Arabidopsis Transcriptome Changes in Response to Phloem-Feeding Silverleaf Whitefly Nymphs. Similarities and Distinctions in Responses to Aphids[1][W][OA]

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Phloem-feeding pests cause extensive crop damage throughout the world, yet little is understood about how plants perceive and defend themselves from these threats. The silverleaf whitefly (SLWF; *Bemisia tabaci* type B) is a good model for studying phloem-feeding insect-plant interactions, as SLWF nymphs cause little wounding and have a long, continuous interaction with the plant. Using the Affymetrix ATH1 GeneChip to monitor the Arabidopsis (*Arabidopsis thaliana*) transcriptome, 700 transcripts were found to be up-regulated and 556 down-regulated by SLWF nymphs. Closer examination of the regulation of secondary metabolite (glucosinolate) and defense pathway genes after SLWF-instar feeding shows that responses were qualitatively and quantitatively different from chewing insects and aphids. In addition to the RNA profile distinctions, analysis of SLWF performance on wild-type and *phytoalexin-deficient4* (*pad4*) mutants suggests aphid and SLWF interactions with Arabidopsis were distinct. While *pad4-1* mutants were more susceptible to aphids, SLWF development on *pad4-1* and wild-type plants was similar. Furthermore, although jasmonic acid genes were repressed and salicylic acid-regulated genes were induced after SLWF feeding, cytological staining of SLWF-infested tissue showed that pathogen defenses, such as localized cell death and hydrogen peroxide accumulation, were not observed. Like aphid and fungal pathogens, callose synthase gene RNAs accumulated and callose deposition was observed in SLWF-infested tissue. These results provide a more comprehensive understanding of phloem-feeding insect-plant interactions and distinguish SLWF global responses.

Phloem-feeding insects are highly specialized in their mode of feeding and present a unique stress on plant fitness. Not only do these insects feed for prolonged periods of time on host photoassimilates, but they also pose a threat as vectors of plant viruses and deposit honeydew encouraging the growth of mold (Brown and Czosnek, 2002; Jones, 2003). Unlike chewing insects that cause swift and extensive tissue damage, most phloem feeders cause minimal tissue damage as they use their stylet to access the vascular tissue to feed. This relationship is more analogous to a plant-biotrophic pathogen interaction, where the pathogen is sustained in a localized area and is dependent on living plant cells. Currently, there remains a paucity of knowledge on how phloem-feeding insects are perceived, the genes involved in defense, and the regulation of resource-allocation genes.

Plants utilize both constitutive and induced defenses for protection against a wide range of biotic threats. Constitutive defenses include physical barriers such as the leaf cuticle, cell walls, and stored metabolites that inhibit the feeding, growth, and development of herbivores (Walling, 2000). For example, the carbohydrate/pectin composition of the cell wall and deposition of cell wall components is important in determining resistance to some biotic threats (Dreyer and Campbell, 1987; Vorwerk et al., 2004). In particular, the deposition of callose at the sites of fungal hyphal insertion is an important factor in susceptibility to these pathogens (Nishimura et al., 2003; Thatcher et al., 2005). Alterations in pectin and callose are also thought to be important in the interactions between plants and phloem-feeding insects (Dreyer and Campbell, 1987; Botha and Matsiliza, 2004).

Induced plant defenses include the activation of both direct and indirect mechanisms to deter herbivores (Walling, 2000; Kessler and Baldwin, 2002). Direct defenses involve the synthesis of secondary metabolites that influence insect attraction/deterrence and inhibit insect growth and development (Baldwin et al., 2001, 2002; Kliebenstein, 2004). In addition, induced proteins, such as proteinase inhibitors, polyphenol oxidases, arginase, and Thr deaminase, inhibit insect

digestive enzymes and/or decrease the nutritive value of the plant tissue (Ryan, 2000; Ussuf et al., 2001; Chen et al., 2005). Indirect defenses include the release of volatiles that signal the location of insects on infested plants to parasitoids and predators of the herbivore (Baldwin et al., 2002; Dicke et al., 2003).

In the Brassicaceae, secondary metabolites called glucosinolates have important roles in pathogen and herbivore interactions. Biosynthesis of glucosinolates is dependent on primary metabolism for the synthesis of amino acids and also secondary metabolism pathway enzymes. In ecotype Columbia plants, glucosinolate amino acid side chains are primarily derived from Met, but homophenylalanine and Trp also contribute to the glucosinolate pools (Hirai et al., 2005). The amino acid-derived glucosinolates are synthesized and stored in vacuoles. Upon cellular damage, myrosinases are released from specialized myrosin cells and hydrolyze the glucosinolate Glc moiety, releasing toxic compounds (such as nitriles, isothiocyanates, epithionitriles, and thiocyanates; Wittstock and Halkier, 2002). Glucosinolates have antibiotic effects on pathogens and aphids and act as attractants of specialist insects (Mewis et al., 2002; Kliebenstein, 2004).

During plant-pathogen and -pest interactions, elicitors present in insect oral secretions or pathogen-secreted effectors activate or suppress a variety of defense signaling pathways (Walling, 2000; Kaloshian and Walling, 2005; Mudgett, 2005; Chisholm et al., 2006). Three major plant hormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), are important in monocot and dicot defense. Microarray and other defense gene expression studies have shown that the SA pathway is primarily activated in response to biotrophic pathogens, while the JA/ET pathway is induced in response to necrotrophic pathogens, wounding, and tissue-damaging insect feeding (Rojo et al., 2003; Glazebrook, 2005; Kaloshian and Walling, 2005; Thompson and Goggin, 2006). While these pathways can function synergistically, cooperatively, or sequentially, negative cross-talk between SA and JA/ET pathways occurs frequently and may modulate the balance of SA and JA defenses (Rojo et al., 2003; Mur et al., 2006). This cross-talk prevents the activation of "unnecessary" defense genes in many biotrophic and necrotrophic pathogen-plant interactions (Glazebrook, 2005).

There are critical limitations in our understanding of phloem-feeding insect defense pathways, as virtually all microarray studies to date have examined phloem-feeding aphids. Furthermore, the majority of the aphid microarray studies reported to date have examined only a select group of genes since small defense-gene-biased cDNA microarrays (100–1,000 genes) were utilized (Moran et al., 2002; Heidel and Baldwin, 2004; Voelckel et al., 2004; Zhu-Salzman et al., 2004; Kaloshian and Walling, 2005; Park et al., 2006; Thompson and Goggin, 2006). A notable exception was a *Myzus persicae* (green peach aphid)-Arabidopsis (*Arabidopsis thaliana*) interaction study published by De Vos et al. (2005). In addition, many of the published microarray

studies have limited biological replications and/or do not measure significance using a statistical method. Collectively, these aphid-plant interaction array experiments have shown that signal intensities are low, and meaningful conclusions can be difficult to ascertain. Despite these limitations, the current transcriptome analyses suggest that changes to aphids are drastically different than those observed by chewing insects (Reymond and Farmer, 1998; Moran et al., 2002; Zhu-Salzman et al., 2004; De Vos et al., 2005; Kaloshian and Walling, 2005; Thompson and Goggin, 2006); aphids tend to induce gene sets more similar to fungal or bacterial pathogens.

To date, it is unknown whether the transcriptome response to aphids is indicative of plant responses to other insects within the order Hemiptera (suborder Sternorrhyncha), which includes aphids, whiteflies, psyllids, and scale insects. This has limited our understanding of plant responses to hemipteran species, as the amount of cellular wounding and duration of feeding can vary depending on species-specific probing behaviors and life histories (Walling, 2000). The silverleaf whitefly (SLWF; *Bemisia tabaci* type B; *Bemisia argentifolii*) is a good model to study plant responses to phloem-feeding insects. SLWFs are stealthy, as they navigate intercellularly and rarely damage epidermal or mesophyll cells prior to puncturing cells of the phloem (Johnson and Walker, 1999; Freeman et al., 2001); in contrast, aphids frequently probe intracellularly (Pollard, 1973). In addition, while aphids have a short and mobile life history, SLWFs have a long and continuous interaction with the plant. The 28-d life cycle of the whitefly is composed of six stages (egg, crawler/first instar, second instar, third instar, fourth instar, adult), only two of which are mobile (crawler, adult). Studies in crop plants (squash [*Cucurbita pepo*] and tomato [*Solanum lycopersicum*]) have shown that plants can perceive differences in the signals from SLWF nymphs and adults, common signals that are delivered by diverse whitefly species, and distinguish signals between closely related biotypes (van de Ven et al., 2000; Walling, 2000). In these crops, both SA- and JA-defense genes and novel defense signaling pathways are activated.

SLWFs are generalists and cause extensive agricultural damage in temperate climates around the world (U.S. Department of Agriculture, 2005). SLWFs are pests of Brassica species (Liu, 2000; McKenzie et al., 2004); therefore, studies in the model plant Arabidopsis are timely and will allow for a more comprehensive understanding of the long-term and intimate interactions that accompany plant responses to phloem-feeding whiteflies. To this end, the changes in the Arabidopsis transcriptome after SLWF second and third nymph feeding were examined using the ATH1 Affymetrix GeneChip. Close examination of the regulation of glucosinolate and defense pathway genes in the ATH1 array showed that Arabidopsis transcriptional reprogramming during SLWF nymph feeding was qualitatively and quantitatively different from that

induced by chewing insects and aphids. These distinguishing events were emphasized by analyzing whitefly development on wild-type and *phytoalexin-deficient4* (*pad4*) plants. While PAD4 is important for susceptibility to aphids, PAD4 did not influence the time for nymphs to reach their fourth instar. Finally, as cell death, callose deposition, and reactive oxygen species (ROS) are defenses induced by some pathogen-plant (Nishimura et al., 2003; Overmyer et al., 2003; Apel and Hirt, 2004) and insect-plant interactions (Bi and Felton, 1995; Botha and Matsiliza, 2004), the biological relevance of the changes in genes important in generation/scavenging ROS and cell wall modification in Arabidopsis-whitefly interactions were examined by staining tissue samples for evidence of hydrogen peroxide (H_2O_2) accumulation, cell death, and callose deposition.

