Parallel Genome-Wide Expression Profiling of Host and Pathogen During Soybean Cyst Nematode Infection of Soybean

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Global analysis of gene expression changes in soybean (*Glycine max*) and *Heterodera glycines* (soybean cyst nematode [SCN]) during the course of infection in a compatible interaction was performed using the Affymetrix GeneChip soybean genome array. Among 35,611 soybean transcripts monitored, we identified 429 genes that showed statistically significant differential expression between uninfected and nematode-infected root tissues. These included genes encoding enzymes involved in primary metabolism; biosynthesis of phenolic compounds, lignin, and flavonoids; genes related to stress and defense responses; cell wall modification; cellular signaling; and transcriptional regulation. Among 7,431 SCN transcripts monitored, 1,850 genes showed statistically significant differential expression across different stages of nematode parasitism and development. Differentially expressed SCN genes were grouped into nine different clusters based on their expression profiles during parasitism of soybean roots. The patterns of gene expression we observed in SCN suggest coordinated regulation of genes involved in parasitism. Quantitative real-time reverse-transcription polymerase chain reaction confirmed the results of our microarray analysis. The simultaneous genome-wide analysis of gene expression changes in the host and pathogen during a compatible interaction provides new insights into soybean responses to nematode infection and the first profile of transcript abundance changes occurring in the nematode as it infects and establishes a permanent feeding site within a host plant root.

Additional keywords: esophageal gland cell, parasitism genes, syncytium.

The soybean cyst nematode (SCN), *Heterodera glycines*, is an obligate, sedentary endoparasite which is consistently the most damaging pest of soybean (Wrather et al. 2001). The nematode life cycle consists of five life stages punctuated by four molts. After the first molt within the egg, SCN second-stage juveniles (J2) hatch into the soil and are attracted to host

plant roots by diffusates. Within hours of arriving at the root surface, the nematode has penetrated through epidermal cells and migrated intracellularly to the vascular cylinder of the root. Once the migratory juvenile reaches the vasculature, it selects an initial syncytial cell to initiate a permanent feeding site called a syncytium. The syncytium supplies the nutritional needs of the parasitic nematode to complete its life cycle (Endo 1986; Hussey and Grundler 1998). Once the juvenile begins to feed from the syncytium, it becomes sedentary and undergoes three more molts to either an adult vermiform male or a pyriform female. The male migrates back out of the root to fertilize the lemon-shaped female, which protrudes from the root, and then dies. The adult female secretes some of her eggs in a gelatinous matrix outside her body. However, the majority of eggs are retained within the uterus. Once the female dies, her body serves as a cyst to protect the eggs in the soil. Under favorable environmental conditions, an entire life cycle is completed in 25 to 30 days. The growth and development of the nematode is dependent on the successful establishment of a feeding cell, such that any impairment in syncytium formation can delay or completely disrupt further development.

Several studies have demonstrated changes in host gene expression during nematode infection of roots, specifically within the developing syncytium (Gheysen and Fenoll 2002; Hussey et al. 2002; Puthoff et al. 2003; Williamson and Gleason 2003). Evidence suggests that the process of syncytium formation involves alterations in basic aspects of programmed plant development, where normal cells dedifferentiate into a highly metabolically active cell type involving alterations to the cell cycle, hormone regulation, and cell wall architecture (Bird 1996; Bird and Koltai 2000; Davis and Mitchum 2005; Goellner et al. 2001; Hussey and Grundler 1998). Some of the processes leading to the establishment of nematode feeding cells are believed to be initiated and modulated by the encoded products of nematode parasitism genes expressed in the esophageal glands and delivered via the nematode's stylet directly into or within the vicinity of the initial feeding cell (Davis et al. 2000, 2004).

Different approaches have been employed to study the plant response to cyst nematode parasitism, including differential display (Hermsmeider et al. 1998, 2000; Vercauteren et al. 2001), promoter-reporter gene fusions (Barthels et al. 1997; Favery et al. 1998; Goddijn et al. 1993; Goverse et al. 1998; Mazarei et al. 2003, 2004; Niebel et al. 1996; Puzio et al. 2000), RNA blotting, protein immunolocalization, in situ hybridization (Goellner et al. 2001; Niebel et al. 1993), and differential library screening (Gurr et al. 1991). Similarly, con-

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struction of cDNA libraries using microaspirated contents of the esophageal gland cells of SCN parasitic stages has led to identification of several genes that may play critical roles in parasitism (Gao et al. 2001, 2003; Wang et al. 2001). Although these approaches have yielded valuable insights into the host response and nematode parasitism, the total number of identified cyst nematode and host plant genes involved in this host-parasite interaction still is relatively small.

