Patterns of Gene Expression Upon Infection of Soybean Plants by *Phytophthora sojae*

Pat Moy,¹ Dinah Qutob,¹ B. Patrick Chapman,¹ Ian Atkinson,² and Mark Gijzen¹

¹Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario, N5V 4T3, Canada; ²London Regional Genomics Centre, Robarts Research Institute, London, Ontario, N6A 5K8, Canada

Submitted 24 February 2004. Accepted 7 June 2004.

To investigate patterns of gene expression in soybean (*Glycine max*) and *Phytophthora sojae* during an infection time course, we constructed a 4,896-gene microarray of host and pathogen cDNA transcripts. Analysis of rRNA from soybean and *P. sojae* was used to estimate the ratio of host and pathogen RNA present in mixed samples. Large changes in this ratio occurred between 12 and 24 h after infection, reflecting the rapid growth and proliferation of the pathogen within host tissues. From the microarray analysis, soybean genes that were identified as strongly upregulated during infection included those encoding enzymes of phytoalexin biosynthesis and defense and pathogenesis-related proteins. Expression of these genes generally peaked at 24 h after infection. Selected lipoxygenases and peroxidases were among the most strongly downregulated soybean genes during the course of infection. The number of pathogen genes expressed during infection reached a maximum at 24 h. The results show that it is possible to use a single microarray to simultaneously probe gene expression in two interacting organisms. The patterns of gene expression we observed in soybean and *P. sojae* support the hypothesis that the pathogen transits from biotrophy to necrotrophy between 12 and 24 h after infection.

Additional keywords: berberine bridge-like enzyme, FAD-linked oxidoreductase, methyl jasmonate, salicylic acid.

Oomycete plant pathogens are widespread and damaging to crops, ornamental plants, and natural environments. Species belonging to the genus *Phytophthora* are especially notorious because of their destructive potential as crop pathogens and invasive organisms. Soybean root rot (*Phytophthora sojae*), potato late blight (*P. infestans*), black pod of cocoa (*P. palmivora* var. *palmivora* and *P. megakarya*), eucalyptus die-back (*P. cinnamomi* var. *cinnamomi* Rands), and sudden oak death (*P. ramorum*) are examples of the many *Phytophthora* spp.

that are current disease problems throughout the world (Erwin and Ribeiro 1996; Kamoun 2000; Stokstad 2004).

A model for molecular and genetic studies of plant-oomycete interactions has been provided by studies on the *Arabidopsis* spp.-downy mildew pathosystem. Several *Arabidopsis* genes controlling resistance to *Peronospora parasitica* have been isolated and host responses to pathogen infection are well characterized (Holub 2001; Slusarenko and Schlaich 2003). Nonetheless, it is unknown whether plant responses may be conserved among different plant-oomycete interactions and there is scarcely any description of molecular mechanisms of oomycete colonization of plant cells (Kamoun 2003; Latijnhouwers et al. 2003; Tyler 2002).

The measurement of expression levels of thousands of genes at once by microarray analysis offers an opportunity to probe overall transcript patterns in particular organs, tissues, or cells. Microarray data provide an overview of gene expression patterns and illuminates targets for further study. For these reasons, microarray-based studies of plant-pathogen interactions are becoming more common. *Arabidopsis* spp. responses to infection by *Alternaria brassicicola* (Schenk et al. 2000; van Wees et al. 2003) *P. parasitica* (Maleck et al. 2000), and *Pseudomonas syringae* (Scheideler et al. 2002; Tao et al. 2003) have highlighted commonalities and differences in host plant defense to these diverse pathogens. There also have been numerous studies that employ other methods of monitoring changes in gene expression profiles in plant-pathogen interactions; for example, by serial analysis of gene expression (SAGE) (Thomas et al. 2002), cDNA amplified fragment length polymorphism (AFLP) (Durrant et al. 2000; van der Biezen et al. 2000), or generating expressed sequence tags (ESTs) (Qutob et al. 2000). The concept of an "interaction transcriptome" has been introduced as a means of describing overall transcriptional profiles of host and pathogen organisms occurring at an infection interface (Birch and Kamoun 2000). Sets of ESTs from mixed host-pathogen cDNA libraries represent a sample of the interaction transcriptome, and various methods for distinguishing host and pathogen genes have been developed (Hrabner and Weller 2001; Huitema et al. 2003a; Qutob et al. 2000). A natural extension of this line of work would be to include both host and pathogen gene targets on a single microarray in order to simultaneously follow changes in the host and pathogen during the course of infection. This is the approach we have applied to further characterize the interaction between soybean and the root rot pathogen, *Phytophthora sojae*.

P. sojae is an aggressive pathogen that is able to infect and kill soybean plants throughout much of the growing season. Annual soybean production losses in North America resulting from *P. sojae* infestations are estimated to exceed 10⁹ kg

Corresponding author: M. Gijzen; Telephone: 1-519-457-1470 ex 280; Fax: 1-519-457-3997; E-mail: gijzenm@agr.gc.ca

Current address of I. Atkinson: Biobasix Solutions, Inc., 326 Carlaw Avenue #202, Toronto, ON, M4M 3N8, Canada.

*The e-Xtra logo stands for "electronic extra" and indicates the HTML abstract available on-line contains five supplemental tables not included in the print edition.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2004.

(Wrather et al. 2001). *P. sojae* is a soilborne pathogen that produces free-swimming zoospores when conditions are suitably cool and wet. Zoospores are attracted to isoflavonoids secreted by soybean roots (Morris et al. 1998). Upon reaching the root surface, the zoospores attach, encyst, and elaborate a germ tube that penetrates into host tissues. Soybean cultivars that carry an effective resistance gene (*Rps*) to an attacking *P. sojae* strain react rapidly with a hypersensitive response (HR). This HR is activated within hours of zoospore attachment and arrests further pathogen growth. In susceptible interactions, no early HR occurs, and *P. sojae* is able to colonize host cells in an initial biotrophic phase of growth that lasts for approximately 12 h (Ward 1990). After this time, the pathogen begins to enter a necrotrophic growth mode, spreading quickly throughout host tissues, causing large, water-soaked, necrotic lesions and leaving dead host cells in its wake. Thick-walled oospores are produced abundantly in infected tissues and are able to survive for years in the soil.

In order to learn more about gene expression patterns in the host and pathogen during a susceptible interaction, we constructed a microarray of soybean and *P. sojae* cDNAs for large-scale transcriptional analysis. Results from this study of the host-pathogen infection site illustrate changes in routes of plant metabolism. Pathways of phenylalanine and phytoalexin biosynthesis, and certain classes of pathogenesis-related proteins were strongly upregulated in the host during infection. Steps in glycolysis and the citric acid and glyoxylate cycles also were upregulated. *P. sojae* genes that were detected during infection included several that encoded necrosis-inducing and elicitor proteins, and hydrolytic enzymes such as glucanases and proteinases. The timing and magnitude of transcriptional responses in the host and pathogen reflected changes in the state and progress of infection as *P. sojae* moved from an initial biotrophic phase to a necrotrophic mode of colonization.

Table 1. Composition of the host-pathogen microarray

Source of transcript, cDNA library ^a	Number of amplicons	Totals
<i>Glycine max</i>		
Root (Gm-r1021)	2,350	...
Cotyledon (Gm-r1030)	1,369	...
Infection site (HA)	186	...
Mixed (Gm-r1070) + Seed coat (SC)	22	3,927
<i>Phytophthora sojae</i>		
Zoospore (ZO)	338	...
Mycelia (MY)	356	...
Infection site (HA)	275	969
...	...	4,896

^a Transcripts from infection site cDNA library (HA) were predicted to originate from soybean or *P. sojae* based on G+C content and BLAST matches.