RESULTS

Analysis of Genes Regulated by SLWF-Instar Feeding

In this study, changes in the Arabidopsis transcriptome profile were examined during SLWF second- and third-instar feeding (21 d postinfestation), as changes in plant defense gene RNAs occur in crop plants in response to these nymphal stages (van de Ven et al., 2000). Four biological replicate experiments containing 10 plants per treatment were performed. RNAs from two biological replicate experiments were pooled, and cRNAs were synthesized and hybridized to two replicate ATH1 GeneChips. To identify genes that were significantly regulated by instar feeding, the data were preprocessed using robust multiarray analysis (RMA) for background adjustment and normalization (Irizarry et al., 2003). Significant Analysis of Microarray (SAM) software was used for differential analysis (Tusher et al., 2001). RMA has been shown to be robust to outliers and has better precision than other methods, especially for low expression values (Irizarry et al., 2003). The quality and reproducibility of the data between the two experiments were examined by comparing all probe sets called "present" (14,815 probe sets). Figure 1 shows the expression values obtained from both experiments are highly correlated as the data points are clustered around the regression line. The correlation coefficient for experiments 1 and 2 was 0.81, supporting the value of using a pooling strategy (Peng et al., 2003). Twenty-seven genes were outliers; they were induced in experiment 2 and not in experiment 1. These genes were found to be heat shock- and stress-induced genes and had high false discovery rates (FDRs [*q* value]; Supplemental Table S1).

Whitefly-regulated genes were identified by performing profile analysis using a SAM δ value of 2.06, which corresponds to a FDR of 3.917%. *PATHOGENESIS-RELATED PROTEIN1* (*PR1*) and *β-GLUCANASE2* (*BGL2*; *PR2*) genes, known to be SLWF induced, verified the use of this FDR (Zarate
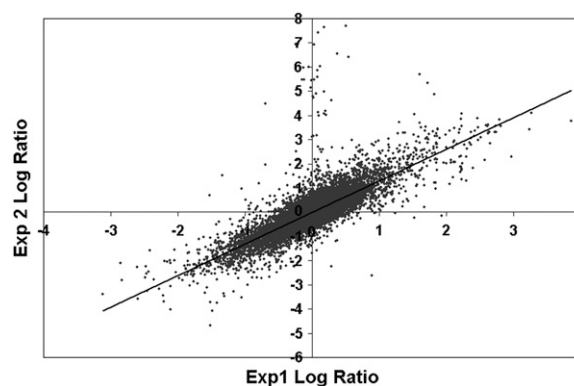
Figure 1. Comparison of log ratios from replicate microarray experiments. Data were filtered to remove "absent" calls, leaving 14,815 probe sets called "present" by MAS 5.0. Background adjustment was performed using RMA. Log ratios were calculated from normalized, log-transformed RMA output (= infested − control). Log ratios from experiment 1 (Exp1) and 2 (Exp2) are displayed.

et al., 2007). Using these parameters, the SAM program identified 1,256 genes as significantly regulated in response to whitefly infestation, including 700 up-regulated and 556 down-regulated genes (Supplemental Table S1). The SAM fold-change values for these genes ranged from −9.6- to 14-fold, and 364 genes (28%) had fold-change values greater than 2-fold. The magnitude of changes in the transcript profile (5.3%) was similar to what has been observed in response to the pathogens and herbivores (De Vos et al., 2005).

For comparison with SAM results, the commonly used Affymetrix Microarray Suite (MAS) 5.0 program was also used for analysis of SLWF array data. MAS 5.0 identified 1,415 genes that were increased or decreased greater than 2-fold on GeneChips after SLWF nymphal feeding. Surprisingly, the overlap between SAM-generated (FDR 3.9%) and MAS 5.0-generated (2-fold change) data sets was only 458 genes, indicating that the programs identified different complements of genes that were considered "significant." The results in this article will focus on the SAM data as FDRs (*q* values) gave an indication of the confidence of conclusions drawn for respective genes.

To understand the extent of similarities and differences of SLWF-induced global expression changes with other plant stresses, publicly available data sets were utilized. When the 1,256 SLWF-regulated transcripts were compared to the 2,181 aphid-regulated transcripts identified by De Vos et al. (2005), overlap of genes up- or down-regulated by both SLWF and *M. persicae* was only 17%. This suggested that the global response to SLWF-instar feeding was distinct from aphid nymph and adult feeding. There was a more compelling overlap with genes regulated during fungal biotroph interactions. The *Erysiphe orontii* 7-d postinfection RNA profile (http://ausubellab.mgh.harvard.edu/imds) had approximately 30% overlap with 1,256 SLWF-regulated genes, perhaps reflecting the long-term interactions of these biotrophs with their

hosts. Although many experimental, technical, and quality variables differ between microarray data sets, general trends in the transcriptome response suggest that the aphid data set and the SLWF data were not as similar as would be expected from two hemipteran transcriptome studies.

Classification of SLWF-Regulated Genes

Gene annotations for the 1,256 whitefly-regulated genes were developed using The Arabidopsis Information Resource (TAIR) and Gene Ontology (GO) descriptions (Ashburner et al., 2000; Rhee et al., 2003). Many genes were of unknown function; GO identified 398 genes (31%) of unknown biological function and TAIR described 264 of these genes (21%) as "expressed proteins." Table I highlights selected genes involved in oxidative stress, cell wall biosynthesis and modification, photosynthesis, signal transduction, and nitrogen and carbohydrate metabolism, as these responses are often regulated in response to insects and pests (Scheideler et al., 2002; Kaloshian and Walling, 2005; Thompson and Goggin, 2006). Similar to what is observed after abiotic, biotic, and other herbivore-interaction stress treatments, a general down-regulation of photosynthesis genes was observed (Table I; Klok et al., 2002; Seki et al., 2002; Zimmermann et al., 2004). The responses of 121 genes involved in nitrogen metabolism and 132 genes involved with carbohydrate metabolism and sugar transport were evaluated for their responses to SLWF infestation (Sheen, 2006). Only four nitrogen- and four carbohydrate-metabolism genes were up-regulated (FDR < 3.97%) after SLWF feeding (Table I). This modest response (at the RNA level) was surprising given the fact that these insects were an additional nutrient sink and a more profound modulation of these metabolic processes might have been anticipated. The increases in β-fructosidase and Gln synthetase RNAs noted after SLWF nymph feeding were likely a general stress response, since these RNAs accumulate in response to abiotic and biotic stress treatments (www.genevestigator.ethz.ch).

The cell wall provides an important barrier to pathogens and pests. Biotic stresses induce genes to strengthen this constitutive defense barrier by altering pectin composition, cell wall cross-linking, and cell wall protein and chemical constituents. After SLWF feeding, many genes encoding proteins that influence the cell wall were modulated. Three α-expansin (EXPA) genes (EXPA4, EXPA11, EXPA16) of the 36 members in the expansin gene family in Arabidopsis were down-regulated; EXPAs have established roles in the rapid extension or stress relaxation of the plant cell wall and have known roles in development (Cosgrove, 2005). In addition, several genes that influence pectin integrity and modification (pectate lyase, pectinacetylesterase), lignin synthesis, an arabinogalactan protein (AGP5), and callose (CALLOSE SYNTHASE1 [CALS1]) were activated, and one pectinesterase gene was repressed (Table I; Supplemental Table S1). Interestingly, AGP5

RNAs increase response to biotic stress and some abiotic stresses (www.genevestigator.ethz.ch).

RNAs for several genes that enable scavenging of ROS and redox homeostasis increased during SLWF-instar feeding (Table I), suggesting that whitefly feeding may induce ROS in planta. While whitefly saliva is poorly characterized, aphids and some caterpillars produce salivary enzymes capable of generating ROS (Miles, 1999; Musser et al., 2002). ROS play important roles in defense due to their antimicrobial activity, importance in altering the quality of proteins in the insect diet, cross-linking the cell wall, and mobility and role as defense signals (Bradley et al., 1992; Bi and Felton, 1995). During Schizaphis graminum and M. persicae infestation of sorghum (Sorghum bicolor) and Arabidopsis, respectively, increases in glutathione S-transferase RNAs have been noted; in contrast, catalase 3-like protein and Fe-superoxide dismutase RNAs declined in sorghum and Arabidopsis, respectively (Moran et al., 2002; Zhu-Salzman et al., 2004). These data suggested that redox gene transcript levels changed modestly by SLWF and aphid feeding, and a strong oxidative stress response was not observed in response to these hemipterans.

Finally, the SLWF microarray data indicated that a set of genes encoding potential signal transduction components, including kinases, phosphatases, and receptor-like kinase genes, was modulated after SLWF infestation (Table I; Fig. 2). In addition, AVIRULENCE-INDUCED GENE1, a reporter of the incompatible interaction between Pseudomonas syringae pv maculicola avrRpt2 and Arabidopsis RPT2, was highly induced (8.6-fold; FDR = 2.82%) by SLWF nymphs (Reuber and Ausubel, 1996).

To date, 863 gene families that include 6,314 Arabidopsis genes have been categorized (Rhee et al., 2003). Of the 1,256 SLWF-regulated genes identified, 369 are members of gene families. Figure 2 shows a subset of these SLWF-regulated gene families categorized by biological function. Many genes that function in signal transduction (mitogen-activated protein kinases, receptor kinases, protein phosphatase 2C phosphatases) were induced, some over 2-fold. Cytochrome P450, transporter, proteins involved in translation, and ROS-metabolism gene families were also generally induced. Sulfurtransferases, potential alkaloid metabolism, cell cycle, antiporter, general transcription factors, and expansin gene family members were repressed. For most gene families, the percentage of genes differentially regulated compared to the total number of family members ranged from 4% to 12%. A few families had a larger percentage of genes that were differentially regulated, including monosaccharide transporters (20%), auto-inhibited Ca^{2+} ATPases (23%), tropinone-metabolism proteins (50%), and blue copper-binding proteins (18%).