Oligonucleotide and cDNA microarrays also have been used to study changes in plant gene expression at a single timepoint during early stages of cyst nematode infection in *Arabidopsis* (Puthoff et al. 2003) and soybean (Khan et al. 2004). A cDNA microarray also was used for developmental expression analysis of 1,358 cDNAs from the esophageal gland cell region of SCN (De Boer et al. 2002b). To our knowledge, a microarray study to simultaneously analyze changes in both plant and cyst nematode gene expression during nematode infection and development in host plant roots has not been conducted. Moy and associates (2004) demonstrated the utility of this approach for analyzing host and pathogen gene expression in the compatible soybean-*Phytophthora* spp. interaction using cDNA microarrays. Availability of cDNA libraries and extensive expressed sequence tag (EST) sequencing efforts in both soybean and SCN have led to the recent development of the GeneChip soybean genome array from Affymetrix, representing 35,611 soybean transcripts and 7,431 SCN transcripts, providing an excellent tool for simultaneous analysis of gene expression changes in both soybean and SCN.

In this study, three timepoints representing different stages of nematode parasitism and development in soybean were chosen for analysis. Our study of the soybean-SCN interaction transcriptome identified a large number of differentially expressed genes in both the host and pathogen. The lack of functional genomics resources in soybean and SCN is a major bottleneck in interpreting the large-scale data generated through the microarray approach. However, earlier studies suggest that homologous soybean and *Arabidopsis* genes share responsiveness to cyst nematode infection (Mazarei et al. 2003, 2004).

Thus, annotation of soybean genes based on sequence similarity to *Arabidopsis* genes with known function is useful for aiding in the identification of key biological processes altered during nematode infection. Using this approach, we were able to annotate 320 of the 429 differentially regulated genes to identify alterations in plant gene expression occurring globally over a time course of infection. The observed patterns of SCN gene expression suggest coordinated regulation of genes involved in parasitism, including cell-wall-modifying enzymes (e.g., cellulases and pectinases) and candidate parasitism genes (Gao et al. 2001, 2003; Wang et al. 2001).

RESULTS

To study the patterns of gene expression changes simultaneously in both the host and pathogen during nematode parasitism in a compatible interaction, we conducted a microarray experiment using Affymetrix GeneChip soybean genome arrays representing probes from both the soybean and SCN genomes. Sampling times at 2, 5, and 10 days postinoculation (dpi) were selected to cover different stages of nematode parasitism. At 2 dpi, the majority of infective J2 (now parasitic J2 [pJ2]) had arrived at the vascular cylinder and selected an initial syncytial cell to begin feeding (Fig. 1A and 1D). By 5 dpi, the sedentary pJ2 body was slightly swollen from feeding, had lost its somatic musculature, and may have had undergone the second molt to the early J3 life stage (Fig. 1B). At this timepoint, several cells have coalesced to form the syncytium (Fig. 1E). By 10 dpi, the syncytia were well developed and nematodes had advanced to early J4 life stages (Fig. 1C and 1F). Infection experiments were conducted in triplicate, with each experiment consisting of two treatments, mock and SCN-infected. To enrich tissues of interest, whole soybean root segments representing the major zone of infection were excised at different timepoints after inoculation and compared with uninfected excised root segments of the same developmental stage. To reduce variability inherent to the system, each root was inoculated with a known number of sterile infective J2 and the infection

Fig. 1. Developmental stages of soybean cyst nematode race 3 (HG type 0) and syncytia in soybean roots of susceptible cv. Williams 82. **A**, Early parasitic second-stage juveniles (J2s) at 2 days postinoculation (dpi). **B**, Late parasitic J2s and early J3s at 5 dpi. **C**, Late parasitic J3s and early J4s at 10 dpi. Soybean root sections showing syncytia at **D**, 2 dpi, **E**, 5 dpi, and **F**, 10 dpi. Nematodes in whole roots were stained with acid fuchsin stain. Soybean root sections were stained with toluidine blue. N = nematode and S = syncytium.

process was synchronized by washing the roots 24 h after the inoculation to remove any juveniles that had not yet penetrated the tissue. Furthermore, at least 30 root segments containing the sedentary nematodes were harvested and bulked for each timepoint. To avoid the early migratory phase of the infection process and the gene expression changes typically associated with this phase of infection, a relatively late initial timepoint of 2 dpi was selected because, at that time, most juveniles had reached the vasculature of the root and initiated feeding.

Statistical analysis was performed separately on soybean and nematode gene expression data derived from the chip analyses. Data from both SCN-infected and mock treatments were used to analyze changes in soybean transcript levels. Data from the SCN-infected treatments were used exclusively for the analysis of nematode gene expression.

Soybean gene expression changes during infection.

