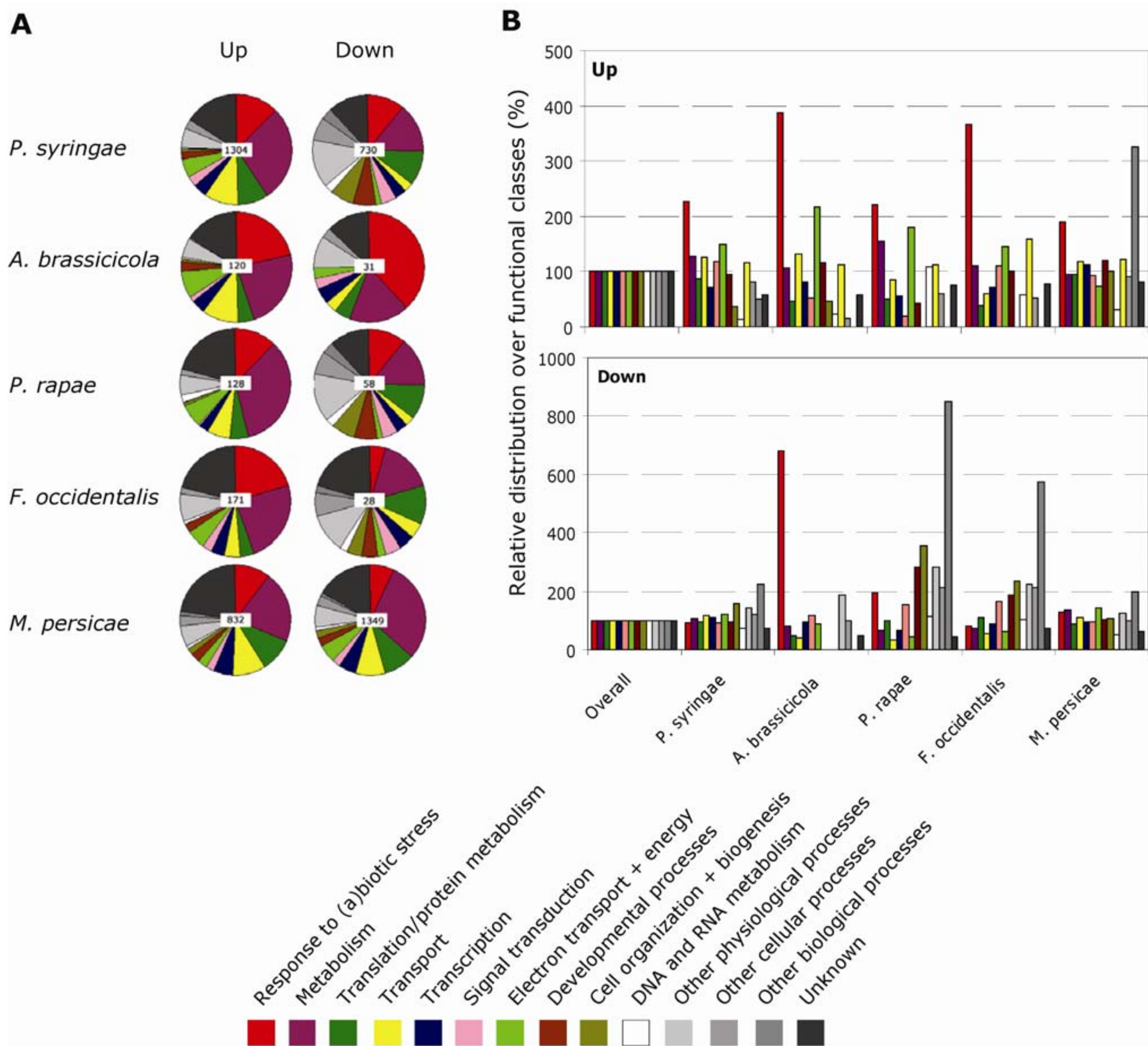

Fig. 6. Functional analysis of differentially expressed gene sets. **A,** Distribution of the differentially expressed genes identified in the *Arabidopsis*-attacker combinations over the functional categories. The number of up- or downregulated genes is given in the center of the respective pies. Classification in functional categories was performed essentially according to the Gene Ontology tool of the *Arabidopsis* Information Resource. Genes belonging to the functional category "response to abiotic and biotic factors" and "response to stress" were grouped in a single functional category designated "response to abiotic and biotic stress". **B,** Degree of overrepresentation of the differentially expressed genes in the functional categories. The distribution of the differentially expressed genes over the functional categories is presented relative to the distribution of all genes on the Affymetrix ATH1 array (set at 100% for each functional category).