Confirmation of Microarray Studies with Six Leu-Rich Repeat Genes

There are approximately 200 Leu-rich repeat (LRR) genes in Arabidopsis (Dangl and Jones, 2001). While

Table I. *Expression level of genes responsive to SLWF second- and third-instar feeding*

| Biological Function	Gene Locus	TAIR Description	Fold-Change	FDR
Oxidative stress				
	At4g32320	Peroxidase family protein (APX6)	−1.61	2.82
	At1g20620	Catalase 3-like protein (SEN2)	1.36	2.82
	At5g40370	Glutaredoxin (GRX)	1.38	3.92
	At1g07890	L-Ascorbate peroxidase 1, cytosolic (APX1)	1.43	3.53
	At5g20230	Blue copper-binding protein (AtBCB)	3.86	2.82
	At4g15680	Glutaredoxin family protein (GRX4)	4.07	3.18
	At1g03850	Glutaredoxin family protein (GRX12)	6.04	2.82
Photosynthesis genes				
	At5g45040	Cytochrome c6 (ATC6)	−3.86	2.82
	At2g44920	Thylakoid lumenal 15-kD protein	−2.60	2.82
	At2g20260	PSI reaction center subunit IV	−2.46	3.61
	At3g21055	PSII 5-kD protein	−2.30	3.07
	At1g44446	Chlorophyll A oxygenase (CAO)	−2.05	3.07
	At1g12250	Thylakoid lumenal protein-related	−2.04	2.82
Cell wall modification				
	At2g39700	Expansin, putative (EXPA4)	−2.38	3.53
	At3g55250	Expressed protein predicted pectate lyase	−2.25	3.90
	At1g20190	Expansin, putative (EXPA11)	−2.12	3.07
	At3g55500	Expansin, putative (EXPA16)	−1.76	3.07
	At1g05570	CALS1 (GSL6, PMR4)	2.23	2.82
	At1g72680	Cinnamyl-alcohol dehydrogenase (CAD1)	2.39	3.92
	At3g49120	Class III peroxidase (PERX34)	2.67	3.47
	At4g19420	Pectinacetylesterase family protein	2.89	2.82
	At4g16260	Glycosyl hydrolase family 17 (β-1,3-glucanase-like)	3.08	3.92
	At4g34230	Cinnamyl-alcohol dehydrogenase (CAD5)	3.55	3.61
	At2g37130	Peroxidase 21 (PER21, P21, PRXR5)	4.35	2.82
	At1g35230	Arabinogalactan protein (AGP5)	4.56	3.25
	At2g45220	Pectinesterase family protein	8.06	3.92
Carbohydrate metabolism				
	At4g15210	β-Amylase (BMY1)	−10.00	2.82
	At4g19840	Phloem protein 2 (ATPP2-A1)	−2.00	3.53
	At1g71880	Suc transporter (SUC1)	1.97	2.82
	At2g02810	UDP-Gal/UDP-Glc transporter	3.00	2.82
	At4g25000	α-Amylase (AMY1)	5.22	2.82
	At3g13790	β-Fructosidase (βFRUCT1)	6.25	3.18
Nitrogen metabolism				
	At5g22300	Nitrilase 4 (NIT4)	−1.90	3.61
	At3g63510	Nitrogen regulation family protein	−1.72	3.25
	At3g44300	Nitrilase 2 (NIT2)	1.18	3.25
	At3g17820	Gln synthetase (GS1)	1.83	2.82
	At5g37600	Gln synthetase (AtGSR2)	2.02	3.07
	At1g09240	Nicotianamine synthase	5.61	3.07
Signal transduction and LRR proteins				
	At5g01920	Protein kinase family protein (STN8)	−2.90	3.92
	At5g01850	Protein kinase	2.04	2.82
	At5g10740	Protein phosphatase 2C-related	2.14	3.90
	At2g39660	Protein kinase (BIK1)	2.54	3.92
	At1g10340	Ankyrin repeat family protein	3.91	3.92
	At4g23140	Receptor-like protein kinase 5 (RLK5)	4.71	3.90
	At1g51890	LRR protein kinase	8.13	3.90
	At5g12940	LRR domain	−3.70	3.52
	At4g18670	LRR domain	−1.43	3.90
	At4g19500	TIR-NBS-LRR domains	−1.37	2.81
	At3g28890	LRR domain	1.37	3.14
	At5g48380	Ser/Thr kinase and LRR domains	2.65	3.90
	At2g32680	LRR domain	5.29	3.90
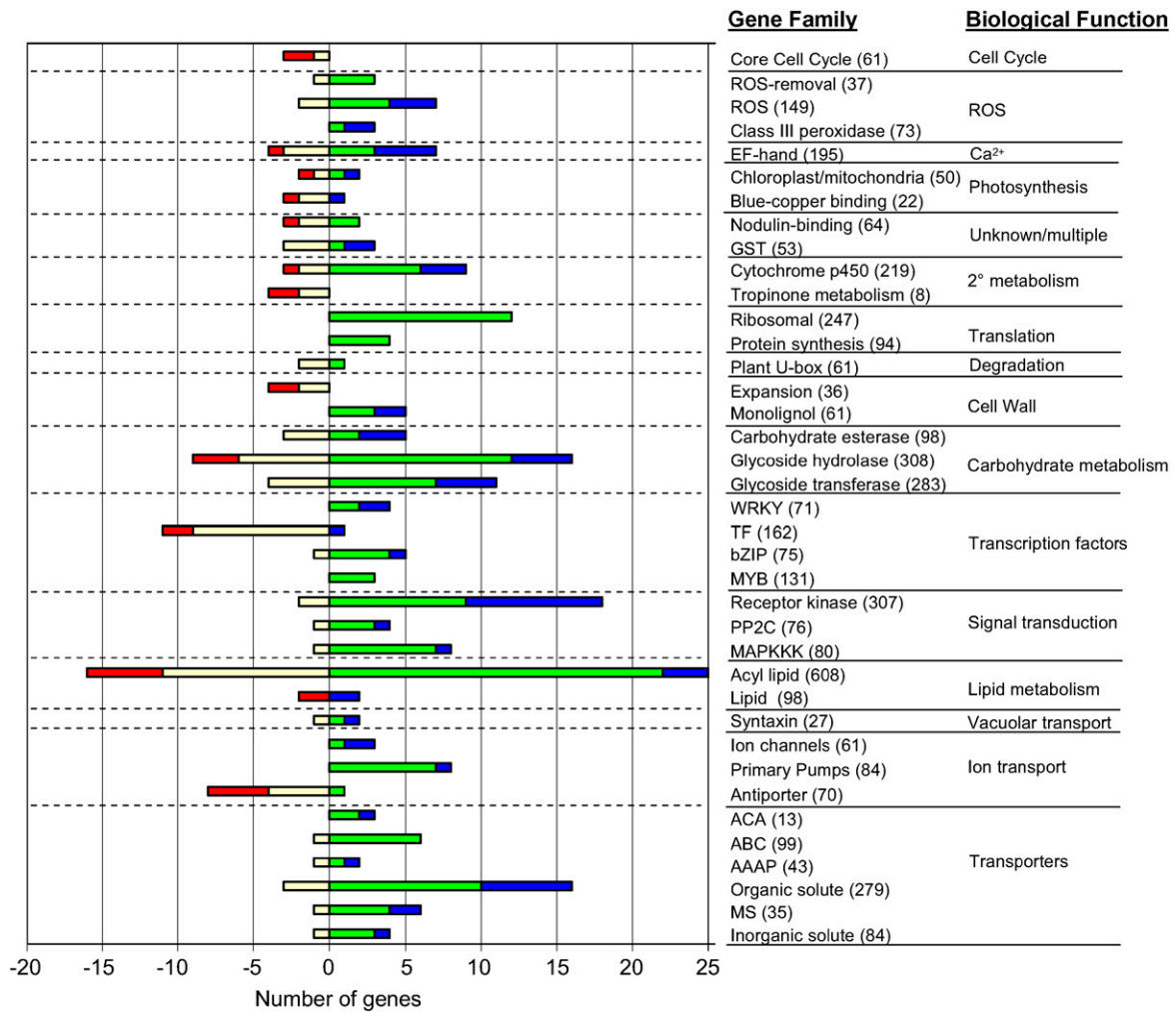
Figure 2. Number of genes induced or repressed after SLWF-instar feeding in respective gene families. The total number of characterized genes in each gene family is shown in parentheses following the gene family name. The postulated biological function(s) of each gene family is shown. Yellow and green signify genes induced or repressed less than 2-fold, respectively. Blue and red show genes induced or repressed greater than 2-fold, respectively.

LRR domains are thought to be important in protein-protein interactions, a subset of LRR genes function as resistance genes in response to pathogens, nematodes, or phloem-feeding insects (Meyers et al., 2003; Kaloshian, 2004). At present, most LRR proteins have unknown biological functions. As many proteins with LRR motifs have been shown to have roles in defense and 30 LRR genes were differentially regulated in response to SLWF feeding (Supplemental Table S2), a group of six genes with predicted LRR domains was used to validate our microarray observations. These genes represented a wide range of predicted changes in RNA levels, including three LRR genes that were up-regulated 1.37- to 5.29-fold (At2g32680, At3g28890, At5g48380) and three genes that showed 1.37- to 3.70-fold decreases in transcripts (At4g18670, At4g19500, At5g12940). FDRs for the LRR genes ranged from 2.81% to 3.90% (Table I).

It is well established that biological variation often accounts for the largest component of variation in a microarray experiment (Zakharkin et al., 2005). Therefore, the reproducibility of microarray results was evaluated in the two pooled RNA samples (experiments 1 and 2) used in the microarray experiments and two pooled RNAs from additional infestations (experiments 3 and 4). Gene-specific primers and PCR were used to monitor these six LRR gene RNAs in infested and noninfested leaf RNA populations. Figure 3 shows that for five of the six LRR genes examined, RNA levels were well correlated in the four biological replications and consistent with gene expression changes detected by the microarray studies. Even genes with expression fold-change values of less than 2-fold as detected by SAM (At4g19500, At4g18670, At3g28890) revealed reproducible changes in RNA levels. This observation stresses the importance

of using statistical methods to identify genes of interest, for even genes with low RNA fold-change values were verified by reverse transcription (RT)-PCR and therefore may warrant study as stress-response genes. It is important to note that although the RT-PCRs used here were not quantitative, there was clearly variation in the magnitude of RNA changes in each infestation. These data highlight the importance of using a pooling strategy to identify significant RNA-response trends.

One LRR gene, At4g19500, which was predicted to have a small decline in its RNA level (−1.37-fold), had a variable response. While its RNA declined in the pooled RNA samples of experiments 2 and 4, its RNA did not change in the pooled RNA samples of experiments 1 and 3. The reason for this variation is not understood at this time. It is possible that At4g19500 may be more transiently expressed in response to SLWF feeding.