Table 2. Disease progression in susceptible soybean hypocotyls after inoculation with *Phytophthora sojae* zoospores^a

Time (h)	Lesion size ^b	Observations ^c
3	NV	Zoospores attach and encyst on the hypocotyl surface. Elaboration of germ tube and penetration into epidermal cells and first three layers of the cortex.
6	NV	Hyphae extend six to eight cell layers deep within the cortex. Most host cells in direct contact with pathogen remain alive. Haustoria abundant.
12	NV	Hyphae penetrate into deep layers of hypocotyl tissues. Early penetrated host cells begin to die, while cells at the margin of infection remain alive.
24	10–20 mm	Hyphae proliferate and are most abundant in the deep layers within the center of the hypocotyl. Host cell death begins to occur throughout infection site. Glyceollin phytoalexins accumulate. Macroscopic lesions apparent.
48	30–50 mm	Large, water-soaked lesions with spreading necrosis, yellow to dark brown in color. Infected tissue around inoculation site is macerated and collapsed. Extensive host cell death.

^a Summarized from the present study and previous work (Bhattacharyya and Ward, 1986; Stössel et al. 1980, 1981; Ward 1990; Ward et al. 1979, 1989).

^b After inoculation with a 10- μ l suspension of 10³ zoospores. NV = no visible lesion.

^c Generalized observations for a susceptible interaction, such as *P. sojae* P6497 on cv. Harosoy. Symptoms may vary depending on *P. sojae* isolate and host cultivar.

RESULTS

To study patterns of gene expression in the host and pathogen during infection of soybean by *P. sojae*, we constructed a high-density microarray containing genes from both organisms. Collections of cDNAs from ESTs provided a template for production of amplicons for spotting to glass slides. The microarray consisted mostly of soybean genes (80%) (Table 1). We chose to track changes in gene expression occurring in a compatible interaction during the course of infection. Sampling times at 3, 6, 12, 24, and 48 h after infection were selected to include early and late infection events, and to cover the transition from biotrophy to necrotrophy. An overview of disease progression during the time course is provided in Table 2. The experimental design is summarized in Figure 1 and Table 3. Three complete biological replicates, performed separately on different dates, provided independent mRNA samples for the many technical replicates that were done. We observed all recommendations of the minimum requirements for a microarray experiment (MIAME) checklist, and deposited our data in the Gene Expression Omnibus, National Center for Biotechnology Information (platform GLP1206; experiment GSE1352).

Soybean and *P. sojae* large subunit rRNAs differ in size.

To estimate the proportion of host and pathogen transcripts present in samples from the infection time course, we examined changing patterns of rRNA composition. Electrophoretic separation of total RNA followed by staining with ethidium bromide indicated that the large subunit of soybean rRNA has

Fig. 1. Schematic illustration of the experimental design is shown. Soybean seedlings were inoculated with water droplets or with water droplets containing zoospores from *Phytophthora sojae*. Inoculation sites were excised and samples of RNA were isolated from the infected and water-treated hypocotyls at the indicated time points.

a slightly higher mobility than its counterpart from *P. sojae* (Fig. 2). Compared with mixtures of soybean and *P. sojae* RNA preparations, we estimate that, at 12 h after infection, *P. sojae* RNA accounts for <10% of the total. However, the proportion of *P. sojae* RNA increases rapidly thereafter and accounts for approximately 40 and 70% of the large subunit rRNA by 24 and 48 h after infection, respectively. Thus, changes in rRNA abundance are in accord with observations of pathogen growth and proliferation shown in Table 2.

Large changes in host gene expression accompany infection.

To measure variability within the microarray experiments, normalized hybridization ratios from technical replicates were averaged in three pools corresponding to biological replicates. Average values from each biological replicate were used to calculate the coefficient of variation and the covariance (Table 4). The coefficient of variation among the biological replicates increased throughout the time course, from 36% at 3 h to 59% at 48 h. However, the covariance showed a strong positive trend throughout the time course. The covariance between data sets is low at early time points when relatively few genes, representing a small fraction of each data set, have changed in expression; whereas, at later time points, when massive shifts in gene expression have occurred, the covariance is high. These results indicate that there may be substantial variation in the averaged normalized hybridization ratios among the biological replicates but that, over time, the values from each data set tend to have a similar pattern of change.

The data normalization presents special considerations because the microarray and the mRNA samples each contain a mix of host and pathogen genes. For example, would it be more appropriate to normalize the soybean data using only the soybean spots? To determine the extent to which different normalization methods affected the selection of genes that change in expression, we compared results from three normalization procedures. A per block, spot, and chip (BSC) normalization method of all spots was compared with LOWESS normalization of all spots and with locally weighted scatter-plot smoothing (LOWESS) normalization of soybean spots alone. Our measure for identifying genes that changed in expression was based on fold change and reproducibility. To be selected, aver-

age normalization ratios for a gene had to show a twofold or greater change in expression in all three biological replicates. Genes consistently showing a twofold or greater increase or decrease in their average hybridization ratios in all three biological replicates were identified as upregulated or downregulated, respectively. Results demonstrate that there is a large overlap in selected soybean genes regardless of normalization method (Fig. 3). Normalization by BSC resulted in selection of 492 soybean genes, whereas LOWESS normalization of all spots or soybean spots alone resulted in selection of 519 and 584 genes, respectively. Normalization using the internal spikes and control spots or separate normalization of *Phytophthora* spots yielded unreliable results (*not shown*). Thus, we chose to retain results from our original BSC normalization procedure.

By this analysis, we identified 1,009 genes that change in expression throughout the time course (summarized in Table 5; complete lists are provided in Supplementary Data). Of the 820 genes that were upregulated, an estimated 517 were from *P. sojae*. All of the 189 downregulated genes corresponded to soybean transcripts. Although these numbers will be influenced by redundancy on the array, and by cross-hybridization of related transcripts, the analysis provides a good estimate of the fraction or percentage of genes that change in expression. Redundant genes that change in expression may be balanced

Fig. 2. Soybean and *Phytophthora sojae* large-subunit rRNAs differ in size. Total RNA purified from axenically grown *P. sojae* mycelia and from uninfected soybean hypocotyls was run individually or mixed in various proportions prior to analysis for comparison with RNA samples from infection sites. Total RNA from infected soybean tissues was collected at 3, 6, 12, 24, and 48 h postinoculation. Shown is an ethidium bromide-stained agarose gel, each lane containing approximately 2.0 µg of total RNA.

Table 3. Experimental design: biological and technical replication

Replicate	Number of slides hybridized per time point (h) ^a					Totals
	3	6	12	24	48	
Biological replicate 1	2	2	2	2	2	10
Biological replicate 2	2	2	2	2	2	10
Biological replicate 3	6	6	6	6	6	30
Total slides hybridized	10	10	10	10	10	50
Data points per amplicon ^b	20	20	20	20	20	100

^a Technical replication included reversing Cy-3 and Cy-5 labels (dye-swapping) between control and treatment samples.

^b Duplicate spots for each amplicon were included in the array.