This experiment followed a split-plot design (Kuehl 2000) with two fixed factors: treatment (infected or uninfected) and time (2, 5, or 10 dpi). For each soybean gene, we conducted a mixed linear model analysis (described below). As part of each mixed linear model analysis, estimates of mean expression on the log scale were obtained for each combination of treatment and time. Each mixed linear model analysis included a test for treatment main effects and a test for treatment-time interaction. The test for treatment main effects tests the null hypothesis of no difference between treatments when averaging over time according to the equation $H_0: (I_2 + I_5 + I_{10})/3 = (U_2 + U_5 + U_{10})/3$, where I_2 , I_5 , I_{10} , U_2 , U_5 , and U_{10} denote the means corresponding to the six combinations of treatment and time. The test for interaction tests the null hypothesis $H_0: I_2 - U_2 = I_5 - U_5 = I_{10} - U_{10}$, which says that the difference between treatment means at each timepoint is equal to a single value common to all timepoints. When controlling the false discovery rate (FDR) at approximately 5%, 618 soybean genes were found to exhibit significant treatment main effects, and no genes were found to exhibit significant interaction. In the absence of interaction, it suffices to average over time and to consider only treatment main effects. However, because some genes exhibited significant interaction at higher FDRs (data not shown), we also conducted separate tests for treatment effects at each timepoint and have provided those results for the 618 differentially expressed genes online in Supplemental Table 1. These time-specific tests are less powerful than the tests for treatment main effects; thus, there are many genes for which none of the time-specific differences were significant despite the significant treatment main effect. The list of 618 statistically significant genes was further filtered by applying a cutoff of a 1.5-fold change between mock and treatment group. The 429 genes exhibiting more than a 1.5-fold change between at least one of the three treatment groups (mock to treatment at 2, 5, or 10 dpi) were considered biologically significant in this analysis. All the tables in this manuscript are coded by highlighting the fold-change values in dark gray for genes that are upregulated ($q = 0.05$ and >1.5 -fold change), light gray for genes that are downregulated ($q = 0.05$ and >1.5 -fold change), and white for genes that show only statistically significant ($q = 0.05$) differential expression but did not pass the 1.5-fold change cutoff.

Very limited functional information is available for the soybean sequences represented on the Affymetrix GeneChip soybean genome array; therefore, we tried to annotate the differentially expressed soybean transcripts by finding orthologs in *Arabidopsis*. By searching the TAIR *Arabidopsis* sequence database using the soybean EST sequences as a query, we were able to assign putative functions for 320 of the 429 differentially expressed soybean genes (Supplemental Table 1). Although the putative functions are based solely on nucleotide or

amino acid similarity and not experimental evidence, the analysis is useful for selecting candidate genes of interest for further work to obtain biologically relevant functional data. A large proportion of differentially expressed genes appear to be involved in basic plant metabolism. Transcripts for genes involved in plant secondary metabolism, including the biosynthesis of phenolic compounds, lignin, and flavonoids, also were upregulated early during nematode infection and remained elevated throughout nematode development. Similarly, genes for proteins related to stress and defense responses such as pathogenesis-related (PR) proteins, disease resistance responsive proteins, polygalacturonase-inhibiting proteins, harpin-induced family proteins, glutathione-S-transferases, peroxidases, and osmotin-like proteins were consistently upregulated after infection at all three timepoints. Subsets of cell-wall-modifying enzymes, including expansins, pectinesterases, xyloglucan endotransglycosylase, and extensins, were either up- or downregulated. However, the majority of these genes were upregulated during nematode infection. Genes possibly involved in plant growth and development, such as the auxin transport protein gene *PIN2* (*EIR1*), and *No Apical Meristem* (*NAM*) also were induced, as were ferritins, iron-binding proteins with a role in maintaining iron homeostasis in plants. Genes involved in cellular signaling and transcriptional activation, such as those encoding bZIP, ERF, MYB, WRKY, and zinc-finger transcription factors, were upregulated. A zinc-finger family transcription factor also was downregulated. Several leucine-rich repeat receptor-like kinases (LRR-RLKs) were both up- and downregulated.

Cluster analysis was performed on the 429 differentially expressed soybean genes based on a shared pattern of differential expression across adjacent timepoints. Separate clustering was done for uninfected (mock) and SCN-infected treatment groups. Based on this gene clustering method, genes could be classified into nine different clusters for each treatment group. The expression profile of each gene is summarized by a uniquely identifiable cluster combination corresponding to the expression pattern in the uninfected and infected roots. Following Vanneste and associates (2005), we presented the results of this clustering in a cross-table format (Fig. 2A). A prototypical expression pattern is depicted at the heading of each row and column in Figure 2A to facilitate interpretation of the counts in the table. The functional relevance of all cluster combinations was evaluated to gain further insight into the dataset. Mock cluster 5–infected cluster 5 is the largest cluster combination, with 75 genes. The expression of genes in this cluster combination do not exhibit any significant change between adjacent timepoints in either mock or infected roots. However, these genes were significantly differentially expressed between mock and infected roots. The majority of genes in this cluster combination are involved in metabolic processes. All genes with an annotated function in the cluster combination mock cluster 5–infected cluster 2 appear to be involved in defense response. Genes in this cluster combination do not show any significant change in their expression between adjacent timepoints in the mock group but are significantly upregulated from 2 to 5 dpi and stay constant between 5 and 10 dpi in infected roots. Although factors like redundancy on the array and the cross-hybridization of gene families should be taken into consideration when interpreting these results, this list provides a good estimate of the global changes in soybean gene expression during nematode parasitism of roots.