SLWFs Suppress JA and Induce SA Defenses

SA, JA, and ET pathways have been shown to be important in regulating defense responses to biotic threats (Rojo et al., 2003). The aphid *M. persicae* induces both of these pathways in Arabidopsis, although JA-regulated defense gene RNAs accumulate to low levels (Moran and Thompson, 2001; Moran et al., 2002). To characterize how these pathways were modulated in response to SLWF nymph feeding, 33 genes known to be involved in the SA, JA, and ET-defense pathways were examined (Glazebrook, 2001; Devoto and Turner, 2003). Only seven of these defense genes were identified using the stringent 3.917% FDR criteria (Supplemental Table S2), suggesting there may be a temporal or quantitative variation in gene expression changes in response to SLWF feeding. To examine the expression of SA-, JA-, and ET-defense genes, the FDR criterion was relaxed; most genes, 25 of the 33 examined, had FDRs of less than 15% (Table II).

Table II shows that SA-biosynthesis and SA-regulated defense genes were up-regulated in response to SLWF-instar feeding. The microarray data showed that several genes upstream of SA accumulation (*SALICYLIC ACID INDUCTION DEFICIENT2* [*SID2*], *ENHANCED DISEASE SUSCEPTIBILITY5* [*EDS5*], *PAD4*) were induced 3.3- to 3.8-fold. Furthermore, genes that respond to SA (*PR1*, *PR5*, *BGL2*) were up-regulated 5.5- to 6.4-fold. Many of the SA genes (12/14) had FDRs <10%, indicating a low chance of false discovery.

In contrast, genes important in JA biosynthesis and regulated by JA were repressed or showed modest to no changes in RNA levels (Table II). This response was drastically different from tissue-damaging insects that primarily induce JA-responsive genes (Reymond and Farmer, 1998). For example, in response to SLWF feeding, the JA-biosynthesis genes *OMEGA-3 FATTY ACID DESATURASE3* (*FAD3*; −2.7-fold) and *FAD7* (−1.89-fold), and JA-responsive defense genes *PLANT DEFENSIN PROTEIN1.2* (*PDF1.2*; −2.7-fold) and *VEGETATIVE STORAGE PROTEIN1* (*VSP1*; −2.3-fold), were repressed. Other JA-defense pathway

genes, *FAD2*, *THIONIN2.1* (*THI2.1*), and *CORONATINE INSENSITIVE1* (*COI1*), showed no change or small changes in RNA levels, −1.17-, 1.02-, and 1.27-fold, respectively. The only exception to this pattern was the JA-regulated and weakly SA-responsive *HEVEIN-LIKE* (*HEL*)/*PR4* (Reymond and Farmer, 1998). *HEL/PR4* RNA levels increased in response to SLWF feeding (2.29-fold). Consistent with the SLWF results, *HEL/PR4* RNAs increased in sorghum leaves after *S. graminum* feeding (Zhu-Salzman et al., 2004). The FDRs for JA-pathway genes were generally higher than genes important in SA-mediated defense; only 12/19 genes had FDRs less than 10%. These genes had small expression level changes (approximately 1-fold), and the FDRs reflected the biological variability in the pooled samples. All ET-pathway genes had FDRs >4.56% with modest changes in RNA levels ranging from −1.39 (*CONSTITUTIVE TRIPLE RESPONSE1* [*CTR1*]) to 1.45 (*ETHYLENE INSENSITIVE3* [*EIN3*]), or RNA levels were not modulated in response to SLWF-instar feeding (Table II). The gene expression values for SA and JA and ET sentinel genes were confirmed by RT-PCR, and the biological relevance of SA- and JA-defense pathways has been investigated using JA- and SA-defense mutants (Zarate et al., 2007).

Whiteflies Did Not Alter Sulfur- and Glucosinolate-Metabolism Gene RNA Levels

To evaluate if SLWFs altered the expression of genes influencing glucosinolate metabolism, the changes in 34 primary sulfur-metabolism genes and 31 glucosinolate-biosynthesis and -catabolism genes were analyzed (Table III; Fig. 4; Bodnaryk, 1994; Hirai et al., 2005). For comparisons, the regulation of these genes was examined after *M. persicae* feeding for 48 and 72 h, *Pieris rapae* (cabbage white caterpillar) fifth-instar feeding for 12 and 24 h (De Vos et al., 2005), and biotrophic fungal pathogen (*Erysiphe cichoracearum*) after 1 d of infection. These additional microarray data sets were obtained at Genevestigator (Zimmermann et al., 2004), which analyzes data using the MAS 5.0 algorithm. The SLWF data were analyzed by both SAM (Table III) and MAS 5.0 (data not shown). The conclusions drawn from both analyses were identical for these sets of genes (data not shown).

Table III shows that SLWFs influenced the RNA levels of a small number of sulfur-metabolism and glucosinolate-metabolism/catabolism genes. If a 2-fold change in RNAs was used as the sole criterion for identification of differentially regulated genes, five genes were induced/repressed by SLWF feeding. When statistically significant changes were evaluated (FDR ≤ 3.917%), two up-regulated and two down-regulated genes were identified. The cytochrome P450 gene *CYP79B2* (At4g39950) and the ATP sulfurylase *APS3* (At5g43780) showed 2.5- and 2.36-fold increases in RNAs, respectively (FDRs, 2.81%; Table III). *CYP79B2* RNAs also increased in response to *P. rapae* feeding and *E. cichoracearum* infection. CYP79B2 catalyzes the

Table II. *Expression of SA-, JA-, and ET-defense and -biosynthesis pathway genes in response to SLWF second- and third-instar feeding*

Pathway	Gene	Gene Locus	Function (TAIR)	Fold-Change	FDR
SA-defense pathway					
"Upstream" genes					
	EDR1	At1g08720	Mitogen-activated protein kinase kinase kinase	1.29	9.08
	PAD4	At3g52430	Lipase-like	3.52	3.17
	EDS1	At3g48090	Lipase-like	1.35	15.03
	SID2	At1g74710	Isochorismate synthase (ICS1)	3.84	4.19
	EDS5	At4g39030	MATE-transporter family	3.25	3.15
"Downstream" genes					
	NPR1	At1g64280	Regulatory protein	1.19	6.61
	PR1	At2g14610	Pathogenesis-related protein	5.58	3.90
	PR5	At1g75040	Pathogenesis-related protein	4.28	2.81
	BGL2	At3g57260	β-1,3-Glucanase	6.37	3.90
	WRKY70	At3g56400	Transcription factor	1.42	5.65
	NIMIN 1	At1g02450	Modulates PR1	2.67	5.65
	TGA2	At5g06950	Transcription factor	1.12	9.08
	NIMIN 3	At1g09415	Kinase interacts with NPR1/PR1	−1.28	18.00
JA-biosynthesis genes					
	PLD1	At3g15730	Phospholipase D α 1	1.27	6.61
	FAD7	At3g11170	Omega-3 fatty acid desaturase	−1.89	4.36
	FAD3	At2g29980	Omega-3 fatty acid desaturase	−2.66	6.23
	FAD2	At3g12120	Omega-3 fatty acid desaturase	−1.17	25.20
	LOX2	At3g45140	Lipoxygenase 2	1.22	9.08
	AOC1	At3g25760	Allene oxide synthase 1	−1.12	3.90
	AOC2	At3g25780	Allene oxide synthase 2	1.40	21.17
	JMT	At1g19640	JA carboxyl methyltransferase	−1.37	7.23
	AOS	At5g42650	Allene oxide synthase	1.13	5.98
	OPR3	At2g06050	12-Oxophytodienoate reductase	1.17	31.88
	SSI2	At2g43710	Stearoyl-ACP desaturase	−1.05	34.40
JA-defense pathway					
	COI1	At2g39940	F-box protein	1.27	9.08
	CEV1	At5g05170	Cellulose synthase	−1.22	13.63
	JAR1	At2g46370	JA amino synthetase	1.05	36.49
	VSP1	At5g24780	Vegetative storage protein	−2.33	9.08
	PDF1.2a	At5g44420	Defensin	−2.67	4.25
	MAPK4	At4g01370	Mitogen-activated kinase	1.15	15.03
	THI2.1	At1g72260	Thionin	1.02	25.21
	HEL/PR4	At3g04720	Hevein-like protein	2.29	5.33
ET-signaling genes					
	ERF1	At3g23240	Transcription factor	1.09	37.79
	ETR1	At1g66340	ET receptor	1.14	12.16
	EIN2	At5g03280	Metal-transporter family	1.13	15.03
	EIN3	At3g20770	Transcription factor	1.45	6.61
	CTR1	At5g03730	Ser/Thr kinase	−1.39	4.56

conversion of Trp to indole-3-acetaldoxime and is involved in the synthesis of both indole glucosinolates and the phytoalexin camalexin (Mikkelsen et al., 2000). Sulfurtransferase proteins are necessary in both primary sulfur- and glucosinolate-metabolism pathways (Fig. 4). SLWF instars influenced three genes implicated in primary sulfur metabolism; two sulfurtransferases were down-regulated >2-fold (At2g03750 and At5g07000) and an ATP sulfurylase RNA increased 10-fold (At3g59760).

Table III shows that similar to the SLWF, the fungal biotroph *E. cichoracearum* caused few changes in glucosinolate or sulfur-metabolism gene expression. Two-fold changes in RNA for only two genes were detected. The RNAs for the sulfurtransferase genes, At2g03770 and At1g13420, increased and declined, respectively, after *E. cichoracearum* infection. These RNAs were not altered after SLWF or aphid feeding. In contrast, caterpillar feeding caused increases in the RNAs encoded by both At2g03770 and At1g13420.