Table 4. Variability among biological replicates

Measure of variation ^a	Time point (h)				
	3	6	12	24	48
Coefficient of variation (%)					
Rep1 × Rep2 × Rep3	36	41	47	52	59
Covariance					
Rep1 × Rep2	0.024	1.5	6.0	13	25
Rep2 × Rep3	0.033	2.0	5.5	26	80
Rep1 × Rep3	0.035	1.6	10	94	83

^a Normalized hybridization ratios from technical replicates were pooled to calculate 4,896 average values for each of the three biological replicates, X_b . Covariance was calculated by comparing pairs of X_b values from each sampling time point. Coefficient of variation was calculated from X_b values from all three biological replicates. Biological replicate 1, Rep1; biological replicate 2, Rep2; biological replicate 3, Rep3.

by redundant genes that did not change. Graphical illustrations of expression levels are shown for selected host and pathogen genes in Figures 4 and 5, respectively.

Most changes were apparent at later stages of infection. Only 22 genes were identified as changing at the 3-h time point, and all of these displayed increases in their hybridization ratios. Early upregulated soybean genes included matches to pathogenesis-related (PR) protein-1a and a flavin adenine dinucleotide (FAD)-linked oxidoreductase encoding a berberine bridge-like enzyme (BBE). Hybridization ratios for these two genes were consistently high. In fact, these two host genes were the only transcripts identified as upregulated in all five sampling times. Thus, host genes encoding PR1a protein and BBE are induced early and build in expression during infection more than any of the other genes on the array. Other host genes that were identified as upregulated early were similar to a putative transcriptional regulator, ADP ribosylation factor-1, β -hydroxysteroid dehydrogenase, and the 20S proteasome α -subunit.

Among the pathogen genes that were expressed early was a match to the yeast T-complex protein. This is an hsp60-type protein thought to be involved in actin and tubulin folding. Other *P. sojae* transcripts present early during infection showed similarity to the crinkling and necrosis-inducing protein CRN2 from *P. infestans* (Torto et al. 2003), and to the nodulin Mtn3 from *Medicago truncatula*. A proliferation-associated protein kinase, similar to counterparts described in yeast and *Cryphonectria* spp, was identified as another *P. sojae* gene

that increased in expression at the 3-h time point. Many other pathogen genes upregulated at 3 h showed no significant similarity to known proteins.

By 6 h after infection, 98 genes changed in their expression and virtually all of these (97) were upregulated. Induced-defense gene expression in the host had grown to include phenylalanine ammonia lyase and matches to other enzymes of phenylpropanoid metabolism. A single host gene declined in expression. This showed similarity to an auxin-repressed, dormancy-associated protein. On the pathogen side, genes with similarity to β -1,4 glucanases and proteinases were among those identified at the 6-h time point.

At 12 h after infection, 231 genes were upregulated (139 host and 92 pathogen). Host genes encoding enzymes of phytoalexin metabolism, such as isoflavone synthase, were prevalent among upregulated transcripts, as were genes with clear similarities to defense and signaling proteins, such as peroxidase, polyphenoloxidase, calmodulin, and protein kinase. Pathogen genes were varied and included several matches to chaperones and other protein-folding enzymes such as hsp60, hsp90, protein disulfide isomerase, and peptidyl-proline cis-trans isomerase.

Many host and pathogen responses peak at 24 h.

The 24-h sampling time represented a clear turning point in the infection process. Most changes in host gene expression after this time result from genes that decline in expression; the number and profile of host genes identified as upregulated at 24 h was similar to that at 48 h. An exception to this was a cluster of mRNAs encoding 40S ribosomal protein that only appeared at 48 h. Expression levels for many upregulated host genes were most intense at 24 h, although this may result partly from a sharp decline in the proportion of host transcripts present in the infection site by 48 h. The profile of upregulated host genes was dominated by matches to enzymes of phytoalexin biosynthesis and defense-related proteins. Many genes that matched enzymes corresponding to steps within central metabolic pathways, such as glycolysis and the citric acid and glyoxylate cycles, also were upregulated and peaked at 24 h. These patterns suggest that host cells rely heavily on β -oxidation of fatty acids as substrates and fuel for the manufacture of phenylpropanoid phytoalexins. The upregulation of the glyoxylate cycle in the host plant during infection by pathogens also has been noted in studies on *Arabidopsis* spp. (Scheideler et al. 2002).

In contrast, many genes on the array with similarity to enzymes of terpenoid metabolism declined or did not change in expression during infection, presenting delineations between phenylpropanoid and isoprenoid pathways. There were exceptions, however, such as soybean genes matching 1-deoxy-D-xylulose 5-phosphate reductoisomerase and tocopherol polyprenyltransferase, both of which were strongly upregulated in

Fig. 3. Venn diagram illustrating results from different normalization methods. Raw data was normalized per block, spot, and chip (BSC) or by the locally weighted scatter-plot smoothing method (GeneSpring 6.0), using all spots on the array or only those identified as soybean genes. Shown are the numbers of soybean genes that change twofold or more in expression in all three biological replicates.

Table 5. Number and percentage of genes that change in expression by twofold or more in all three biological replicates^a

Time (h)	Upregulated				Downregulated			
	Host		Pathogen		Host		Pathogen	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
3	8	0.19	14	1.3	0	0	0	0
6	35	0.89	62	6.2	1	0.025	0	0
12	139	3.5	92	9.4	0	0	0	0
24	164	4.2	424	43	34	0.83	0	0
48	189	4.8	326	34	173	4.2	0	0
Total ^b	303	7.7	517	53	189	4.6	0	0

^a Infected plants were compared with water-inoculated control plants. The total array consisted of 4,896 amplicons, including an estimated 3,927 host and 969 pathogen genes.

^b All time points. Differs from the sum of values from each time point due to redundancy.

step with genes such as isoflavone synthase. These genes may participate together in the biosynthesis of glyceollins, because these phytoalexins are prenylated isoflavonoids and, thus, would recruit components from each pathway for synthesis.

The 24-h sampling time also was a turning point from the pathogen perspective. The number of *P. sojae* genes identified

at 24 h was more numerous than at any other sampling time. This was surprising because we expected this to occur at 48 h, the time point at which the pathogen had grown and colonized the plant to the greatest extent. These results, along with the soybean data indicating that many host responses also peak at 24 h, suggest that *P. sojae* recruits a greater diversity of genes

Fig. 4. Expression levels for selected soybean genes that consistently showed a twofold or greater change in hybridization ratios. IN all, 52 genes were chosen from 492 soybean genes that changed in expression by twofold or more in all three biological replicates at any time point. Normalized hybridization ratios averaged from three biological replicates are represented in color, GenBank accession numbers, matches from BLASTX searches, and expect values. Matches to known soybean genes are highlighted in blue.

while the host is still actively waging a vigorous defense. It also is possible that the breadth of gene expression in *P. sojae* is maximal during transition from a biotrophic to a necrotrophic phase of growth. Each of these pathogen growth modes would be represented in the 24-h infection sites. Associations between the host and pathogen still may be at various stages of development at 24 h; whereas, by 48 h, the infection has progressed well into necrotrophy.

The spectrum of pathogen genes identified at the 24- and 48-h time points included several that plausibly are involved in facilitating infection or countering host defenses. Pathogen genes with strong similarity to proteinases, glucanases, and cutinases were expressed at these times. Two *P. sojae* genes similar to the cellulose-binding elicitor (CBEL) protein from *P. parasitica* (Gaulin et al. 2002) were highly expressed during infection and peaked at 24 h. Expression of the *P. sojae* necro-

Fig. 5. Expression levels for selected *Phytophthora sojae* genes that consistently showed a twofold or greater change in hybridization ratios. In all, 52 genes were chosen from 517 *P. sojae* genes that changed in expression by twofold or more in all three biological replicates at any time point. Normalized hybridization ratios averaged from three biological replicates are represented in color, GenBank accession numbers, matches from BLASTX searches, and expect values. Matches to oomycete genes are highlighted in blue.

sis-inducing protein occurred late during infection, results that were in accord with past findings (Qutob et al. 2002). The elicitor-like protein SOJY showed a similar pattern of expression, appearing at 48 h after infection. Previous studies have shown that the SOJY transcript is particularly abundant in zoospores (Qutob et al. 2003).