Quantitative real-time reverse-transcription polymerase chain reaction validation of soybean microarray results.

Quantitative real-time reverse-transcription polymerase chain reaction (qPCR) was used to validate the microarray results.

Fig. 2. A, Cross-table representation of the expression profiles within mock and treatment groups for the 429 differentially expressed soybean genes. The frequencies of each cluster combination within the dataset are indicated in each square. Lines in top and left squares show trend lines for changes in expression pattern across three timepoints. Numbers on the top row and left column indicate the cluster numbers. **B**, Cluster analysis of differentially expressed soybean cyst nematode (SCN) genes. Genes are classified into nine different clusters based on the significance of their expression changes across adjacent timepoints. The patterns represent early (clusters 2 and 8), late (clusters 4 and 6), transient (clusters 3 and 7), and continuous (clusters 1 and 9) changes during SCN development. Cluster 5 includes transcripts that exhibited significantly nonconstant expression across the three timepoints but with no significant changes in expression between adjacent timepoints. The genes in this cluster exhibit more gradual increases or decreases in expression than the genes with similar patterns in clusters 1 and 9. For visualization purposes, each gene's estimated mean log-scale expression profile was standardized to have mean 0 and variance 1 prior to plotting.

The 19 genes and primers used for qPCR validation to represent different expression patterns and their putative functions are listed in Supplemental Table 2. Total RNA from the same biological replicates used for microarray analysis was used for qPCR. These experiments included triplicate technical replication for each biological replicate. The quantification of gene expression was performed using the relative quantification ($\Delta\Delta C_T$) method and comparing the data with internal controls. The quantitative PCR results, by and large, are in agreement with DNA array hybridization results (Table 1). The estimated direction of the treatment effect was consistent for 49 of the 57 comparisons (approximately 86%), and the correlation between the $\log_2$ fold changes estimated by microarray and qPCR was 0.863. If we average over time, as was done in our microarray analysis due to the absence of significant interaction, the agreement between the microarray and qPCR results improves. The estimated direction of the treatment main effect was identical for all genes examined, and the correlation between the $\log_2$ fold changes averaged over time was 0.887. The differences between qPCR and microarray results include genes encoding an ethylene response factor (*ERF*), LeMir homolog, chalcone synthase, polygalacturonase-inhibiting protein 1 (*PGIP-1*), and PR protein 1 (*PR1*), which all were found to be upregulated in our microarray analysis at 2 dpi but were slightly downregulated in qPCR analysis. However, the microarray and qPCR results were qualitatively in agreement for these genes at later timepoints. We also observed differences for a gene encoding an expansin family protein which was found to be downregulated in microarray analysis at 2 dpi but shown to be upregulated by qPCR. Observed differences between microarray and qPCR were confined to the 2 dpi timepoint, with the exception of genes encoding a MATE efflux family protein and a cytochrome P450 which showed differences only at 10 dpi. This is likely due to the high level of variability inherent to the 2 dpi timepoint as a result of the migratory phase of the nematode life cycle, inability to control the number of nematodes in the roots, and the small size of syncytia. Differences in the magnitude of expression for several genes as detected by the two different methods also were observed (Table 1). The differences

between the microarray results and some of the qPCR results are likely attributed to the differences in sensitivity of the two different approaches, possibly due to cross hybridization of closely related gene families in microarray experiments, as well as the variability associated with the inability to control the number of nematodes within the root, which affects the number of host cells responding to infection in separate experiments.

Expression profile of nematode genes during infection.

Nematode gene expression data from the SCN-infected treatments at three different timepoints were compared because there were no nematode transcripts present in mock-infected tissues for pairwise comparison. Thus, nematode genes identified as differentially expressed across these timepoints are the genes that are induced or suppressed during nematode infection and development. In total, 1,871 genes were identified as differentially expressed during nematode development at a 5% FDR, of which 1,850 genes exhibited a 1.5-fold or greater difference in expression between at least one of the three time combinations used (2 to 5, 5 to 10, or 2 to 10 dpi). These 1,850 genes were grouped into nine clusters based on changes in their expression across the three timepoints (Fig. 2B). The first row of Figure 2B contains genes whose expression at 2 dpi was significantly less than at 5 dpi. The second row contains genes whose expression levels at 2 and 5 dpi did not differ significantly. The third row contains genes whose expression at 2 dpi was significantly greater than at 5 dpi. A list of all differentially expressed nematode genes with their predicted functions and the cluster information is available online in Supplemental Table 3.