Unlike the trends in glucosinolate pathway gene regulation observed with SLWF and *E. cichoracearum*, aphid feeding caused a 2.2- to 9-fold repression of 13 glucosinolate genes and up-regulated *CYP79A2* (cytochrome P450), *GSH2* (glutathione synthase), and two sulfurtransferase-like protein genes (Atg26280 and At1g28170). None of the genes induced/repressed by SLWF feeding was modulated after aphid feeding. Responses to caterpillar feeding contrasted with the responses to the three biotrophs (SLWF, *M. persicae*, *E.*

Table III. *Expression levels of glucosinolate-metabolism and primary sulfur-metabolism genes in response to SLWF, M. persicae, E. cichoracearum, and P. rapae.*

Function	Gene	Gene Locus	Treatment[a]			
			SLWF	MP	EC	PR
Methylthioalkylmate synthase						
	MAM-I	At5g23010	−1.30 (12.16)	−1.79	1.09	**2.06**
	MAM-L	At5g23020	1.69 (5.98)	**−2.38**	1.54	1.56
Cytochrome P450						
	CYP79F1	At1g16410	−1.18 (12.16)	**−3.57**	−1.09	**2.79**
	CYP79F2	At1g16400	A	**−3.57**	−1.09	**2.79**
	CYP79A2	At5g05260	A	**2.17**	1.38	1.50
	CYP79B2	At4g39950	**2.50 (2.81)***	−1.96	**2.16**	**2.41**
	CYP79B3	At2g22330	−1.14 (26.41)	−1.12	1.10	**2.58**
	CYP83A1	At4g13770	−1.19 (19.53)	**−3.45**	−1.07	**1.92**
	CYP83B1	At4g31500	1.58 (2.81)*	−1.27	1.62	1.57
C-S lyase						
	SUR1	At2g20610	A	**−2.22**	1.13	1.60
S-Glucosyltransferase (S-GT)						
	UGT74B1	At1g24100	1.05 (15.03)	**−4.55**	1.25	1.59
Sulfurtransferases						
	AtST5a	At1g74100	1.27 (4.19)	**−3.23**	1.23	1.75
	AtST5b	At1g74090	−1.53 (6.61)	**−5.56**	1.07	1.79
	AtST5c	At1g18590	A	**−5.26**	1.31	**2.51**
		At2g03750	**−2.66 (2.81)***	−1.33	−1.28	1.03
		At1g13420	A	−1.37	**−3.33**	**3.80**
		At5g43690	A	1.39	−1.23	**3.88**
		At5g07000	**−2.26 (4.74)**	−1.61	−1.20	−1.16
		At5g07010	1.33 (15.03)	**−4.76**	−1.75	1.06
		At3g45070	1.01 (24.19)	1.75	1.03	**2.31**
		At4g26280	A	**2.85**	1.10	**2.02**
		At2g03770	A	1.55	**2.07**	**5.83**
		At1g28170	A	**2.02**	−1.14	1.78
		At2g14920	−1.11 (3.62)*	**−2.70**	−1.18	−1.18
		At2g27570	A	1.04	−1.11	1.25
		At2g03760	−1.36 (12.16)	1.64	1.15	1.42
2-Oxoglutarate-dependent dioxygenase						
	AOP1	At4g03070	1.17 (19.53)	**−3.45**	−1.06	1.04
	AOP2	At4g03060	−1.39 (15.03)	**−6.67**	−1.05	**2.46**
	AOP3	At4g03050	A	1.89	−1.10	**3.70**
Myrosinases						
	TGG2	At5g25980	−1.88 (12.16)	1.94	1.23	1.05
Epithioester protein						
	ESP	At1g54040	−1.47 (4.69)	−1.54	−1.37	**3.05**
ATP sulfurylases						
	APS1	At3g22890	1.01 (39.15)	−1.16	1.07	1.83
	APS2	At1g19920	−1.67 (7.23)	**−2.86**	−1.14	−1.06
	APS3	At5g43780	**2.36 (2.81)***	1.07	1.27	1.44
	APS4	At4g14680	−1.75 (9.08)	−1.56	1.04	1.78
APS kinases						
	AKN1	At2g14750	−1.09 (9.08)	**−3.70**	1.26	**2.19**
	AKN2	At4g39940	−1.21 (5.65)	**−2.70**	1.29	**2.78**
		At5g67520	1.31 (12.16)	−1.72	−1.67	**2.57**
		At3g03900	−1.19 (12.16)	−1.67	1.17	1.52
APS reductases						
	APR1	At4g04610	−1.06 (32.95)	**−5.56**	−1.14	**2.28**
	APR2	At1g62180	1.14 (12.16)	**−9.09**	−1.40	1.01
	APR3	At4g21990	1.09 (19.53)	**−2.56**	1.09	1.03
Sulfite reductase (SIR)						
	SIR	At5g04590	−1.10 (29.74)	−1.82	1.04	1.54

(*Table continues on following page.*)

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

Function	Gene	Gene Locus	Treatment[a]			
			SLWF	MP	EC	PR
O-Acetyl-serine(thiol)-lyases (Bsas)						
	OASA1	At4g14880	−1.00 (4.81)	−1.00	1.09	1.09
	OASB	At2g43750	−1.99 (4.19)	−1.61	−1.28	1.00
	OASC	At3g59760	***−10.00 (9.08)***	−1.35	1.24	1.88
	AtCYSD1	At3g04940	−1.40 (4.66)	−1.49	−1.61	−1.52
	AtCYSD2	At5g28020	1.31 (9.08)	***−3.70***	−1.47	−1.14
		At5g28030	1.09 (25.20)	***−9.09***	1.00	1.30
		At3g22460	1.65 (4.81)	**3.25**	−1.01	−1.02
	CS26	At3g03630	−1.30 (4.74)	1.11	−1.09	1.37
		At1g55880	A	1.09	1.16	1.20
Cystathionine γ synthases (CGS)						
		At1g33320	A	1.98	−1.23	1.42
	CGS1, MTO1	At3g01120	−1.38 (12.16)	−1.82	1.01	1.26
Cystathionine β-lyase (CBL)						
	CBL	At3g57050	−1.12 (5.33)	−1.03	1.10	1.14
Met synthases (Mets)						
	ATMS3	At5g20980	1.21 (13.63)	1.21	−1.32	1.36
	ATMS2	At3g03780	−1.27 (21.17)	***−2.22***	1.00	1.29
Glutamyl-Cys synthetase (GSH1)						
	GSH1	At4g23100	−1.17 (4.69)	1.00	−1.06	1.07
Glutathione synthases (GSH2)						
	GSH2	At5g27380	−1.12 (24.10)	**4.30**	1.14	1.14
Ser acetyltransferases (Serat)						
	AtSERAT2.1	At1g55920	1.44 (9.08)	**5.88**	−1.40	−1.18
	AtSERAT3.1	At2g17640	1.07 (21.17)	1.05	−1.05	−1.15
	AtSERAT2.2	At3g13110	1.13 (12.16)	***−3.125***	1.25	1.75
	AtSERAT3.2	At4g35640	A	1.61	1.24	1.54
	AtSERAT1.1	At5g56760	1.08 (12.16)	1.00	−1.22	1.85

[a] Responses of glucosinolate-metabolism and primary sulfur-metabolism genes to SLWF second and third instars, *M. persicae* (MP) adults and nymphs, *E. cichoracearum* (EC), and *P. rapae* (PR) larvae are shown. MP, EC, and PR data were collected from Genevestigator. Genes induced greater than or less than 2-fold are highlighted in bold or bold and italic, respectively. Genes considered "significant" in the SLWF microarray are marked with an asterisk (FDR < 3.917%), and FDRs are shown in parentheses after the fold-change value. Genes called "absent" on SLWF array are denoted with "A."

cichoracearum). *P. rapae* caused 14 glucosinolate-biosynthesis/catabolism gene RNAs to increase. This was not surprising, as JA treatments induce both primary and secondary sulfur-metabolism genes (Jost et al., 2005) and responses to JA and tissue-damaging herbivores overlap significantly (Reymond and Farmer, 1998; Rask et al., 2000; Reymond et al., 2000). When aphid and caterpillar gene expression patterns were compared, six genes displayed reciprocal regulation patterns (i.e. induced by caterpillar feeding and repressed by aphid feeding).

PAD4 Did Not Affect the Rate of SLWF Nymph Development

To further compare Arabidopsis phloem-feeding defenses to aphids and SLWFs, the expression of *PAD4* and stress-induced senescence genes was examined. *PAD4* encodes a lipase thought to function in SA accumulation. A recent study by Pegadaraju et al. (2005) shows that *PAD4* RNAs accumulate after aphid feeding and *PAD4* positively regulates senescence-associated genes (*SAG*s) in a SA-independent manner.

On the *pad4-1* mutant, *M. persicae* population growth rates were increased compared to wild type, suggesting that PAD4 regulates cellular metabolism to decrease susceptibility to aphids in wild-type plants.

Similar to *M. persicae*, *PAD4* RNAs increased in response to SLWF-instar feeding (3.2-fold, 3.17% FDR; Table II). To examine whether SLWF development was influenced by *PAD4*-dependent processes, the regulation of stress-induced *SAG*s and SLWF developmental rates on wild-type and *PAD4* mutant plants were evaluated. Table IV shows that, similar to the *M. persicae* (Pegadajaru et al., 2005), *SAG13* and *SAG21* RNAs increased during SLWF nymph feeding. In addition, SLWFs caused *SAG12* RNAs to increase. *SAG12* is not a stress-induced *SAG* and is not modulated after *M. persicae* infestation (Pegadaraju et al., 2005).