All of the selected downregulated genes corresponded to host transcripts, and virtually all of these were identified from the 24- and 48-h time points. Genes that declined in expression at 24 h included matches to enzymes of methionine, asparagine, and cellulose biosynthesis. Components of the photosynthetic apparatus also were downregulated. Surprisingly, many genes with possible roles in defense were strongly downregulated during infection. Prevalent among these were lipoxygenases, peroxidases, and metallothioneins. Transcripts encoding the cationic peroxidase 2 (Prx2; represented on the array by several ESTs, including GenBank accession number AW508620) declined by 10-fold or more during the infection time course. Transcripts encoding Prx2 are present in most soybean tissues, but reach their highest level of expression in seed coats (Gijzen et al. 1999a). There are several closely related transcripts that share a high degree of sequence similarity to Prx2, so it is possible that these genes decline collectively or that one or more predominant transcripts are downregulated. In addition to Prx2, many other class III peroxidases represented on the microarray showed varying patterns of expression, including several that were upregulated during infection. In contrast, expression patterns of arrayed lipoxygenase genes were mostly synchronous and showed consistent declines during infection. This data contrasts with findings of other studies that demonstrate that lipoxygenases are induced during pathogen infection of legumes and other plants (Porta and Rocha-Sosa 2002).

The possibility that lipoxygenases and other genes identified as downregulated simply resulted from a declining ratio of soybean to *P. sojae* RNA was considered but, overall, the results suggest otherwise. Altogether, only 189 of an estimated 3927 host genes were identified as downregulated. More massive shifts would be expected here if the declining proportion of soybean RNA dramatically affected hybridization ratios. We

also noted that many genes that are considered to be constitutively expressed, and often are used to normalize hybridization signals in conventional RNA blots, did not change in their expression. For example, a selection of soybean genes were identified as upregulated, unchanging, and downregulated, based upon our screening criteria (Fig. 6). Genes matching actin, β -tubulin, polyubiquitin, and 60S ribosomal protein displayed hybridization ratios that were near unity throughout the time course.

To confirm expression results obtained from microarray hybridizations, select genes were further analyzed by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Primers were designed to target soybean genes identified as upregulated and downregulated, and transcript levels were determined from independent mRNA samples isolated from *P. sojae*-infected tissues at 3, 24, and 48 h after infection. Additional treatments of wounding, methyl-jasmonate (MeJA), salicylic acid (SA), AgNO₃, and infection of soybean plants resistant to *P. sojae* strain P6497 were included for comparison with water-inoculated control plants. Applying AgNO₃ to soybean hypocotyls is known to induce defense responses and phytoalexin accumulation (Bhattacharyya and Ward 1986). Representative results from this analysis for three different genes are shown in Figure 7. Genes encoding BBE and a class III peroxidase were strongly upregulated during infection, confirming results from the microarray analysis. These genes also were induced in soybean plants resistant to *P. sojae* strain P6497, and after treatment with AgNO₃. Wounding, MeJA, and SA treatments were less effective in inducing these two genes. Measurement of lipoxygenase expression levels by real-time RT-PCR indicated that abundance of this transcript declines by more than 60-fold within 48 h after infection with a virulent *P. sojae* strain. Again, these results are congruent with the microarray data. In contrast, lipoxygenase expression levels did not change appreciably in soybean plants resistant to this strain of *P. sojae*. Treatment with AgNO₃ or MeJA, and wounding caused moderate increases in lipoxygenase expression by 48 h, whereas SA appeared to have no effect. Thus, the only treatment that resulted in strong downregulation of this gene was infection with a virulent *P. sojae* strain.

Fig. 6. Graph of microarray hybridization results for 12 soybean genes over the time course of infection. Four genes identified as upregulated are shown in red, four genes that did not change in expression are shown in black, and four genes identified as downregulated are shown in green. Putative identity and GenBank accession numbers are provided for each gene. Data values are averages of all three biological replicates.

DISCUSSION

Aside from the data emerging from recent EST studies, there is little information on overall transcriptional responses occurring in host and pathogen cells during infection of soybean by *P. sojae*. The availability of EST data and corresponding cDNA clones has made microarray experiments feasible for this host–pathogen interaction. We chose to analyze a susceptible interaction over a 48-h time course to focus on the disease process and to monitor early and late events during infection. This period covers the transition from biotrophy to necrotrophy and includes events during different stages of pathogen colonization and host defense.

It is worthwhile to consider the limitations of this study. The genes on the array represent only a sample of the complete gene-set of each organism. From the pathogen side, the experiment can be considered a single-dye hybridization, because there were no pathogen transcripts present in control tissues used for the pairwise comparison. Thus, pathogen genes identified as upregulated are genes that are expressed during infection, but are not necessarily infection-specific. Indeed, many transcripts originating from zoospore and axenic-mycelia cDNA libraries were among the expressed pathogen genes identified.

Changes in ratios of host and pathogen mRNA levels in treatment versus control samples over the time course also can

affect normalized hybridization ratios independent of actual expression levels, especially for host genes. In this regard, we devised an original method for estimation of the proportion of host and pathogen transcripts by comparison of large subunit rRNA ratios. These results show that *P. sojae* RNA is barely detectable during the first 12 h of infection, whereas it comprises nearly half of the total RNA by 24 h, and two-thirds or more by 48 h. The 48-h estimate is in close agreement with measurements of host and pathogen transcript ratios by analysis of G+C content of ESTs (Qutob et al. 2000). From the host side, a declining proportion of host mRNA present in infection sites can lead to two types of errors in interpreting microarray data. First, marginally upregulated genes may be missed if their increase is simply offset by the overall decline in the proportion of host mRNA. Second, the same factors can lead to false positives in the identification of downregulated host genes. One must be careful in drawing conclusions about downregulated host genes, especially at later time points. Nevertheless, many so-called housekeeping genes did not change in expression, and several host genes were identified that declined in expression by a factor of 10-fold or more. It is likely that these represent bona fide downregulated genes, because changes of this magnitude cannot be accounted for by the shift in the overall ratio of host and pathogen mRNA abundance.

Cross hybridization among related transcripts from a gene family also will occur on microarrays, similar to conventional RNA blot hybridizations. Thus, distinguishing expression patterns of closely related genes requires follow up with more discriminating methods. This may be especially important for host genes, because soybean is considered an allotetraploid, with many genes represented in highly conserved pair-sets. Cross hybridization between host and pathogen genes is less problematic. Plants and oomycetes are phylogenetically distinct and belong to different groups of crown eukaryotes. It also is known that soybean and *Phytophthora* genes differ significantly in G+C content and codon usage. Thus, it is unlikely that cross hybridization between soybean and *P. sojae* genes have seriously corrupted the data. There also is evidence to support this, because many soybean genes identified as upregulated were compared with sequences from the *P. sojae* whole genome and in no case did we discover significant similarity. Furthermore, consider the 14 *P. sojae* genes that were identified at 3 h. These were the only pathogen genes that showed any consistent hybridization signal at 3 h. All of these cDNAs match *P. sojae* genomic sequences, and none of them match any known soybean sequences. Thus, hybridization signals for these genes surely arise from expression by *P. sojae*, and not by cross hybridization of soybean transcripts.