A well-accepted hypothesis is that modulation of the different defense signaling pathways involved plays an important role in this process (Reymond and Farmer 1998). Although ample information is available on the role of SA, JA, and ET in the response of plants to certain pathogens and insects, the information is often highly specific for a given plant-pathogen or plant-insect interaction. Moreover, the different studies often are characterized by unique experimental conditions. Here, we attempted to gain insight into the dynamics of the response of a single plant species (*Arabidopsis thaliana*) to a variety of microbial and herbivorous attackers under identical conditions. This approach allowed us to compare the dynamics of signal production and the transcriptional reprogramming of *Arabidopsis* upon attack by pathogens and insects with very different modes of attack.

Correlation between signal signature and marker gene expression.

Gene expression profiles and SA, JA, and ET production were examined simultaneously during the entire period between inoculation or infestation and the occurrence of the resulting severe symptoms or damage (Fig. 1). Because aphids did not cause any visible symptoms, the response of *Arabidopsis* to this attacker was monitored over a 72-h time course. All other attackers caused a significant increase in the production of one or more of the signals tested (Fig. 2). The accumulation patterns of SA, JA, and ET during the different *Arabidopsis*-attacker interactions clearly differed in composition, magnitude, and timing. This so-called signal signature was reflected in the expression patterns of the well-characterized marker genes *PR-1*, *VSP2*, *PDF1.2*, and *HEL* (Fig. 3). For instance, *P. syringae* infection caused a considerable increase in SA, JA, and ET production, and was associated with the subsequent activation of all the SA-, JA-, and ET-responsive marker genes tested. Furthermore, *A. brassicicola* infection caused a significant increase in both JA and ET levels, resulting in the activation of the JA- and ET-responsive marker genes *PDF1.2* and *HEL.* However, in some *Arabidopsis*-attacker combinations, the signal signature correlated to only a limited extent with the expression patterns of the marker genes. The high levels of JA produced by *Arabidopsis* in response to infection by *A. brassicicola* resulted in the activation of the JA-responsive gene *PDF1.2*, but not in that of the JA-responsive gene *VSP2*. Moreover, although *Pieris rapae* and *F. occidentalis* induced comparable levels of JA in *Arabidopsis*, *VSP2* was activated in the *Arabidopsis*-*Pieris rapae* interaction, whereas *PDF1.2* was not. Conversely, *F. occidentalis* triggered the expression of *PDF1.2* but not *VSP2.* Hence, it must be concluded that the signal signature of a given plant-attacker combination plays a primary role in the orchestration of the plant's defense response, but additional layers of regulation lead to differential marker gene expression.

Attacker-induced transcriptional changes.

The goal of the microarray analysis was to explore the complexity of the transcriptional reprogramming initiated by the different pathogens and insects in relation to the observed *Arabidopsis*-attacker signal signatures, and to identify robust sets of attacker-responsive genes. To this end, we applied stringent selection criteria to identify genes that show a consistent change in expression during pathogenesis and herbivore feeding. Depending on the *Arabidopsis*-attacker combination, 151 to 2,181 genes showed a consistent change in expression over time. Surprisingly, aphid feeding triggered the largest number of consistent changes in gene expression, even though these insects caused the least symptoms of all attackers tested and did not induce detectable changes in SA, JA, and ET levels (Figs. 1 and 2). In contrast to the other four *Arabidopsis*-attacker combinations, a large proportion of the differentially expressed genes in the *Arabidopsis*-aphid inter-

Table 3. Analysis of probe sets showing a consistent twofold change in time in *Arabidopsis* leaves upon infection or infestation with *Pseudomonas syringae*, *Alternaria brassicicola*, *Pieris rapae, Frankliniella occidentalis*, or *Myzus persicae*

Attacker	Signal signature[a]			Consistent changes[b]			Overlap (%)[c]				
	SA	JA	ET	Up	Down	Total	*P.s.*	*A.b.*	*P.r.*	*F.o.*	*M.p.*
P. syringae	+++	+++	+++	1,304	730	2,034	100	5	5	7	12
A. brassicicola	–	+++	+++	120	31	151	68	100	5	22	13
Pieris rapae	–	++	+	128	58	186	52	4	100	39	7
F. occidentalis	–	++	–	171	28	199	72	17	36	100	18
M. persicae	–	–	–	832	1,349	2,181	12	1	1	2	100

[a] Relative amounts of signal molecules produced in *Arabidopsis* in response to pathogen or insect attack; SA = salicylic acid, JA = jasmonic acid, ET = ethylene, +++ = high levels, ++ = moderate levels, + = low levels, and – = no change compared with control.
[b] Number of probe sets representing attacker-responsive genes with a consistent greater than twofold change over time in the same direction (up or down).
[c] Pairwise comparison of the percentage of overlap between probe sets. Percentages are presented relative to the total number of changes induced by the attacker given in the same row (e.g., 68% of all *A. brassicicola*-induced changes are also induced by *P. syringae*).