The biological role of *PAD4* in resistance to SLWF was investigated using a no-choice developmental assay, which measured the rate of nymph development (Fig. 5). Thirty adult SLWFs were caged on either *pad4-1* or wild-type Columbia plants and removed after 2 d to synchronize egg hatching and nymph
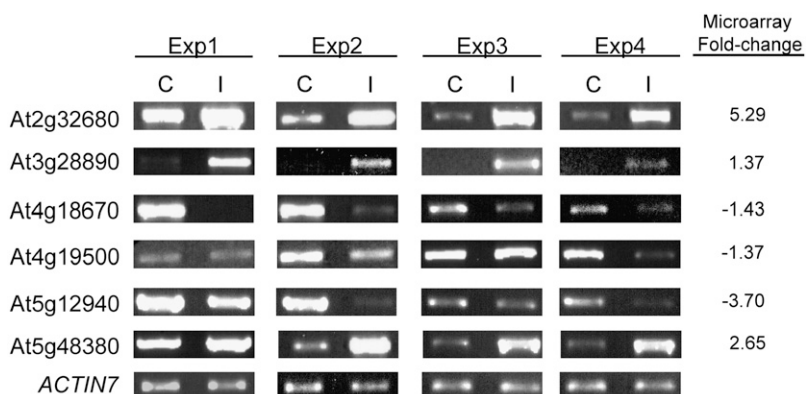
Figure 3. Confirmation of microarray data with RT-PCR. Gene-specific primers were designed for six LRR genes responsive to SLWF feeding with FDRs ≤3.917%. *ACT7* primers were used as a control for cDNA synthesis (20 cycles). PCR was performed using 30 to 35 cycles; for gene-specific primers, see "Materials and Methods." Experiment 1 to 4 (Exp1–Exp4) RNAs are pools from two biological replicates, representing a total of eight independent infestation experiments. Fold-change values are based on experiment 1 and 2 microarray data as calculated by the SAM program. At2g32680 and At3g28890 encode LRR proteins with similarity to the disease resistance genes Cf2.2 and HCr2-08, respectively. At4g18670 encodes an LRR extensin. At4g19500 encodes a TIR-NBS-LRR protein. At5g12940 encodes an LRR protein. At5g48380 encodes an LRR-kinase domain protein.

development. Ten replicate infestations were performed for each line and the experiment was repeated twice. The percentage of fourth instars was calculated 21 d after infestation. Figure 5 shows PAD4 did not influence SLWF development. This contrasts to the influence of PAD4 on aphid population growth (Pegadaraju et al., 2005) and other SA and JA mutants, which exhibit statistically significant differences in SLWF development (Zarate et al., 2007). It should be noted that the role of PAD4 on other SLWF life-history parameters cannot be discounted. The results of this bioassay along with the examination of glucosinolate and primary sulfur-metabolism genes suggested that aphid-plant and SLWF-plant interactions are distinct.

Cytological Examination of the Hypersensitive Response, ROS Accumulation, and Callose Deposition in SLWF-Infested Leaves

Hypersensitive response (HR), microHRs, and H_2O_2 accumulation are detected during pathogen infection and are important in modulating localized defense responses (Dempsey et al., 1999). Chewing insects can also induce oxidative changes in plants (Bi and Felton, 1995). To examine whether microHRs occurred and H_2O_2 accumulated during SLWF infestation, SLWF-infested and control leaves were examined after cytological staining. Positive staining near SLWF nymphs would indicate these defense responses were induced locally, as SLWFs tend to insert their stylets and navigate directly to the vascular tissue (Freeman et al., 2001). Figure 6 shows the results of trypan blue dye staining, which was used to monitor cell death. HR-associated cell death was characterized by localized areas of dark blue staining and was clearly observed during infection by avirulent *Hyaloperonospora parasitica* Hiks1 (positive control; Fig. 6, B and E). This

pattern of staining was not observed in the untreated control tissue (Fig. 6, A and D) or in SLWF nymph-infested tissue (Fig. 6, C and F). The SLWFs themselves stained light blue and empty egg casings appeared yellow on infested leaves (Fig. 6, C and F). These results show that localized cell death did not occur in response to the prolonged SLWF-instar feeding.

3,3'-Diaminobenzidine tetrahydrochloride (DAB) staining was used to monitor the production of H_2O_2. Mechanical wounding was used as a positive control and H_2O_2 was clearly detected as brown staining at wound sites (Fig. 6, H and K). Untreated tissue showed no DAB staining (Fig. 6, G and J). Similar to controls, DAB staining was not observed in the immediate area where SLWF second- and third-instar nymphs were feeding, indicating that at 21 dpi H_2O_2 accumulation was not associated with the established and prolonged feeding activity of SLWF nymphs (Fig. 6, I and L), despite increases in RNAs for several genes important in ROS (Table I). It should be noted that we cannot discount the possibility that H_2O_2 was produced transiently or at earlier time points in SLWF nymph-Arabidopsis interactions.

Callose deposition is observed in response to biotrophic fungal infection at papillae sites and in sieve elements in response to aphids in crop plants (Nishimura et al., 2003; Botha and Matsiliza, 2004).

Table IV. *Regulation of senescence genes after SLWF feeding*

Gene Name	Locus	Fold-Change	FDR
			%
SAG13	At2g29350	9.32	3.52
SAG21	At4g02380	2.03	5.98
SAG12	At5g45890	2.13	13.62
SAG27	At2g44300	A[a]	A

[a]Letter "A" designates gene called "absent" on ATH1 GeneChip.
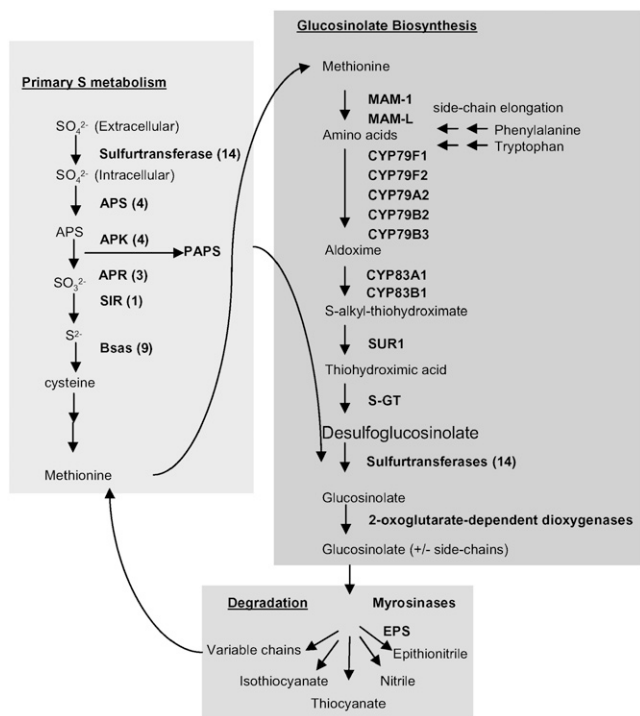
Figure 4. Primary sulfur- and glucosinolate-metabolism pathways in Arabidopsis. This figure was modeled after Hirai et al. (2005). Genes are listed in Table III.

CALS1 (*PMR4*, *GSL5*) is important for the synthesis of callose after wounding, during papillae formation, and in pollen development and fertility (Jacobs et al., 2003; Nishimura et al., 2003; Enns et al., 2005). Furthermore, *CALS1* mutants exhibit an increased resistance to fungal pathogens (Nishimura et al., 2003). The SLWF microarray data indicated that *CALS1* was the only member of the *GLUCAN-SYNTHASE-LIKE* (*GSL*) gene family whose RNAs increased after SLWF feeding, implicating callose deposition as part of Arabidopsis' induced defenses to whitefly feeding (Table I). To detect whether callose was deposited at feeding sites, SLWF nymph-infested tissue was stained with aniline blue. Wounding was used as a positive control; callose was clearly detected as blue fluorescence at the sites of razor incisions (Fig. 6N). The vascular tissue of untreated plants exhibited a light yellow fluorescence (Fig. 6M). In SLWF-infested leaves, callose deposits were detected as bright blue fluorescence directly beneath nymphs where feeding sites were likely established (Fig. 6O). In addition, callose deposits were also observed in vascular tissue in close proximity to nymphs in infested leaves (Fig. 6P).

DISCUSSION

Phloem-feeding insects are major agricultural and horticultural pests throughout the world, yet limited knowledge exists on how plants respond at the molecular level to these insects. Current knowledge

is primarily based on *M. persicae*, *Myzus nicotianae*, *S. graminum*, and *Macrosiphum euphorbiae* interactions with Arabidopsis, *Nicotiana attenuata*, sorghum, and tomato, respectively (Kaloshian and Walling, 2005; Thompson and Goggin, 2006). A small number of studies have examined interactions with other hemiptera at the molecular level (Kaloshian and Walling, 2005; Thompson and Goggin, 2006). This article presents a transcriptome analysis examining the expression of a significant proportion of Arabidopsis genes (approximately 22,000) in response to a phloem-feeding insect other than aphids.

In this study, 1,256 genes (FDR < 3.917%) were found to be differentially regulated in response to SLWF second- and third-instar feeding. Many of these genes have biological functions that are typically regulated in response to biotic stress, such as cell wall, oxidative stress, signal transduction, and nitrogen- and carbohydrate-metabolism genes, as well as genes with unknown functions. The SAM program proved to be a good method for differential analysis as even genes with low fold-change values (1.37-fold) showed detectable changes in RNA levels when monitored by RT-PCR. While some aphid microarray studies have reported problems with low signal intensities (Voelckel et al., 2004; Thompson and Goggin, 2006), this was not a limitation with the SLWF data set, for 14,815 probe sets had "present" calls and gene expression was as high as 14-fold.

Prior to this experiment, it had been assumed that, despite the disparate life histories of SLWFs and aphids, Arabidopsis defense responses to these phloem-feeding insects would be similar. Unlike many tissue-damaging insects, which induce production of JA, ET, and JA/ET-responsive genes, aphids primarily activate the SA-dependent pathway in Arabidopsis (Moran and Thompson, 2001; Ellis et al., 2002; Moran et al., 2002; De Vos et al., 2005). Published studies suggest that the expression of SA-pathway genes is variable as increases in specific *PR* RNAs are not observed in all studies (Moran and Thompson, 2001; Ellis et al., 2002; Moran et al., 2002; De Vos et al., 2005). The data presented here showed that another hemipteran, the SLWF, induced
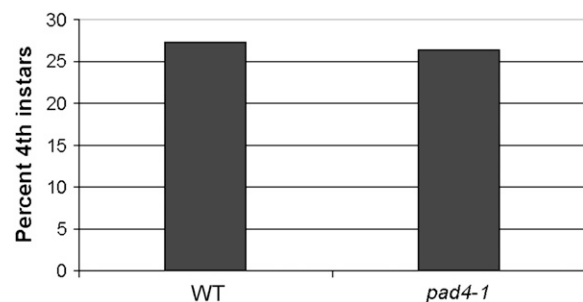