Overall, the limitations of this work need to be considered when drawing conclusions, but the large amount of biological and technical replication that went into the experimental design makes the results meaningful and reliable. We also applied stringent criteria for identification of genes that change in expression. Targets selected for verification by real-time RT-PCR confirmed sequence identity and pattern of expression in every case. The expression profiles of many genes on the array also could be predicted from past studies. For example, soybean genes encoding phenylalanine ammonia lyase, chalcone synthase (Ebel and Grisebach 1988), isoflavone synthase (Dhaubhadel et al. 2004; Steele et al. 1999), chitinase (Gijzen et al. 2001), basic peroxidase (Yi and Hwang 1998), and many others have been well characterized and are known to be transcriptionally induced upon infection with *P. sojae*. Our microarray hybridization data is congruent with results from previous studies that employed more traditional methods of expression analysis for these

Fig. 7. Analysis of transcript levels in soybean hypocotyls by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Fold changes in transcript abundance were calculated by comparison with water-inoculated control plants via real time monitoring of PCR product formation using dye-labeled, gene-specific primers. Treatments were as follows: Wounded, wounding with a razor blade; MeJA, application of 50 μ M methyl jasmonate; SA, application of 1.0 mM salicylic acid; AgNO₃, application of 1.0 mM AgNO₃; *P. sojae* - R, inoculation of soybean plants cv. Harosoy 63, resistant to *Phytophthora sojae* P6497; and *P. sojae* - S, infection of soybean plants cv. Harosoy, susceptible to *P. sojae* P6497.

genes. These comparisons provide an additional “verification set” that validates the microarray data. Thus, we have a high degree of confidence in our data.

Generally, infection with *P. sojae* appears to hyperactivate SA-type pathways of defense such as phenylpropanoid metabolism and PR1a protein. The BBE also may be controlled by SA-dependent mechanisms because the pattern of expression of this transcript was nearly identical to *PR1a*. Transcripts encoding BBes appear to be widespread in plants, but the catalytic functions of most of the corresponding enzymes have not been determined. Those BBes that have been characterized generate H₂O₂ as a reaction product and are considered FAD-linked oxidoreductases. Recently, a tobacco BBE with glucose oxidase activity has been described, and a role has been suggested for BBes as defensive enzymes that produce H₂O₂ to inhibit microbial growth (Carter and Thornburg 2004). This proposal would fit well with our observations of BBE expression in soybean during *P. sojae* infection.

In contrast to SA-dependent transcription, pathways of MeJA appear to remain dormant or are downregulated, as evidenced by declines in lipoxygenases, certain proteinase inhibitors, and terpenoid metabolism. There is other data suggesting that oomycetes are poor inducers of MeJA and lipoxygenase pathways, because past studies on *Arabidopsis* spp. have shown that *Peronospora parasitica* does not induce lipoxygenase transcripts, whereas these genes are strongly induced during *Pseudomonas* infection (Slusarenko and Schlaich 2003). It has been suggested that biotrophic pathogens tend to activate SA-dependant pathways while necrotrophs induce MeJA-dependant networks (Nimchuck et al. 2003). Our work shows that *Phytophthora sojae*, a hemibiotroph, triggers a biotrophic-type of defense response from the host, perhaps because the pathogen initially grows as a biotroph during the early stages of infection. Once host defenses have been committed to a biotrophic-type response, it may not be possible to reverse or modify this to keep step with the pathogen’s shifting mode of growth. It is conceivable that plant-pathogenic *Phytophthora* spp. have evolved this strategy to confound or bypass plant defense responses. A study on tobacco demonstrated that lipoxygenases are necessary components of plant resistance to *P. parasitica* (Rancé et al. 1998), and nonhost resistance of *Arabidopsis* spp. to *P. infestans* has been associated with activation of MeJA pathways (Huitema et al. 2003b). Taken together, the results suggest that, in susceptible interactions, *Phytophthora* spp. suppress or evade activation of host MeJA pathways during infection because they are particularly prone to those types of defenses.

A more prosaic hypothesis is that the soybean lipoxygenase and peroxidase (*Prx2*) genes that we observe to decline in expression have a constitutive rather than defensive function, and are simply downregulated by the host to divert resources to more pressing needs. A study on gene expression during somatic embryogenesis in soybean would tend to support this view, because investigators found that expression of lipoxygenase and peroxidase (*Prx2*) genes more closely paralleled genes involved in storage protein and lipid biosynthesis, rather than defense-related genes (Thibaud-Nissen et al. 2003).

Although it is not yet clear why specific soybean genes such as lipoxygenases and *Prx2* decline dramatically, one can predict that certain changes in host gene expression result from the targeted action of pathogen virulence effectors. It is probable that oomycetes have evolved factors that are able to neutralize or interfere with host metabolism and defense, because these strategies are well known among bacterial pathogens of plants and animals. One would expect these pathogen effectors to be expressed during infection. Other *P. sojae* genes that contribute to invasion, ingress, and nutrient acqui-

sition during infection also will be expressed and are likely to be represented in abundance on the genes lists we have compiled here. Just considering the matches to known *Phytophthora* proteins, new and relevant data regarding the expression pattern of many genes of interest has been delineated. *P. sojae* transcripts matching the *P. infestans* CRN2 and the *P. parasitica* CBEL proteins tended to appear earlier during infection than transcripts encoding the *P. sojae* necrosis-inducing protein or the elicitor-like protein, SOJY. Expression of a *P. sojae* counterpart of the *P. infestans* thioredoxin peroxidase may have a role in detoxification of H₂O₂ produced at the infection site. The strong expression of transcripts matching the *P. infestans* H⁺-translocating ATPase suggests that these molecular pumps drive many transport processes required during plant infection.

In addition to providing expression data for individual genes, whether host or pathogen, this study presents a quantitative overview of transcriptional changes occurring during the course of infection. The results demonstrate that it is feasible to monitor changes in host and pathogen transcription simultaneously using microarrays comprising genes from each organism. To summarize, the greatest changes in *P. sojae* gene expression occurred between 12 and 24 h, coinciding with the rapid increase in the proportion of *P. sojae* rRNA present in infection sites. Many host defense responses also peaked or leveled off at 24 h, suggesting that a turning point in the infection process was past. Overall, it is striking how well this data fits the model of infection that has been proposed for *P. sojae*, and how these patterns can be interpreted in the context of hemibiotrophy.

MATERIALS AND METHODS

Biological material.

Soybean (*Glycine max* (L.) Merr.) cvs. Harosoy (*Rps7*) and Harosoy 63 (*Rps7*, *Rps1a*) were obtained from a collection at Agriculture and Agri-Food Canada in Harrow, Ontario. Seed were grown in the dark at 24°C for 16 h and 16°C for 8 h in water-soaked vermiculite for 7 to 9 days prior to all experimental treatments. *P. sojae* (M.J. Kaufmann & J.W. Gerde-mann) race 2, isolate P6497, is avirulent on Harosoy 63 and virulent on Harosoy, and was from the *Phytophthora* culture collection at the University of California, Riverside (Förster et al. 1994). Standard procedures were followed for culture and storage of *P. sojae* on vegetable juice (V8) agar at 25°C in the dark (Ward et al. 1979). Conditions for propagation of axenic-grown mycelia and zoospores were followed as described by Qutob and associates (2000).

Experimental treatments.