Table 4. Overlap of jasmonic acid (JA)-responsive genes showing a consistent twofold change in time in *Arabidopsis* leaves after infection or infestation with JA-inducing attackers *Pseudomonas syringae*, *Alternaria brassicicola*, *Pieris rapae*, or *Frankliniella occidentalis*

Attacker	Consistent changes[a]		Overlap of JA-responsive genes (%)[b]			
	Total	JA-responsive	*P. syringae*	*A. brassicicola*	*Pieris rapae*	*F. occidentalis*
P. syringae	2,034	652 (32%)	100	9	9	17
A. brassicicola	151	67 (44%)	85	100	9	34
Pieris rapae	186	103 (55%)	66	6	100	54
F. occidentalis	199	138 (69%)	80	17	41	100

[a] Total number of probe sets with consistently more than twofold change (up or down) over time in response to the attacker and the number of genes from the "total" list that showed a more than twofold change in the same direction in response to treatment with 0.05 mM methyl jasmonate. The percentage of these JA-responsive genes is given in parentheses.
[b] Percent overlap between the JA-responsive genes among the selected attacker-induced probe sets. Percentages are given relative to the total number of JA-responsive genes induced by the attacker given in the same row (e.g., 85% of all JA-responsive, *A. brassicicola*-induced changes are also *P. syringae* responsive).

action was downregulated (62 versus 14 to 36% in the other combinations). A relatively large fraction of the downregulated genes is involved in plant metabolism, confirming previous findings that demonstrate that aphids are major manipulators of plant physiology and nutrition status (Davies et al. 2004). Previously, Moran and co-workers (Moran et al. 2002; Moran and Thompson 2001) identified 19 *M. persicae*-responsive genes in *Arabidopsis* by Northern blot and small-scale microarray analysis. Of these, 13 genes (68%) were among the 2,181 identified as being consistently responsive to *M. persicae* in our GeneChip analysis, including the SA-responsive genes *PR-1* (At2g14160) and *PR-2* (β-1,3-glucanase; At3g57260). Although *PR-1* transcript levels were barely detectable on the Northern blots (Fig. 3), they were clearly expressed in the cells surrounding the feeding sites on the main veins of the *PR-1:GUS* reporter line (Fig. 4). These results indicate that significant local changes in gene expression can be identified by microarray analysis while escaping from identification by Northern blot analysis.

A large proportion of the gene sets identified in our study as being attacker responsive also had been identified in comparable studies (Glazebrook et al. 2003; Reymond et al. 2000; Tao et al. 2003; Van Wees et al. 2003; Verhagen et al. 2004). For instance, Reymond and associates (2000) identified 17 genes showing a more than twofold increase in expression level in response to *Pieris rapae* feeding using a small dedicated microarray with probes for 150 *Arabidopsis* genes. Of the genes also represented on the ATH1 chip, 59% showed a consistent more than twofold increase in our *Arabidopsis–Pieris rapae* data sets, even though different time points after infestation (3 h in the study by Reymond and associates versus 12 and 24 h in our study) and different larval stages (L4 to L5 in the study of Reymond and associates versus L1 to L2 in our study) were tested. Furthermore, 65% of the *P. syringae*-responsive genes that were identified in our study (and were present on both the ATH1 and the Affymetrix 8k array) also were identified by Tao and associates (2003). Similarly, 79% of the *A. brassicicola*-responsive genes also were identified by Van Wees and associates (2003), who also used the susceptible phytoalexin-deficient mutant *pad3* to study the *Arabidopsis–A. brassicicola* interaction. Together, these data indicate that the gene sets that were selected in this study are, to a large extent, representative for the different *Arabidopsis*-attacker combinations used. It must, however, be noted that, to achieve a maximal response of *Arabidopsis* to *P. syringae* infection, we made use of an avirulent strain of the pathogen. Although it has been suggested that the difference in the transcriptional response of *Arabidopsis* to virulent and avirulent strains of *P. syringae* is predominantly quantitative (Tao et al. 2003), it cannot be excluded that a small proportion of the selected genes are specific for the incomepatible interaction.