Figure 5. SLWF development on wild-type and *pad4-1* mutant lines. SLWF nymphs were counted 21 d after infestation. The percentage of fourth instars relative to the total number of nymphs was calculated. The rates of SLWF development in wild-type and *pad4-1* lines were not significantly different in a Student's *t* test (0.457).
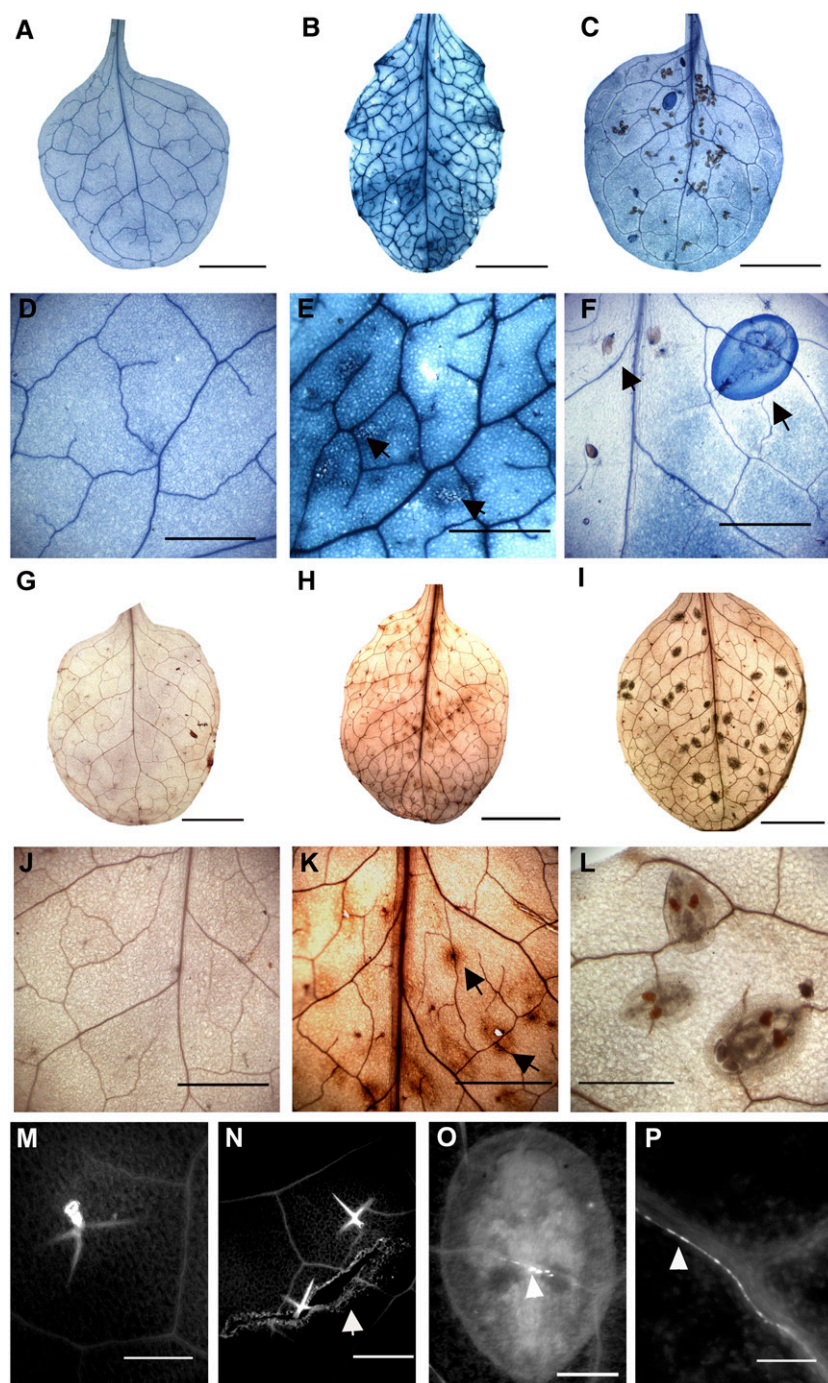
Figure 6. Cytological examination of Arabidopsis SLWF-infested tissue for microscopic lesions, H_2O_2 accumulation, and callose deposition. A to F, Trypan blue staining for HR. Untreated wild-type control plants (A and D); *H. parasitica* Hiks1-infected plants (B and E); and SLWF-infested plants (C and F) are shown. Eggs, second, and third instars are observed in C. Eggs and a third-instar nymph are shown in F. A to C, Bars = 2 mm. D to F, Bars = 500 μm. G to L, DAB staining to detect H_2O_2. Untreated wild-type control plants (G and J); wounded plants (H and K); and SLWF-infested tissue (I and L) are shown. SLWF second-instar (smaller) and third-instar (larger) nymphs are seen in I and L. G to I, Bars = 2 mm. J to L, Bars = 500 μm. M to S, Aniline blue staining for callose deposition. Callose was observed by fluorescence microscopy. Untreated wild-type control plants (M); wounded leaves (N); and SLWF-infested leaves (O and P) are shown. Callose was observed after wounding and in the vasculature in infested leaves. Callose deposition beneath a SLWF third-instar nymph is shown. Trichome fluorescence was also noted. M and N, Bars = 100 μm. O and P, Bars = 50 μm.

many genes in the SA pathway over 2-fold, including SA-biosynthesis genes and downstream SA-responsive *PR* genes. Expression of these genes was confirmed with RT-PCR (Zarate et al., 2007). The prolonged and continuous SLWF nymph-Arabidopsis interaction (21 d) may explain the consistent detection of many SA-pathway genes in these experiments.

Despite the similar induction of the SA pathway by SLWFs and aphids, many differences in the Arabidopsis transcriptome response and potential defenses were observed in this study. Examination of the over-lap of global responses, glucosinolate gene changes, expression of *PDF1.2* transcript, and *PAD4* bioassays suggested that aphid and SLWF-Arabidopsis interactions were genus specific. Of interest, when comparing global expression changes, only 17% of the gene changes observed in response to aphids were also observed in the SLWF microarray.

Changes observed in JA-regulated and glucosinolate-biosynthesis gene transcripts were different between SLWF and aphids. In *M. persicae*-Arabidopsis interactions, aphids increased JA-responsive RNAs, such

as *PDF1.2*, approximately 2-fold (Moran et al., 2002). Aphid species that more frequently puncture cells, such as the specialist *Brevicoryne brassicae*, cause *PDF1.2* RNAs to accumulate to higher levels (Moran et al., 2002). Consistent with SLWFs performing fewer cellular punctures and triggering elevated SA-regulated gene expression, the SLWF microarrays show that *PDF1.2* RNAs declined rather than increased in response to nymph feeding (Johnson and Walker, 1999; Freeman et al., 2001). These data suggested that SLWFs may evade JA-induced defenses by avoiding the tissue damage that activates JA responses or introduce effectors that suppress JA-dependent defenses (Zarate et al., 2007).

Further distinctions in the Arabidopsis response to SLWFs were discerned by evaluation of the glucosinolate-metabolism gene expression profiles after SLWF feeding and in response to three biotic threats, including a fungal pathogen (*E. cichoracearum*), chewing insect (*P. rapae*), and aphid (*M. persicae*). Consistent with minimal tissue damage introduced, SLWF nymphs and *Erysiphe* induced few changes in glucosinolate synthesis/metabolism gene RNAs. In contrast, the *M. persicae* microarray data sets suggest that aphids actively repressed many of these genes (De Vos et al., 2005). However, small changes in the aliphatic glucosinolate profile have been noted after *M. persicae* infestation (Mewis et al., 2005). The disparate patterns in glucosinolate- and sulfur-metabolism gene expression changes by these phloem-feeding hemipterans suggested that SLWFs and *M. persicae* are perceived differently or have developed different mechanisms to avoid the enhanced production of these toxic compounds. While glucosinolates actively deter aphids (Mewis et al., 2005), their potential role in host choice or nymph development is not yet known for whiteflies.

The unique species-specific interactions between phloem feeders and Arabidopsis were reinforced with a bioassay using the *pad4-1* mutant. SLWF development was comparable on *pad4-1* and wild-type plants (Fig. 5). In contrast, aphid population growth rate was increased on *pad4-1* plants (Pegadaraju et al., 2005). This was rather surprising as the microarray data showed that stress-induced *SAG* genes were induced after SLWF feeding in wild-type plants (Table IV). Examination of the expression profile of a larger set of senescence genes in Arabidopsis suggested that SLWF tends to change transcript levels of fewer genes than aphids (data not shown). Future studies examining the senescence genes regulated by *PAD4* may provide insight into how defenses to aphids differ from SLWFs.

The role of other defense responses, such as the HR (microscopic lesions), ROS accumulation (H_2O_2), and callose deposition, in Arabidopsis has not been well characterized in response to hemipterans. In this study, localized cell death and H_2O_2 were not detected during SLWF second and third nymph feeding despite the prolonged interactions with their feeding site. These data suggest that SLWF is perceived in a manner similar to many compatible pathogens; the HR and ROS that characterize pathogen gene-for-gene interactions were not seen. Interestingly, neither HR nor an oxidative burst is observed in insect gene-for-gene resistance in wheat (*Triticum aestivum*)-Hessian fly (*Mayetiola destructor*) interactions (Giovanini et al., 2006). Similarly, no HR is observed in compatible and incompatible *M. euphorbiae*-tomato interactions, although some ROS accumulate after 24 h (Martinez de Ilarduya et al., 2003).

Unlike HR and ROS, callose deposits were observed in the major and minor veins near SLWF nymph feeding sites. This was consistent with the 2-fold increase in *CALS1* RNAs observed in the SLWF microarray experiments. Callose plugs have been observed previously in the vascular tissue after aphid feeding on wheat (Botha and Matsiliza, 2004) and at the site of fungal penetration (Jacobs et al., 2003; Nishimura et al., 2003). While it had been postulated that callose plugs would impede fungal penetration, analysis of *CALS1* mutants indicates that when callose is absent in papillae, there is an enhanced resistance to virulent fungal pathogens (Jacobs et al., 2003; Nishimura et al., 2003). It has been proposed that callose may aid fungal infection by functioning as a structural support for hyphae, facilitating nutrient uptake, or functioning as a barrier for plant recognition of pathogen elicitors (Jacobs et al., 2003; Vorwerk et al., 2004). The role of callose in the establishment or maintenance of the intimate SLWF nymph-Arabidopsis interaction is not presently known. Future studies using callose synthase mutants are needed.