Inoculation of soybean hypocotyls with *P. sojae* zoospores was carried out as described by Qutob and associates (2002). For wounding, seedlings were cut with a razor blade by scarring the surface 1 to 2 mm deep along the hypocotyl length. Treatments with 50 µM MeJA (Bedoukian Research Inc, Danbury, CT, U.S.A.), 1 mM SA (Sigma-Aldrich, Oakville, ON, Canada), or 1 mM AgNO₃ were performed by placing 10-µl drops of the indicated solutions on soybean hypocotyls. Five to eight drops were placed along each hypocotyl length. Control seedlings were treated with sterile distilled water. Each treatment contained at least 20 hypocotyls. All treated seedlings were incubated in the dark at 25°C and tissue samples of treated hypocotyls were harvested after 3, 6, 12, 24, and 48 h. Excised tissue was flash frozen in liquid N₂ and stored at –80°C or immediately used for RNA isolation. Biological replicates were performed at different times with independent sets of soybean plants and *P. sojae* zoospores.

RNA extraction.

Total RNA from control, wounded, chemically treated, *P. sojae*-infected soybean hypocotyls was extracted according to the method of Wang and Vodkin (1994). Total RNA was quantified using a spectrophotometer and separated on denaturing 1.5% (wt/vol) agarose-formaldehyde gels to assess concentration and integrity. Samples of 10 µg of total RNA were used for linear amplification of mRNA using an in vitro transcription kit (RiboAmp, Arcturus, Mountain View, CA, U.S.A.) according to the manufacturer's protocol. Briefly, this included a first-strand reaction that yields cDNA incorporating a T7 promoter, a second-strand synthesis using exogenous primers to produce double-stranded cDNA, purification of double-stranded cDNA, synthesis of antisense mRNA (aRNA) by T7 RNA polymerase, and purification of aRNA. Quality and titer of the aRNA was determined monitoring absorbance at 260 nm after electrophoresis, using an automated instrument (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA, U.S.A.).

Amplification of target cDNAs for arraying.

Soybean and *P. sojae* cDNAs and ESTs generated from large-scale sequencing projects (Gijzen et al. 1999b; Qutob et al. 2000; Shoemaker et al. 2002) were from collections at Agriculture and Agri-Food Canada or were purchased from commercial sources (Incyte Genomics, St. Louis). The array was designed to be enriched for unigenes, and especially for transcripts originating from soybean root cDNA libraries because *P. sojae* is primarily a root pathogen. A total of 45 cDNAs with matches to enzymes of phenylpropanoid metabolism were selected to be included on the array, but otherwise transcripts were chosen at random. We estimate that the 4,896 spot array represents in excess of 4,000 cDNA transcripts.

Individual cDNAs were amplified from master EST library stocks by PCR using modified M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse primers (5'-AACAGCTATGACCATG-3') for the Gm-r1030-, Gm-r1070-, and Gm-r1021-specific clones. All others were amplified using T3 (5'-ATTAAACCCTCACTAAAGGA-3') and T7 (5'-TAATACGACTCACTATAGGG-3') primers. Amplification by PCR was performed in a thermocycler (ABI 9700; Applied Biosystems, Foster City, CA, U.S.A.) with each 50-µl reaction containing 2 µl of bacterial suspension, 2 mM MgCl₂, 0.25 mM each dNTP, 1 µM each primer, and 2.5 U of *Taq* DNA polymerase in 1× reaction buffer (Invitrogen, Carlsbad, CA, U.S.A.). Cycling conditions was 94°C for 1 min of initial denaturation; followed by 40 cycles of 92°C for 30 s, 56°C for 45 s, 72°C for 3 min 30 s; and terminated by a 5-min extension at 72°C. PCR products, arranged in 96-well format, subsequently were purified by gel filtration (Sephadex G-50; Amersham Biosciences, Piscataway, NJ, U.S.A.) and analyzed on 1.0% (wt/vol) agarose gels. Amplicons producing a single strong band were dried under vacuum (Speed Vac; Savant Instruments, Holbrook, NY, U.S.A.), redissolved in 20 µl of 50% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON, Canada) to a final DNA concentration of 100 to 200 ng µl⁻¹, and re-racked onto 384-well microtiter plates (Microarray Technology Microplates; Amersham Biosciences). Plates were sealed and stored at -80°C prior to printing.

Microarray design.

PCR products were printed onto glass reflective slides (type 7, Amersham Biosciences) using a spotting robot (Generation III; BIOGEM Molecular Dynamics, Sunnyvale, CA, U.S.A.). Each slide was designed to contain two identical arrays (amplicons represented in duplicate) of 12 blocks each, for a total of 24 subarrays. External controls (Lucidea Microarray Score-

Card 1.0 System, Amersham Biosciences) were included on each subarray to assess labeling and hybridization, but were not used in normalization. Prior to hybridization, slides were air dried and UV crosslinked at 50 mJ cm⁻².

Preparation of labeled probes.

Fluorescent-labeled cDNA probes were prepared by random-primed reverse transcription in the presence of cyanine-3 (Cy3)-dCTP or cyanine-5 (Cy5)-dCTP (Amersham Biosciences). Reverse transcription was performed in a 20-µl reaction volume containing 10 µg of aRNA; 5 µl of a control mRNA spike standard (Amersham Biosciences); 2 µg of random nonamers; 1 µg of oligo (dT); 1 µl of a dNTP mix containing 12 mM each dATP, dTTP, and dGTP and 3 mM dCTP; 3 µl of 1 mM Cy3- or Cy5-dCTP; 2 µl of 0.1 M dithiothreitol 1× reaction buffer; and 100 units of reverse transcriptase (SuperScript II, Invitrogen). Primers and aRNA were denatured at 70°C for 10 min, chilled on ice, and incubated for an additional 10 min at 25°C prior to addition of the remaining reaction components. Reverse transcription was carried out at 42°C for 2 h. Following cDNA synthesis, the RNA was degraded by adding 2 µl of 2.5 M NaOH to the mix and incubating at 37°C for 15 min. The reaction was neutralized with the addition of 10 µl of 2 M HEPES free acid (Research Organics Inc., Cleveland, OH). Unincorporated nucleotides were removed from the Cy3- or Cy5-labeled targets by absorption chromatography (QIAquick PCR purification kit; Qiagen, Valencia, CA, U.S.A.). Light absorption at 550 and 650 nm was measured using a spectrophotometer (Beckman DU 640; International MI-SS, Inc., Corona, CA, U.S.A.) to calculate incorporation of Cy3 and Cy5, respectively, into cDNA.

Microarray hybridization.

Slides were incubated for 20 min at 55°C in prewarmed pre-hybridization buffer (3.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.2% [wt/vol] sodium dodecyl sulfate [SDS], 0.1% [wt/vol] bovine serum albumin), rinsed three times in sterile distilled water, and dried by application of a gentle stream of nitrogen gas. Equal quantities (approximately 20 to 30 pmol) of each fluorescent Cy3- or Cy5-labeled probe was combined, dried under vacuum (Speed Vac, Savant Instrument, Holbrook, NY, U.S.A.), and resuspended in 30 µl of hybridization solution containing 50 ng of Poly(A) DNA in 50% (wt/vol) formamide and 25% (vol/vol) microarray hybridization buffer (Amersham Biosciences). The hybridization mix was denatured for 2 min at 92°C, cooled to room temperature, and applied to the array under a 24-by-60-mm coverslip (VWR Scientific, West Chester, PA, U.S.A.). Slides, placed in a sealed hybridization chamber containing 10 µl of water, were immersed in a 42°C water bath for approximately 16 h. After hybridization, the coverslip was removed in 0.2× SSC and washed on a rotary shaker at room temperature in 0.2× SSC, 0.1% (wt/vol) SDS for 3 min, 0.2× SSC for 3 min, and in 0.05× SSC for 10 s. Slides were dried immediately by nitrogen gas following the final wash. In all, 50 slide hybridizations were prepared using RNA from three independent biological replicates. For each of the 3-, 6-, 12-, 24-, and 48-h time points there were 10 hybridizations with probes derived from water-treated and *P. sojae*-infected seedlings reciprocally labeled with both Cy3 and Cy5 nucleotides.