Genes showing a more than twofold change at a single time point are either part of a transient response or false positives and, thus, are unlikely to be identified consistently when bioassays are performed under different experimental conditions. Although some of these genes may play an important role in the response of *Arabidopsis* to the attacker involved, the scope of this study was not to provide a qualitative in-depth analysis of individual gene sets that are differentially expressed in the different *Arabidopsis*-attacker combinations, but to explore the complexity of the transcriptional changes in the response of *Arabidopsis* to attack by different pathogens and insects. Therefore, we limited our analysis to those genes that showed a robust change in expression and disregarded all others. The selected robust gene sets obtained with the whole-genome ATH1 arrays can be related to actual SA, JA, and ET levels and will be of value for more detailed analyses of individual *Arabidopsis*-attacker interactions.

Stress-related genes are overrepresented in all *Arabidopsis*-attacker combinations.

To gain insight into the function of the differentially expressed genes, we categorized their biological function essentially according to the Gene Ontology tool of TAIR. Some of these functional categories cover a relatively large proportion of the *Arabidopsis* genome (e.g., genes in the functional category "metabolism" represent 21.7% of all annotated genes, whereas genes in the category "response to abiotic and biotic stress" represent only 5.6% of the genome). Thus, information on the percentage of selected genes in a given functional category is biased by the degree of representation of this category in the genome. To identify functional categories in which a relatively large proportion of the genes show a consistent change in expression in response to pathogen or insect attack, we compared the number of identified genes in a given functional category with the degree of representation of this category in the whole genome. In this way, functional categories that are overrepresented in the selected differentially expressed genes sets were readily identified (Fig. 6B). In all *Arabidopsis*-attacker combinations tested, the number of upregulated genes predicted to be involved in the response to biotic and abiotic stress was two- to fourfold higher than expected on the basis of representation of this category in the genome. Evidently, differential expression of a large proportion of genes from this category plays an important role in the response of *Arabidopsis* to pathogen and insect attack. However, when looking at the absolute percentages of representation of the genes in the different functional categories (Fig. 6A), the contribution of stress-related genes in the investigated interactions is not immediately clear. For instance, of all consistently upregulated genes in the different *Arabidopsis*-attacker combinations, 10.6 to 21.7% belongs to the functional category response to abiotic and biotic stress, whereas a considerably larger proportion of the genes (20.8 to 33.8%) fall into the functional category metabolism (Fig. 5A). Thus, assessment of the distribution of the identified gene sets over the different functional classes as a function of the degree of representation of these functional categories in the genome makes it is possible to better weigh the importance of a given functional category in the plant response studied.

Complexity of transcriptional reprogramming upon pathogen and insect attack.

To explore the complexity of transcriptional changes induced by the different *Arabidopsis* attackers used, we compared the overlap between gene sets. Because both *P. syringae* and *M. persicae* induced, by far, the largest number of consistent changes (10- to 14-fold more genes than *A. brassicicola, Pieris rapae*, and *F. occidentalis*), it is evident that the transcriptional response of *Arabidopsis* to these very different attackers is highly complex. In the case of *P. syringae*, this may be related to the fact that infection of *Arabidopsis* by this pathogen results in the production of high levels of SA, JA, and ET, each of which may activate different sets of genes. In the case of *M. persicae* feeding, however, none of these signals tested was detectable. Evidently, the onset of the large transcriptional reprogramming elicited by these phloem-feeding insects is not based on the production of high overall levels of SA, JA, or ET, suggesting that the responses of *Arabidopsis* to *P. syringae* and *M. persicae* is highly unrelated. Indeed, most of the transcriptional changes induced by *P. syringae* or *M. persicae* were unique. Nonetheless, 253 genes (141 upregulated genes and 112 downregulated genes) (data not shown) of

all consistently induced changes in the *Arabidopsis-P. syringae* and the *Arabidopsis-M. persicae* interaction overlapped. Thus, although both attackers have very different modes of action and trigger a highly dissimilar signal signature, a large number of *Arabidopsis* genes are recruited in response to both attackers. However, these overlapping genes represent only 12% of the total number of consistent changes identified in both interactions and, thus, may contribute only to a limited extent to the overall defense reaction.