Clusters 1, 2, and 4 include transcripts that exhibit a significantly increasing pattern of expression over the course of infection. Most of the transcripts in these clusters appear to be involved in nematode development and metabolism. Members of clusters 6, 8, and 9 show a significantly decreasing pattern of expression over the course of infection. The majority of previously identified parasitism gene candidates that are expressed in the nematode esophageal gland cells and encode products containing a signal peptide fall into these three clusters (Table 2). Cluster 3 represents transcripts that were significantly

Table 1. Soybean microarray data validation by quantitative real-time reverse transcription-polymerase chain reaction (qPCR)

Affymetrix probe ID	GenBank accession	Putative Function	Fold change ^a					
			2 dpi micro-array	qRT-PCR	5 dpi micro-array	qRT-PCR	10 dpi micro-array	qRT-PCR
GmaAffx.47493.1.S1_at	AW307476	Auxin transport protein (EIR1) PIN2	1.32	1.21	6.02	6.97	2.38	3.21
Gma.12330.2.S1_at	BI972758	ERF (ethylene response factor)/AP2 transcription factor family protein.	1.70	-1.47	2.31	2.79	1.89	1.21
Gma.2505.1.S1_a_at	AB062754	Ferritin	1.74	1.42	2.88	4.26	3.11	2.21
GmaAffx.55267.1.S1_at	BE020105	MATE efflux family protein	1.19	1.24	1.76	7.84	1.22	-1.35
GmaAffx.50677.1.S1_s_at	BI971744	Proline-rich extensin-like family protein	1.84	3.18	4.93	15.42	1.40	1.74
GmaAffx.91749.1.S1_s_at	CF807474	Leucine-rich repeat transmembrane protein kinase	1.48	2.08	20.03	5.99	-1.94	-4.54
Gma.11026.2.S1_s_at	BQ080041	LeMir (miraculin homolog) trypsin and protease inhibitor	1.20	-1.31	11.84	17.61	-1.76	-2.12
Gma.5283.1.S1_at	BM091956	Expansin family protein (EXPR3)	-1.06	4.86	6.79	75.66	11.67	91.38
GmaAffx.92774.1.S1_at	CF808124	Polygalacturonase inhibiting protein 1 (PGIP1)	1.46	-1.09	3.36	5.72	-1.06	-1.06
Gma.4332.1.S1_at	A1442841	Disease resistance-responsive family protein	5.37	2.78	26.48	18.23	2.79	3.23
GmaAffx.60533.1.S1_at	BQ453624	Laccase family protein	2.08	1.87	3.95	10.32	1.49	2.88
GmaAffx.90538.1.A1_s_at	CF806263	Pathogenesis-related protein 5 (PR-5)	3.03	4.07	17.57	27.62	11.72	44.79
GmaAffx.88845.1.S1_at	CK605838	Pathogenesis-related protein, similar to PR-1a protein	1.55	-1.08	16.29	20.26	8.11	10.48
Gma.4300.1.S1_at	S46989	Chalcone synthase	1.03	-2.06	2.28	4.74	1.12	1.65
Gma.2499.1.S1_at	CD407677	Gibberellin-regulated family protein, similar to GAST1 protein precursor	-1.19	-1.58	-1.68	-1.85	-1.34	-1.56
Gma.7227.1.S1_at	BU551112	Ubiquitin-conjugating enzyme 2 (UBC2)	-3.25	-1.69	-3.89	-1.78	-3.25	-2.85
Gma.4837.1.S1_at	BI969343	Nitrate transporter (NTP2)	-1.21	-1.85	-2.04	-2.32	-2.07	-2.43
GmaAffx.32345.1.A1_s_at	BI970277	Pectinesterase family protein	-2.29	-5.2	-1.83	-1.15	-3.05	-4.34
Gma.7418.1.S1_at	BU547276	Cytochrome P450	-1.13	-1.69	-2.37	-10.00	-3.06	2.61

^a Fold changes for each gene at each timepoint in soybean cyst nematode (SCN)-infected roots were determined by comparison to corresponding mock samples. The qPCR results are compared with microarray results at each timepoint. Negative values indicate that the gene is suppressed in SCN-infected roots. Dark gray boxes indicate genes upregulated by at least 1.5-fold, Light gray boxes indicate genes downregulated by at least 1.5-fold. The white boxes indicate genes with changes less than 1.5-fold between two timepoints.

Table 2. The 73 soybean cyst nematode gland-specific genes showing significant differential expression during development