Scanning and data acquisition.

Arrays were sequentially scanned for control channel (Cy3) and data channel (Cy5) fluorescence emissions using a laser scanner (Virtek, Waterloo, ON, Canada). Data normalization per BSC was performed as follows. Spot intensities were quantified individually using software (Array Vision 4.0; Imaging

Research, St. Catharines, ON, Canada). A preliminary normalizing script for standardizing Cy3 and Cy5 intensities on each of the 12 blocks (duplicated subarrays) was performed using a Perl script program. The algorithm calculated the mean in each pen area of the log10 ratios for Cy5 or Cy3 data after excluding zero and infinity values. A normalization factor was derived to bring the global (pen area) mean of the log10 ratio to 1. This factor was applied to the raw Cy5 or Cy3 ratios in the first step of normalization. The resulting values were consolidated in spreadsheet files (Excel; Microsoft, Redmond, WA), and imported into microarray analysis software (GeneSpring 6.0; Silicon Genetics, Redwood City, CA, U.S.A.). Further normalization of the values were performed using the per spot (data channel divided by control channel) and per chip (value of each spot divided by the 50th percentile) functions of the program (GeneSpring 6.0). Alternate methods of data normalization also were performed to compare results. Raw data from the laser scanner was normalized using LOWESS (GeneSpring 6.0), and this was repeated after selecting only the soybean spots.

Real-time quantitative PCR.

For quantitative RT-PCR experiments, 5 µg of total RNA was reverse transcribed to cDNA with random hexamers (ThermoScript Reverse Transcriptase Kit, Invitrogen). Real-time PCR was performed on a thermocycler (LightCycler; Boehringer Mannheim/Roche Diagnostics, Laval, PQ, Canada) using fluorescently labeled primers. Cycling conditions were performed using 2 µl of 1:10, 1:100, or 1:1,000 (vol/vol) dilutions of first-strand cDNA in a final volume of 20 µl, using buffers and conditions supplied by the manufacturer (FastStart SYBR Green I, Boehringer Mannheim/Roche Diagnostics). Gene-specific oligonucleotides for BBE, lipoxygenase, and peroxidase targets were as follows (5' to 3' sequences): BBE, forward CAAACAACCTGGGCTGCAG, reverse AGCAAGAGTTCTAGAGGGTC; lipoxygenase, forward CCGAGAGCATCCAAATACAA, reverse GCTCTATTATCGTTTGGACA; peroxidase, forward ACACCTACGTTTACGGCA, reverse, CCGGTGGTCACACAAT. Annealing temperatures and cycling conditions were optimized for each target, and are available upon request. Amplification rate for each target was evaluated from cycle threshold numbers obtained from serial cDNA dilutions. Amplification products were analyzed by agarose (0.8% wt/vol) gel electrophoresis as well as by analysis of dissociation (melting) curves following each run to determine optimal template and primer concentration. Amplification profiles monitoring relative amounts of PCR product were visualized on graphs plotting log of fluorescence against the number of cycles. Relative fold change in accumulation of any target under a given experimental treatment was standardized against cDNA derived from water-inoculated soybean tissue.

ACKNOWLEDGMENTS

We thank A. Gaidauskas-Scott for technical assistance; J. Brandle and I. van Grinsven for DNA sequencing; V. Poysa for soybean seed; and M. McLeod and C. Biltcliffe for assistance with labeling, hybridization, scanning, and normalization.

LITERATURE CITED

Bhattacharyya, M. K., and Ward, E. W. B. 1986. Resistance, susceptibility, and accumulation of glyceollins I-III in soybean organs inoculated with *Phytophthora megasperma* f. sp. *glycinea*. *Physiol. Mol. Plant Pathol.* 29:227-237.

Birch, P. R. J., and Kamoun, S. 2000. Studying interaction transcriptomes: Coordinated analyses of gene expression during plant-microorganism interactions. Pages 77-82 in: *New Technologies for Life Sciences: A*

Trends Guide. R. Wood, ed. Elsevier Science, London.

Carter, C. J., and Thornburg, R. W. 2004. Tobacco nectarin v is a flavin-containing berberine bridge enzyme-like protein with glucose oxidase activity. *Plant Physiol.* 134:460-469.

Dhaubhadel, S., McGarvey, B., Williams, R., and Gijzen, M. 2004. Isoflavonoid biosynthesis and accumulation in developing soybean seeds. *Plant Mol. Biol.* 53:733-743.

Durrant, W. E., Rowland, O., Piedras, P., Hammond-Kosack, K. E., and Jones, J. D. 2000. cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell* 12:963-977.

Ebel, J., and Grisebach, H. 1988. Defense strategies of soybean against the fungus *Phytophthora megasperma* f. sp. *glycinea*: a molecular analysis. *Trends Biochem. Sci.* 13:23-27.

Erwin, D. C., and Ribeiro, O. K. 1996. *Phytophthora Diseases Worldwide*. American Phytopathological Society Press, St. Paul, MN, U.S.A.

Förster, H., Tyler, B. H., and Coffey, M. D. 1994. *Phytophthora sojae* races have arisen by clonal evolution and by rare outcrosses. *Mol. Plant-Microbe Interact.* 7:780-791.

Gaulin, E., Jauneau, A., Villalba, F., Rickauer, M., Esquerre-Tugayé, M. T., and Bottin, A. 2002. The CBEL glycoprotein of *Phytophthora parasitica* var. *nicotianae* is involved in cell wall deposition and adhesion to cellulosic substrates. *J. Cell Sci.* 115:4565-4575.

Gijzen, M., Kuflu, K., Qutob, D., and Chernys, J. T. 2001. A class I chitinase from soybean seed coat. *J. Exp. Bot.* 52:2283-2289.

Gijzen, M., Miller, S. S., Bowman, L. A., Batchelor, A. K., Boutilier, K., and Miki, B. L. A. 1999a. Localization of peroxidase mRNAs in soybean seeds by *in situ* hybridization. *Plant Mol. Biol.* 41:57-63.

Gijzen, M., Miller, S. S., Kuflu, K., Buzzell, R. I., and Miki, B. L. 1999b. Hydrophobic protein synthesized in the pod endocarp adheres to the seed surface. *Plant Physiol.* 120:951-959.

Holub, E. B. 2001. The arms race is ancient history in Arabidopsis, the wildflower. *Nat. Rev. Genet.* 2:516-527.

Hraber, P. T., and Weller, J. W. 2001. On the species of origin: diagnosing the source of symbiotic transcripts. *Genome Biol.* 2:R37.

Huitema, E., Torto, T. A., Styer, A., and Kamoun, S. 2003a. Combined ESTs from plant-microbe interactions: using GC counting to determine the species of origin. *Methods Mol. Biol.* 236:79-84.