Compared with *P. syringae* and *M. persicae*, *A. brassicicola, Pieris rapae*, and *F. occidentalis* induced only a relatively low number of consistent changes in gene expression (151 to 199 up- or downregulated genes). A small number of these genes ($n = 6$) showed a consistent change in all three *Arabidopsis*-attacker combinations (data not shown). Pairwise comparison of the differentially expressed gene sets revealed an overlap of 4% (*Pieris rapae* versus *A. brassicicola*), 17% (*F. occidentalis* versus *A. brassicicola*), and 39% (*Pieris rapae* versus *F. occidentalis*). In these three *Arabidopsis*-attacker interactions, JA is a dominant component of the signal signature produced. Indeed, 44 to 69% of all differentially expressed genes identified in these three *Arabidopsis*-attacker combinations also were found to be responsive to exogenous application of MeJA (Table 4), indicating that JA-responsive gene expression plays a central role in the response of *Arabidopsis* to infection or infestation by all three attackers. However, the majority (94 to 46%) of these MeJA-responsive genes showed an attacker-specific expression pattern in pairwise comparisons between the differentially expressed gene sets. This may be explained partly by differences in sampling time points; however, on all time points tested, JA levels clearly were elevated. Hence, the sets of JA-responsive genes that are differentially activated or repressed in the different *Arabidopsis*-attacker combinations are highly divergent, suggesting that so-far-unidentified regulatory processes play an important role in modulating the final outcome of the defense response. A model of how invasion by JA-inducing attackers may result in the activation of differential sets of JA-responsive genes is shown in Figure 7. Similar models can be drawn for genes that are regulated by other defense-related signals such as SA and ET, resulting in a network of interconnecting signaling pathways that provides the plant with a powerful regulatory potential to fine tune its defense response.

In conclusion, we demonstrated that *Arabidopsis* is highly adapted in its response to pathogens and herbivorous insects with very different modes of attack. Depending on the *Arabidopsis*-attacker combination, the signal molecules SA, JA, and ET are produced with large differences in both quantity and timing. We identified differentially expressed gene sets that, over time, show a consistent change in expression for each of the *Arabidopsis*-attacker combinations. In all cases, stress-related genes are clearly overrepresented in the gene sets identified. In four of the five *Arabidopsis*-attacker combinations tested, JA plays an important role in the differential regulation of a large proportion of the activated or repressed genes. Nevertheless, the vast majority of the JA-responsive changes are specific for each plant-attacker combination. Evidently, signal molecules such as JA play an important role in the primary response of the plant to pathogen and insect attack. However, additional layers of regulation obviously shape the outcome of the defense reaction. Pathway cross-talk or effects of so-far-unidentified regulatory factors may play an important role in the fine tuning of the plant's response to pathogens and insects. The nature and importance of these regulatory processes will be a challenging topic for future research.

MATERIALS AND METHODS

Cultivation of plants.

Seeds of *Arabidopsis* accession Col-0 and the phytoalexin-deficient Col-0 mutant *pad3-1* (Glazebrook and Ausubel 1994) were sown in quartz sand. Two-week-old seedlings were transferred to 60-mL pots containing a sand-and-potting soil mixture that was autoclaved twice for 20 min. Plants were cultivated in a growth chamber with an 8-h day (200 µE m^{-2} s^{-1} at 24ºC) and 16-h night (20ºC) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon 1938) containing 10 µM Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

Pathogen bioassays.

Inoculations with the bacterial leaf pathogen *P. syringae* pv. *tomato* DC3000 were performed as described previously (Van Wees et al. 1999). Briefly, *P. syringae* pv. *tomato* DC3000 with the plasmid pV288 carrying avirulence gene *avrRpt2* (Kunkel et al. 1993) was cultured overnight at 28ºC in liquid King's medium B (King et al. 1954), supplemented with kanamycin at 25 mg $liter^{-1}$ to select for the plasmid. Subsequently, bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$ to a final density of 10^7 CFU ml^{-1}. Wild-type Col-0 plants were inoculated by pressure infiltrating a suspension of *P. syringae* pv. *tomato* DC3000(*avrRpt2*) at 10^7 CFU ml^{-1} into all fully expanded leaves of 5-week-old plants.