Affymetrix probe ID	GenBank accession	Homolog accessions ^a	Homolog description	E value	Q value	Fold change (dpi) ^b		
						2 to 5	5 to 10	2 to 10
Cluster 1								
HgAffx.10157.1.S1_at	CB378344	AAP30770	DG pioneer protein 17G01	1E-27	0.0002	4.46	17.15	76.56
HgAffx.13278.1.S1_at	CD748618	AAR01198	SvG cellulose binding protein 3B05	5E-6	0.0018	9.96	5.33	53.09
HgAffx.19469.1.S1_at	CB278984	AAR01198	SvG cellulose binding protein 3B05	4E-9	0.0004	5.50	13.38	73.63
HgAffx.22522.2.S1_x_at	CK351795	AAL78214	DG cell pioneer protein SB84 (Hgg-20)	9E-8	0.0041	4.56	7.60	34.65
HgAffx.2890.2.S1_at	CK351817	AAL78214	DG pioneer protein SB84 (Hgg-20)	4E-16	0.0009	5.71	7.05	40.27
Cluster 2								
HgAffx.15851.1.S1_at	AF500034	AAP30773 AAN32886 AAP30754	DG pioneer protein 24A12 DG pioneer protein 2D01 DG pioneer protein 11A06	5E-72 6E-65 3E-57	0.0019	25.65	-1.16	22.18
HgAffx.15851.2.S1_x_at	AF500015	AAP30754 AAN32886 AAP30773	DG pioneer protein 11A06 DG pioneer protein 2D01 DG pioneer protein 24A12	4E-75 5E-72 6E-49	0.0278	31.75	-1.55	20.44
HgAffx.15851.3.S1_at	CD748340	AAP30754 AAN32886 AAP30773	DG pioneer protein 11A06 DG pioneer protein 2D01 DG pioneer protein 24A12	2E-76 2E-69 3E-48	0.0011	6.07	1.24	7.54
HgAffx.19290.1.S1_x_at	CK348866	AAL78214	DG pioneer protein SB84 (Hgg-20)	7E-10	0.0098	3.27	1.45	4.73
HgAffx.23868.1.S1_at	CD748226	AAR01198	SvG cellulose binding protein 3B05	2E-10	0.0055	5.56	1.37	7.62
HgAffx.24001.1.S1_at	AF070225	AAR85527	DG 14-3-3b protein [<i>Meloidogyne incognita</i>]	7E-13	0.0298	2.03	1.22	2.48
HgAffx.24001.2.S1_at	CK351582	AAR85527	DG 14-3-3b protein [<i>M. incognita</i>]	2E-73	0.0319	1.97	1.01	1.99
HgAffx.2849.1.S1_at	AF490248	AAO85456 AAN32892 AAP30755	DG pioneer protein 18H08 Secretory protein 4D06 Putative gland protein 29D09	3E-62 3E-5 9E-5	0.0136	4.21	-1.46	2.89
HgAffx.4006.1.S1_at	CB825557	AAN15806	SvG pioneer protein 5G05 (SP26) [<i>M. incognita</i>]	4E-40	0.0494	1.83	1.10	2.00
Cluster 3								
HgAffx.4037.1.S1_at	AF469058	AAR01198	SvG cellulose binding protein 3B05	7E-61	0.0011	23.42	-2.43	9.64
HgAffx.4135.2.S1_at	AF500020	AAM50038 AAO33476 AAP30759 AAP30760 AAP30761	DG pioneer protein 27D09 DG pioneer protein 6E07 DG pioneer protein 13A06 DG pioneer protein 10A07 DG pioneer protein 20G04	2E-39 3E-40 4E-40 9E-27 2E-25	0.0072	9.69	-4.73	2.05
Cluster 4								
HgAffx.19033.1.S1_x_at	AF500028	AAO85453 AAP30767	SvG pioneer protein 16A01 DG pioneer protein 21E12	4E-5 3E-4	0.0303	-2.89	9.05	3.14
HgAffx.2243.1.S1_at	CK351480	AAL78214	DG pioneer protein SB84 (Hgg-20)	1E-17	0.0122	-4.30	44.06	10.24
HgAffx.22522.2.A1_x_at	CK351795	AAL78214	DG pioneer protein SB84 (Hgg-20)	9E-8	0.0396	-6.18	30.88	5.00
HgAffx.10011.1.S1_at	CB378490	AAP30770	DG pioneer protein 17G01	1E-13	0.0234	-1.45	6.62	4.58
HgAffx.11249.1.S1_at	CB935201	AAP30770	DG pioneer protein 17G01	4E-9	0.0067	-1.48	10.61	7.19
HgAffx.16883.1.S1_at	CB279474	AAL78214	DG pioneer protein SB84 Hgg-20	9E-13	0.0034	1.10	28.53	31.35
HgAffx.19033.1.S1_s_at	AF500028	AAO85453 AAP30767	SvG pioneer protein 16A01 DG pioneer protein 21E12	4E-5 3E-4	0.0002	-1.67	597.88	358.60
HgAffx.19033.2.S1_at	AF500027	AAP30766 AAO85453 AAP30767	DG pioneer protein 30D08 DG pioneer protein 16A01 DG pioneer protein 21E12	2E-11 6E-7 2E-6	0.0130	-1.16	91.77	79.42
HgAffx.19033.4.S1_s_at	CK350593	AAP30766 AAP30767 AAO85453	DG pioneer protein 30D08 DG pioneer protein 21E12 DG pioneer protein 16A01	4E-5 4E-5 4E-5	0.0219	-1.09	16.11	14.83
HgAffx.1945.1.S1_at	CK351851	AAL78214	DG pioneer protein SB84 (Hgg-20)	6E-4	0.0136	-1.13	12.26	10.88
HgAffx.22522.2.S1_at	CK351795	AAL78214	DG pioneer protein SB84 (Hgg-20)	9E-8	0.0194	1.35	10.05	13.60
HgAffx.2890.1.S1_at	CK350408	AAL78214	DG pioneer protein SB84 (Hgg-20)	8E-81	0.0276	1.36	14.89	20.28
HgAffx.2890.2.S1_s_at	CK351817	AAL78214	DG pioneer protein SB84 (Hgg-20)	6E-36	0.0024	1.06	53.53	56.88
HgAffx.3143.1.S1_at	CK351245	AAL78214	DG pioneer protein SB84 (Hgg-20)	7E-6	0.0379	1.54	3.43	5.28
HgAffx.5473.1.S1_at	CD747951	AAR85527	DG 14-3-3b protein [<i>M. incognita</i>]	1E-41	0.0028	-1.25	5.24	4.20
HgAffx.6623.1.S1_at	CB825588	AAR85527	DG 14-3-3b protein [<i>M. incognita</i>]	6E-39	0.0126	-1.86	11.47	6.15
HgAffx.6660.1.S1_at	CB825551	AAP30770	DG pioneer protein 17G01	2E-24	0.0012	1.53	56.62	86.54
HgAffx.9769.1.S1_at	CB378732	AAP30770	DG pioneer protein 17G01	2E-18	0.0069	1.12	54.29	60.94
HgAffx.7996.1.S1_at	CB824215	AAP30770	DG pioneer protein 17G01	1E-18	0.0040	2.15	27.77	59.59