The induction of Arabidopsis defenses in response to SLWF nymphal feeding is unique to what has been observed in response to biotrophic pathogens and aphids. In general, while many defenses such as glucosinolate metabolism, HR, and H_2O_2 are induced by pathogens and aphids, these defenses do not appear to be induced by SLWF. Transcriptome analysis will provide a helpful tool to identify SLWF plant defenses and targets of insect manipulation. In particular, examination of repressed transcripts may prove insightful as effectual SLWF defense pathways have been shown to be repressed during SLWF feeding (Zarate et al., 2007).

MATERIALS AND METHODS

Plant Growth and Insect Maintenance

The SLWF colony (*Bemisia tabaci* type B; *Bemisia argentifolii* Bellows and Perring) was maintained on *Brassica napus* cv 'Florida Broad Leaf' grown under fluorescent and incandescent lights (180 μE m^{-2} s^{-1}) at 27°C and with 55% relative humidity under long-day (16 h light:8 h dark) conditions in the Insectory and Quarantine Facility at the University of California, Riverside. Brassica seeds were sown in 6-inch-diameter pots containing UC Soil Mix Number 3 and fertilized as needed with Miracle-Gro all-purpose water-soluble plant food according to manufacturer's instructions. Adult whiteflies were collected from infested plants by aspiration into 15-mL falcon tubes.

Individual Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia plants were grown for 21 d in 4-inch-diameter round pots under fluorescent and incandescent lights (180 μE m^{-2} s^{-1}) with 50% relative humidity, 23°C, and

an 8-h-light/16-h-dark cycle. One hundred adult whiteflies were collected into each 15-mL falcon tube, and a tube was placed upright in each pot. Plants were individually encased with 5- × 10-inch nylon bags that were secured to each pot with a rubber band. The whiteflies were released by unscrewing the falcon tube. After 7 d, the adult whiteflies were removed from the plants by aspiration. The infested and noninfested plants were caged for the remainder of the experiment to ensure any adults that escaped aspiration could not reach the plants. Rosette tissue was collected after 21 d, when second and third instars were observed on wild-type Columbia plants. Developmentally matched leaves were harvested from uninfested plants. Infestations were performed in two growth chambers; each chamber contained one experimental block, which included 10 control and 10 infested plants. This experiment was repeated for a total of eight biological replicate experiments.

SLWF Developmental Bioassay

For the no-choice nymph developmental assay performed with wild-type Columbia-0 and *pad4-1* plants, 10 plants/line were grown as described above. Thirty adult whiteflies were collected and caged on 2-week-old wild-type and *pad4-1* plants. Infestations were performed at 23°C. In an attempt to synchronize whitefly development, adults were removed after 2 d by aspiration and plants were recaged. At 21 d postinfestation, the number of nymphs (first, second, third, and fourth instars) per plant was tabulated and percentage of fourth instars was calculated (number of fourths/total nymphs). The experiment was repeated twice for a total of three experiments. Each infested plant had approximately 100 nymphs; this level of infestation is similar to that observed for field-grown Brassica (Liu, 2000) and infestation rates used in SLWF-Arabidopsis studies (Zarate et al., 2007).

RNA Isolation

Total RNA from the eight biological replicates was isolated using the RNAwiz protocol (Ambion) and purified using a RNAeasy column (Qiagen). RNA from the two biological replicates performed in each growth chamber were pooled to eliminate variance due to different environmental factors. This yielded the infested and control RNA pools used in the microarrays (experiments 1 and 2) and RT-PCRs (experiments 3 and 4). The quality of the RNA was determined by A_{260}/A_{280} absorbance readings. RNA integrity (1 μg) was verified by fractionation on a 1% formaldehyde gel.

Hybridization

Biotin-labeled cRNAs were synthesized from infested and control RNAs for experiments 1 and 2 at the University of California, Irvine, Microarray Facility using the Affymetrix Eukaryotic One-Cycle Target Labeling Assay protocol (Affymetrix GeneChip Expression, Analysis Technical Manual; Affymetrix). The labeled cRNA was hybridized to Affymetrix Arabidopsis genome ATH1 Chip arrays, washed, and scanned using a Hewlett-Packard Genearray scanner.

Data Analysis

The quality of the two replicate GeneChips and normality of the data were tested by plotting the signal log ratios of experiment 1 against experiment 2. Quantile normalization and background adjustment was performed using RMA in the Bioconductor program (Irizarry et al., 2003; Gentleman et al., 2004). Genes with "absent" calls, determined by MAS 5.0, in both replicate experiments were filtered out. Significant genes were identified using SAM (Tusher et al., 2001). The *PR1* gene was known to be induced by SLWF feeding prior to this experiment and was used as a cutoff for significant genes (FDR, 3.90%). A workable number of genes (1,256) with low FDR (*q* value < 3.917%) was identified by selecting a δ value of 2.06 (Supplemental Table S1).

For comparison, MAS 5.0 was performed using the standard parameters (Affymetrix GeneChip Expression, Analysis Technical Manual; Affymetrix) Genes with "absent" calls in replicate experiments were removed from further analysis. Genes were considered "significant" if their signals "increased" or "decreased" in both experiments and gene expression was >2- fold or <−2- fold (data not shown).

The MAS 5.0 files and four CEL files are submitted to GEO (http://www.ncbi.nlm.noh.gov/geo). Gene lists for Table I were compiled using gene lists for nitrogen-metabolism genes at Dr. Jen Sheen's Integrated Arabidopsis Gene Functional Annotation Web site (www.nyu.edu/fas/dept/biology/n2010/SupplementalData/table2.htm) and ROS genes from a review (Mittler et al., 2004). To examine general trends in the overlap of SLWF and *Erysiphe orontii* 7-d postinfection RNA profiles (http://ausubellab.mgh.harvard.edu/imds), a FDR < 4% was used to select for *E. orontii* genes (approximately 1,300). The *Myzus persicae* infestation data set of De Vos et al. (2005) and *Pieris rapae* data were accessed through Genevestigator (Zimmermann et al., 2006).

RT-PCR

Total RNA was DNase treated using TURBO-*free* DNase (Ambion). Oligo(dT)$_{21}$ primer (0.5 μg) was added and RNA denatured for 5 min at 70°C. RT was performed using ImProm-II reverse transcriptase and RNasin as indicated in the manufacturer's instructions (Promega).

PCR (95°C 5 min, 95°C 35 s, 55°C–64°C 35 s, 72°C 2 min; 20 cycles, final extension time 72°C 10 min) using *ACTIN7* (*ACT7*) primers was used to check the cDNA synthesis and equalize cDNA amounts between reactions (25 mM MgCl$_2$, 8 μM forward primer, 8 μM reverse primer, 1 unit Taq polymerase, 8 mM dNTPs). *ACT7* primers were designed to span intron 4 (1892–1989) to verify that cDNAs were free of genomic DNA contamination. Gene-specific primers were designed for each LRR gene by designing primers to unique segments of each gene. BLASTN was used to confirm that primers were gene specific (http://www.ncbi.nih.gov/BLAST/). For the RT-PCR reactions monitoring RNA from LRR genes, 30 cycles were used to detect induced RNAs and 35 cycles for suppressed RNAs. The following primers were used: *ACT7*, At5g09810: 5′-CTCATGAAGATTCTCACTGAG-3′ and 5′-ACAACAGATA-GTTCAATTCCCA-3′; At5g48380: 5′-ATTAGTCGTTGGGGTTGTTTTGT-3′ and 5′-ATTGGTTCTTGAATACTCGGGA-3′; At4g19500: 5′ -CGTAGCAGAT-TGTGGGACTC-3′ and 5′-TTCAAGGTTCTCCTGATTATTTC-3′; At2g32680: 5′-TCCTCTAATGGCTTTTCTGGT-3′ and 5′ -GCTTCCTGTAAACTTATTGT-CA-3′; At3g28890: 5′-ACCTTTCTCAACTTACCCGTCTC-3′ and 5′-TCTCA-CAATCTCGTCAAGTCAATG-3′; At5g12940: 5′-CATCGCTGATTGGAAG-GGAA-3′ and 5′ -ACACAAATGGTTATGGCTCAAG-3′; and At4g18670: 5′-GGTGGGGATGGAGGAGAGTA-3′ and 5′-GGTTGCCTGGGTTTGATGAT-3′.

Wounding and Hyaloperonospora Infection

Arabidopsis Columbia-0 plants were grown under short-day conditions as described. Three-week-old plants were infected with the avirulent pathogen *Hyaloperonospora parasitica* Hiks1 as described (Eulgem et al., 2004). Infected leaves were collected for staining at 7 dpi. As a positive control for H_2O_2 accumulation, 3-week-old leaves were wounded by crushing the leaf lamina using needle-nosed pliers and immediately stained (Jacobs et al., 2003). For callose deposition, tissue was wounded with a razor and collected after 24 h (Adam and Somerville, 1996).

Microscopy

Leaves were collected from SLWF-infested (21 dpi), *H. parasitica*-infected (7 dpi), wounded, and uninfested plants. For visualization of callose, leaves were cleared with 95% ethanol and stained with 150 mM K$_2$P0$_4$ (pH 9.5), 0.01% aniline blue for 2 h (Koch and Slusarenko, 1990). The leaves were examined for UV fluorescence using a Leica MZII fluorescence stereoscope at the Center for Plant Cell Biology at the University of California, Riverside (365 nm excitation, 396 nm chromatic beam splitter, 420 nm barrier filter). Images were captured using a SPOT RT CCD camera.

HR was visualized by staining with lactophenol-trypan blue (Martinez de Ilarduya et al., 2003). Whole leaves were stained in 90°C lactophenol-trypan blue for 2 min and allowed to incubate at room temperature for 2 h. The tissue was destained using chloral hydrate (2.5 g/mL) for 4 d. Leaves were mounted in 50% glycerol and examined under bright-field microscopy using a Leica MZII stereoscope. Images were captured as described above.

H_2O_2 accumulation was visualized by staining whole Arabidopsis leaf tissue with 2.8 mM DAB (pH 3.68). DAB was added to tissue and vacuum infiltrated for 20 min, then incubated at 37°C for 5 h (Martinez de Ilarduya et al., 2003). The DAB solution was removed and boiling 95% ethanol used to clear the tissue. Leaves were examined under bright-field microscopy as above.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Stress-response genes preferentially expressed in experiment 2.

Supplemental Table S2. Genes differentially expressed after SLWF nymph feeding.

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