Bioassays with the fungal leaf pathogen *A. brassicicola* MUCL 20297 were carried out as described by Ton and associates (2002). Briefly, *A. brassicicola* was grown on potato

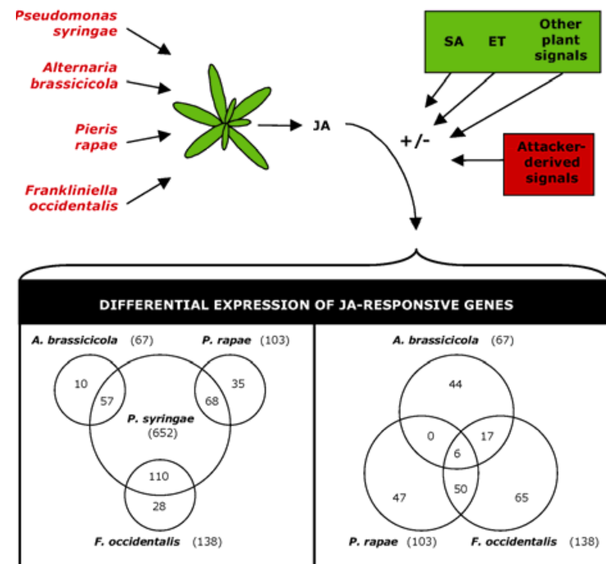

Fig. 7. Differential expression of jasmonic acid (JA)-responsive genes upon attack by JA-inducing pathogens and insects. Attack on *Arabidopsis* by *Pseudomonas syringae, Alternaria brassicicola, Pieris rapae,* or *Frankliniella occidentalis* resulted in a strong increase in the production of JA, and a concomitant change in the expression of a large number of JA-responsive genes (numbers are given between parenthesis). Nevertheless, the overlap among the JA-responsive genes between the different *Arabidopsis*-attacker combinations was relatively low (number of overlapping genes between the indicated *Arabidopsis*-attacker combinations are given in the Venn diagrams). Salicylic acid (SA) and ethylene (ET) have been demonstrated to cross-communicate with the JA pathway. Hence, depending on the amount and timing of their production, SA and ET may have positive or negative effects on the expression of specific sets of JA-responsive genes. In addition, so-far-unidentified plant- or attacker-derived signals, or physiological conditions that are inflicted by the attacker, may be involved in modulating JA-responsive gene expression.

dextrose agar plates for 2 weeks at 22ºC. Subsequently, conidia were collected as described by Broekaert and associates (1990). Five-week-old susceptible pad3-1 plants were challenge inoculated by applying 3-µl drops of 10 mM $MgSO_4$ containing 10^6 spores/ml onto all fully expanded leaves of 5-week-old plants.

Insect bioassays.

Tissue-chewing larvae of the small cabbage white butterfly *Pieris rapae* were reared on Brussels sprout plants *(Brassica oleracea gemmifera* cv. Cyrus) in a growth chamber with a 16-h day and 8-h night cycle (21ºC, 50 to 70% relative humidity), as described previously (Van Poecke et al. 2001). Infestation of *Arabidopsis* Col-0 plants was carried out by transferring five first-instar larvae of *Pieris rapae* to each plant using a fine paintbrush.

The population of the Western flower thrips *F. occidentalis* originated from a greenhouse infestation on chrysanthemum. This virus-free population was reared on *Phaseolus vulgaris* cv. Prelude pods, supplied with *Pinus* pollen, in glass jars that were placed at 25ºC in a growth chamber with a 16-h day and 8-h night cycle as described (Kindt et al. 2003). Thrips infestations were performed by transferring 20 larvae of *F. occidentalis* to each *Arabidopsis* Col-0 plant.

Phloem-feeding green peach aphids (*M. persicae*) were maintained on *B. chinensis* L. cv. Granaat under greenhouse conditions (25ºC, 50 to 70% relative humidity). The 16-h light period prevented sexual reproduction, keeping the population clonal. *Arabidopsis* Col-0 plants were infested with *M. persicae* by transferring 40 nymphs and apterous adults to each plant (Van Poecke et al. 2003).

All insect populations used consisted of fairly immobile stages, such that individuals remained on the plants to which they were transferred.

MeJA treatment.

Induction treatment with MeJA was performed by dipping 5-week-old Col-0 plants in an aqueous solution containing 0.05 mM MeJA (Serva, Brunschwig Chemie, Amsterdam, The Netherlands) and 0.01% of the surfactant Silwet L-77 (Van Meeuwen Chemicals B.V., Weesp, The Netherlands) as described previously (Pieterse et al. 1998). Plants were harvested at 0, 1, 3, and 6 h after induction treatment and immediately frozen in liquid nitrogen.

ET quantification.