(Continued on next page)

^a BLASTX hits. All homologs are from *Heterodera glycines* unless indicated otherwise. Protein accession numbers and E-values are given for all the significant BLASTX matches to gland cell-specific genes. DG = dorsal gland; SvG = subventral gland.

^b Dark gray boxes indicate genes upregulated by at least 1.5-fold. Light gray boxes indicate genes downregulated by at least 1.5-fold. The white boxes indicate genes with changes less than 1.5-fold between two timepoints.

Table 2. (Continued from preceding page)

Affymetrix probe ID	GenBank accession	Homolog accessions ^a	Homolog description	E value	Q value	Fold change (dpi) ^b		
						2 to 5	5 to 10	2 to 10
Cluster 5								
HgAffx.17301.1.S1_at	CB935153	AAL78214	DG cell pioneer protein SB84 (Hgg-20)	1E-10	0.0372	2.63	2.30	6.04
HgAffx.19033.4.A1_at	CK350593	AAP30766	DG pioneer protein 30D08	4E-5	0.0418	-1.45	-1.72	-2.49
		AAP30767	DG pioneer protein 21E12	4E-5				
		AAO85453	DG pioneer protein 16A01	4E-5				
HgAffx.22856.1.S1_at	AF006052	AAC48327	SvG beta-1,4-endoglucanase-1	2E-29	0.0396	-1.63	-1.73	-2.83
HgAffx.4139.2.A1_x_at	CK348925	AAP30775	DG pioneer protein 32E03	1E-10	0.0425	-1.96	-2.78	-5.46
HgAffx.2032.1.S1_at	CB378359	AAL78214	DG pioneer protein SB84 (Hgg-20)	2E-14	0.0350	2.54	3.30	8.37
HgAffx.3706.1.S1_at	CB374919	AAL40720	SvG/DG calreticulin [<i>M. incognita</i>]	5E-40	0.0487	1.57	1.68	2.64
Cluster 6								
HgAffx.16985.1.S1_at	CB279371	AAP30757	DG pioneer protein 30G12	2E-11	0.0169	-1.91	-2.87	-5.49
		AAO33477	DG pioneer protein 4G05	2E-11				
		AAP30758	Putative gland protein 25A01	2E-11				
HgAffx.20336.1.S1_at	AF044210	AAC15708	SvG beta-1,4-endoglucanase-2	4E-26	0.0106	-2.04	-4.78	-9.75
HgAffx.21086.1.S1_at	AF490250	AAO85458	DG pioneer protein 19C07	4E-43	0.0108	-1.78	-2.66	-4.74
HgAffx.22005.4.S1_x_at	AF344863	AAL78212	DG pioneer protein SB89 (Hgg-25)	1E-47	0.0123	-1.77	-2.44	-4.32
		AAP30776	DG pioneer protein 34B08	4E-27				
HgAffx.3020.1.S1_at	AF468679	AAN14978	SvG chitinase 3D11	4E-63	0.0026	-1.28	-2.45	-3.13
HgAffx.4275.4.S1_s_at	AF345800	AAL78228	SvG pioneer protein GSB3 (Hgg-24)	3E-130	0.0039	-1.53	-3.51	-5.37
		AAP30764	DG pioneer protein 28B03	2E-94				
HgAffx.3578.1.S1_at	AF469059	AAN32888	DG annexin 4F01	3E-60	0.0325	1.43	-2.10	-1.47
HgAffx.14339.1.S1_at	AF490246	AAP30774	DG pioneer protein 30E03	1E-88	0.0243	1.04	-3.05	-2.93
		AAO85454	DG pioneer protein 16B09	1E-88				
		AAP30768	DG pioneer protein 22C12	4E-84				
HgAffx.20813.1.S1_at	AF502393	AAP30836	DG pioneer protein 30C02	2E-77	0.0242	1.33	-3.59	-2.71