Huitema, E., Vleeshouwers, V. G. A. A., Francis, D. M., and Kamoun, S. 2003b. Active defence responses associated with non-host resistance of *Arabidopsis thaliana* to the oomycete pathogen *Phytophthora infestans*. *Mol. Plant Pathol.* 4:487-500.

Kamoun, S. 2000. *Phytophthora*. Pages 237-265 in: *Fungal Pathology*. J. Kronstad, ed. Kluwer Academic Press, Dordrecht, The Netherlands.

Kamoun, S. 2003. Molecular genetics of pathogenic oomycetes. *Eukaryotic Cell* 2:191-199.

Latijnhouwers, M., de Wit, P. J. M., and Govers, F. 2003. Oomycetes and fungi: similar weaponry to attack plants. *Trends Microbiol.* 11:462-469.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K. A., Dangel, J. L., and Dietrich, R. A. 2000. The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* 26:403-410.

Morris, P. F., Bone, E., and Tyler, B. M. 1998. Chemotropic and contact responses of *Phytophthora sojae* hyphae to soybean isoflavonoids and artificial substrates. *Plant Physiol.* 117:1171-1178.

Nimchuk, Z., Eulgem, T., Holt, B. F., and Dangel, J. L. 2003. Recognition and response in the plant immune system. *Annu. Rev. Genet.* 37:579-609.

Porta, H., and Rocha-Sosa, M. 2002. Plant lipoxygenases. Physiological and molecular features. *Plant Physiol.* 130:15-21.

Qutob, D., Hraber, P. T., Sobral, B. W. S., and Gijzen, M. 2000. Comparative analysis of expressed sequences in *Phytophthora sojae*. *Plant Physiol.* 123:243-253.

Qutob, D., Huitema, E., Gijzen, M., and Kamoun, S. 2003. Variation in structure and activity among elicitors from *Phytophthora sojae*. *Mol. Plant Pathol.* 4:119-124.

Qutob, D., Kamoun, S., and Gijzen, M. 2002. Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. *Plant J.* 32:361-373.

Rancé, I., Fournier, J., and Esquerre-Tugayé, M. T. 1998. The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. *Proc. Natl. Acad. Sci. U.S.A.* 95:6554-6559.

Scheidler, M., Schlaich, N. L., Fellenberg, K., Beissbarth, T., Hauser, N. C., Vingron, M., Slusarenko, A. J., and Hoheisel, J. D. 2002. Monitoring the switch from housekeeping to pathogen defense metabolism in *Arabidopsis thaliana* using cDNA arrays. *J. Biol. Chem.* 277:10555-10561.

Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C., and Manners, J. M. 2000. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proc. Natl. Acad. Sci. U.S.A.* 97:11655-11660.

- Shoemaker, R., Keim, P., Vodkin, L., Retzel, E., Clifton, S. W., Waterston, R., Smoller, D., Coryell, V., Khanna, A., Erpelding, J., Gai, X., Brendel, V., Raph-Schmidt, C., Shoop, E. G., Vielweber, C. J., Schmatz, M., Pape, D., Bowers, Y., Theising, B., Martin, J., Dante, M., Wylie, T., and Granger, C. 2002. A compilation of soybean ESTs: generation and analysis. *Genome* 45:329-238.
- Slusarenko, A. J., and Schlaich, N. L. 2003. Downy mildew of *Arabidopsis thaliana* caused by *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*). *Mol. Plant Pathol.* 4:159-470.
- Steele, C. L., Gijzen, M., Qutob, D., and Dixon, R. A. 1999. Molecular characterization of the enzyme catalyzing the aryl migration reaction of isoflavonoid biosynthesis in soybean. *Arch. Biochem. Biophys.* 367:146-150.
- Stokstad, E. 2004. Nurseries may have shipped sudden oak death pathogen nationwide. *Science* 303:1959.
- Stössel, P., Lazarovits, G., and Ward, E. W. B. 1980. Penetration and growth of compatible and incompatible races of *Phytophthora megasperma* var. *sojae* in soybean hypocotyls tissues differing in age. *Can. J. Bot.* 58:2594-2601.
- Stössel, P., Lazarovits, G., and Ward, E. W. B. 1981. Electron microscope study of race-specific and age-related resistant and susceptible reactions of soybeans to *Phytophthora megasperma* var. *sojae*. *Phytopathology* 71:617-623.
- Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H. S., Han, B., Zhu, T., Zou, G., and Katagiri, F. 2003. Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15:317-30.
- Thibaud-Nissen, F., Shealy, R. T., Khanna, A., and Vodkin, L. O. 2003. Clustering of microarray data reveals transcript patterns associated with somatic embryogenesis in soybean. *Plant Physiol.* 132:118-136.
- Thomas, S. W., Glaring, M. A., Rasmussen, S. W., Kinane, J. T., and Oliver, R. P. 2002. Transcript profiling in the barley mildew pathogen *Blumeria graminis* by serial analysis of gene expression (SAGE). *Mol. Plant-Microbe Interact.* 15:847-856.
- Torto, T. A., Li, S., Styer, A., Huitema, E., Testa, A., Gow, N. A., van West, P., and Kamoun, S. 2003. EST mining and functional expression assays identify extracellular effector proteins from the plant pathogen *Phytophthora*. *Genome Res.* 13:1675-1685.
- Tyler, B. M. 2002. Molecular basis of recognition between *Phytophthora* pathogens and their hosts. *Annu. Rev. Plant Pathol.* 40:137-167.
- van der Biezen, E. A., Juwana, H., Parker, J. E., and Jones, J. D. 2000. cDNA-AFLP display for the isolation of *Peronospora parasitica* genes expressed during infection in *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* 13:895-898.
- van Wees, S. C., Chang, H. S., Zhu, T., and Glazebrook, J. 2003. Characterization of the early response of Arabidopsis to *Alternaria brassicicola* infection using expression profiling. *Plant Physiol.* 132:606-617.
- Wang, C. S., and Vodkin, L. O. 1994. Extraction of RNA from tissues containing high levels of procyanidins that bind RNA. *Plant Mol. Biol. Rep.* 12:132-145.
- Ward, E. W. B. 1990. The interaction of soya beans with *Phytophthora megasperma* f. sp. *glycinea*: pathogenicity. Pages 311-327 in: *Biological Control of Soilborne Plant Pathogens*. B. Hornby, ed. CAB International, Wallingford, U.K.
- Ward, E. W. B., Cahill, D. M., and Bhattacharyya, M. K. 1989. Early cytological differences between compatible and incompatible interactions of soybeans with *Phytophthora megasperma* f. sp. *glycinea*. *Physiol. Mol. Plant Pathol.* 34:267-283.
- Ward, E. W. B., Lazarovits, G., Uwin, C. H., and Buzzell, R. I. 1979. Hypocotyl reactions and glyceollin in soybeans inoculated with zoospores of *Phytophthora megasperma* f. sp. *glycinea*. *Phytopathology* 69:951-955.
- Wrather, J. A., Stienstra, W. C., and Koenning, S. R. 2001. Soybean disease loss estimates for the United States from 1996 to 1998. *Can. J. Plant Pathol.* 23:122-131.
- Yi, S. Y., and Hwang, B. K. 1998. Molecular cloning and characterization of a new basic peroxidase cDNA from soybean hypocotyls infected with *Phytophthora sojae* f. sp. *glycinea*. *Mol. Cells* 8:556-564.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Microarray Gene Expression Data Society, minimum information about a microarray experiment:
www.mged.org/Workgroups/MIAME/miame.html
 National Center for Biotechnology Information, Gene Expression Omnibus:
www.ncbi.nlm.nih.gov/geo/