Immediately after pathogen inoculation or transfer of insect populations to the shoots, rosettes were detached from the roots, weighed, and placed individually in 35-ml gas-tight serum flasks ($n = 10$) that subsequently were incubated under climate chamber conditions. At different time intervals, 1-ml gas samples were withdrawn through the rubber seal. The concentration of ET was measured by gas chromatography as described by De Laat and Van Loon (1982).

JA and SA quantification.

All leaves from 20 plants per treatment were frozen in liquid nitrogen and pulverized with mortar and pestle. For each JA extraction, a sample of 1 g was taken from the frozen leaf material and transferred to a 50-ml centrifuge tube. To the frozen samples were added 100 ng of the internal standard 9,10-dihydrojasmonic acid, 10 ml of saturated NaCl solution, 0.5 ml of 1 M citric acid, and 25 ml of diethylether containing 0.005% (wt/vol) butylated hydroxytoluene as antioxidant. Subsequently, extraction and gas chromatography-mass spectrometry quantification of JA was carried out as described by Mueller and Brodschelm (1994).

For each SA extraction, a sample of 0.5 g of ground leaf tissue was transferred to a 1.5-ml microfuge tube and 100 µl of the internal standard *ortho*-anisic acid (1 µg ml^{-1}) and 0.5 ml of 70% ethanol were added. Subsequently, extraction and quantification of SA were carried out as described by Meuwly and Métraux (1993).

Northern blot analysis.

Total RNA was extracted as described previously (Van Wees et al. 1999). For Northern blot analysis, 15 µg of RNA was denatured using glyoxal and dimethyl sulfoxide (Sambrook et al. 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-N^+ membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. Northern blots were hybridized with gene-specific probes for *PR-1*, *PDF1.2*, *VSP2*, and *HEL* as described previously (Pieterse et al. 1998). To check for equal loading, the blots were stripped and hybridized with a probe for 18S rRNA. The AGI numbers for the genes studied are At2g14610 (*PR-1*), At5g24770 (*VSP2*), At5g44420 (*PDF1.2*), and At3g04720 (*HEL*). Probe for 18S was derived from an *Arabidopsis* cDNA clone (Pruitt and Meyerowitz 1986).

Q-RT-PCR.

Q-RT-PCR analysis basically was performed as described previously (Czechowski et al. 2004). RNA (2 µg) was digested with Turbo DNA-free (Ambion, Huntingdon, U.K.) according to the manufacturer's instructions. To check for genomic DNA contamination, a PCR with primers designed on intron sequences of *ACT7* (At5g09810; *ACT7*-FOR; 5′-GAC ATG GAA AAG ATA TGG CAT CAC AC-3′; *ACT7*-REV; 5′-AGA TCC TTC CTG ATA TCG ACA TCA C-3′) was carried out. Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT_{20} primers (Invitrogen, Breda, The Netherlands), 10 mM dNTPs, and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Efficiency of cDNA synthesis was assessed by Q-RT-PCR using primers of the constitutively expressed gene *UBI10* (At4g05320; *UBI10*-FOR; 5′ AAA GAG ATA ACA GGA ACG GAA ACA TAG T-3′; *UBI10*-REV; 5′-GGC CTT GTA TAA TCC CTG ATG AAT AAG-3′). Gene-specific primers were designed for five *Arabidopsis* genes, each of which showed an attacker-specific expression pattern in one of the five *Arabidopsis*-attacker interactions studied. The corresponding AGI numbers and primers are At1g30700, FOR 5′-TCC GTA ACC TCC GCT TCA AC-3′, REV 5′-CGT GGC CTC CAC TTC TGA TT-3′ (*Arabidopsis–P. syringae*); At4g26150; FOR 5′-GGA TTT GGA GAC CCAGAG CA-3′, REV 5′-TGG CAG CCT CCT TCT CAT CT-3′ *(Arabidopsis–A. brassicicola*); At4g15210, FOR 5′-GAC GGC CTA CAA AAC GCT GT-3′, REV 5′-CCA TTG TGG GAT CGG GAT AG-3′ (*Arabidopsis-Pieris rapae*); At1g72260, FOR 5′-CTG CCC TTC CAA CCA AGC TA-3′, REV 5′-TGG CAT CCA CTC ACT TGC AT-3′ (*Arabidopsis-F. occidentalis*); and At5g62360, FOR 5′-CAA ACA AGC CCC AAG CTC AT-3′, REV 5′-CGC ACC ATC ATT GCT GAA GT-3′ (*Arabidopsis-M. persicae*). Q-RT-PCR analysis was done in optical 96-well plates with an MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands), using SYBR Green to monitor double-stranded (ds)DNA synthesis. Each reaction contained 1 µl of cDNA, 0.5 µl of each of the two gene-specific primers (10 pmol/ µl), and 10 µl of 2× iQ SYBR Green Supermix reagent (Bio-Rad) in a final volume of 20 µl. The following PCR program was used for all PCR reactions: 95ºC for 3 min, followed by 40 cycles of 95ºC for 30 s, 59.5ºC for 30 s, and 72ºC for 30 s. Threshold cycle (C_T) values were calculated using Optical System Software, version 1.0 for MyIQ (Bio-Rad). Sub-

sequently, C_T values were normalized for differences in dsDNA synthesis using the *UBI10* C_T values. Normalized transcript levels of the five genes in each of the five *Arabidopsis*-attacker combinations were compared with those of the respective mock-treated controls and the fold change in expression level was calculated.

GUS assays.

Transgenic *Arabidopsis PDF1.2:GUS* and *PR-1:GUS* lines, containing a translational fusion of the *PDF1.2* or the *PR-1* promoter with the *uidA* reporter gene in the Col-0 background (provided by Y. Plotnikova, Massachusetts General Hospital, Boston, MA, U.S.A.), were grown in soil as described above. Insects were transferred to 5-week-old plants as described above. After 24 h of caterpillar feeding or 72 h of thrips or aphid feeding, leaf tissues were harvested and GUS activity was assessed by transferring the seedlings to GUS staining solution (1 mM X-Gluc, 100 mM NaP_i buffer, pH 7.0, 10 mM EDTA, and 0.1% [vol/vol] Triton X-100) as described previously (Spoel et al. 2003). After overnight incubation at 37°C, the leaf tissues were destained by repeated washes in 70% ethanol and evaluated for staining intensity.

Sample preparation and microarray data collection.

For isolation of RNA, whole rosettes were harvested at different time intervals during each *Arabidopsis*-attacker interaction or at several time points after MeJA treatment, and immediately frozen in liquid nitrogen. For all time points, every *Arabidopsis*-attacker combination, and the MeJA treatment, appropriate mock-treated plants were harvested. RNA was prepared from four biological replicates, each consisting of five plants, as described above and cleaned using RNeasy Plant Mini Kit columns (Qiagen Benelux BV, Venlo, The Netherlands). These replicates were pooled to reduce noise arising from biological variation. In retrospect, it is now recognized that pooling RNA samples of biological replicates is not optimal. If the experiments would have been done today, each biological replicate would have been used for hybridization of a GeneChip. Synthesis of cRNA probes, hybridization to GeneChips, and collection of data from the hybridized GeneChips were performed as described previously (Verhagen et al. 2004; Zhu et al. 2001). Hybridizations with labeled cRNAs were conducted with *Arabidopsis* ATH1 full-genome GeneChips (Affymetrix, Santa Clara, CA, U.S.A.), containing a total of 22,810 probe sets representing approximately 23,750 *Arabidopsis* genes (Redman et al. 2004). On this GeneChip, each gene is represented by at least one probe set consisting of 11 25-mer oligonucleotides. Probe preparations and GeneChip hybridizations were carried out by ServiceXS (Leiden, The Netherlands) and the Affymetrix service station of Leiden University Medical Center, where they passed all internal quality checks.

Expression profiling.

GCOS (Affymetrix) was used to globally normalize the expression data on each GeneChip to an average value of 200 so that hybridization intensity of all chips was equivalent. In addition, expressed genes were identified by GCOS, which uses statistical criteria to generate a present or absent call for genes represented by each probe set on the array. Microarray data files then were analyzed using GeneSpring 6.1 (Silicon Genetics, Redwood, CA, U.S.A.). Default settings used during the data analyses were per chip = normalize to 50th percentile and per gene = normalize to specific samples. The *P* values from the Pearson correlation tests run for GeneChips that were hybridized with probes from four biological replicates of nontreated control plants ranged between 0.92 and 0.97. This is in good agreement with the high correlation coefficients previously reported for independent biological samples (Redman et al. 2004), indicating that the GeneChip hybridizations and microarray data collections were performed in a technically sound manner.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Arabidopsis Information Resource (TAIR): www.Arabidopsis.org

Nottingham Arabidopsis Stock Centre International Affymetrix service (NASCarrays): affymetrix.Arabidopsis.info