HgAffx.22770.1.S1_at	AF469060	AAN32889	DG ubiquitin extension protein 4G06	7E-44	0.0327	-1.15	-2.13	-2.46
HgAffx.22811.1.S1_at	AF343567	AAL73347	SvG venom-allergen protein (Vap-1) GSB9 (SP13)	1E-65	0.0256	-1.27	-2.89	-3.67
HgAffx.24067.1.S1_at	AF520565	AAO63754	DG chorismate mutase 45D07	1E-80	0.0041	-1.30	-3.97	-5.14
HgAffx.3111.1.S1_at	AF500026	AAP30765	SvG pioneer protein 15A10	5E-73	0.0029	1.05	-4.18	-3.97
HgAffx.3699.1.S1_s_at	AF473827	AAO33474	DG CLE protein 4G12	1E-58	0.0115	-1.27	-2.22	-2.82
		AAG21331	DG CLE protein SYV46 (SP1)	1E-56				
HgAffx.4135.1.S1_at	AF500021	AAM50038	DG pioneer protein 27D09	3E-31	0.0470	1.40	-3.68	-2.62
		AAP30761	DG pioneer protein 20G04	2E-74				
		AAP30760	DG pioneer protein G10A07	2E-73				
HgAffx.4135.3.A1_at	CK349109	AAM50038	DG pioneer protein 27D09	11E-3	0.0475	1.28	-9.32	-7.30
HgAffx.4139.1.S1_at	AF500036	AAP30775	DG pioneer protein 32E03	4E-32	0.0124	1.10	-2.52	-2.28
HgAffx.4275.1.S1_at	AF500025	AAP30764	DG pioneer protein 28B03	8E-123	0.0161	-1.55	-2.74	-4.25
		AAL78219	DG pioneer protein SB26 (Hgg-26)	2E-117				
		AAL78228	SvG pioneer protein GSB3 (Hgg-24)	6E-66				
HgAffx.4275.2.S1_at	AF500024	AAP30763	DG SKP-1 like protein 8H07	3E-166	0.0212	-1.47	-2.55	-3.75
HgAffx.4275.4.A1_at	AF345800	AAL78228	SvG pioneer protein GSB3 (Hgg-24)	3E-130	0.0236	-1.63	-4.65	-7.58
		AAP30764	DG pioneer protein 28B03	2E-94				
Cluster 8								
HgAffx.22005.5.S1_s_at	BF013581	AAL78212	DG pioneer protein SB89 (Hgg-25)	2E-25	0.0157	-3.01	-1.86	-5.60
		AAP30776	DG pioneer protein 34B08	9E-16				
HgAffx.2130.1.S1_at	AY101191	AAM50039	SvG cellulase 26D05	0	0.0222	-4.22	-1.91	-8.05
HgAffx.22005.2.S1_at	AF500037	AAP30776	DG pioneer protein 34B08	1E-46	0.0278	-3.31	-1.92	-6.36
HgAffx.22045.1.S1_at	BG310823	AAM21970	SvG pectate lyase 2 [<i>Globodera rostochiensis</i>]	3E-23	0.0409	-5.20	-2.28	-11.88
HgAffx.23342.1.S1_at	BF014507	CAC83611	SvG expansin EXPB1 [<i>Globodera rostochiensis</i>]	7E-39	0.0230	-6.76	-2.33	-15.72
HgAffx.99.1.A1_at	AF273735	AAG21337	DG protein SYV42 (SP8)	3E-55	0.0067	-2.46	-1.24	-3.06
Cluster 9								
HgAffx.20336.2.S1_s_at	AY043224	AAP88024	SvG beta-1,4-endoglucanase-4	2E-79	0.0022	-2.40	-11.78	-28.29
HgAffx.22005.1.S1_at	AF502392	AAP30835	DG proline-rich glycoprotein 33E05	5E-125	0.0006	-3.55	-2.57	-9.12
		AAL78211	proline-rich glycoprotein GSB24 (Hgg-15)	9E-54				
		AAP30776	DG pioneer protein 34B08	4E-34				
HgAffx.22014.1.S1_at	AF469061	AAN32890	DG pioneer protein 4D09	6E-46	0.0011	-2.45	-3.95	-9.68
HgAffx.3764.1.S1_at	AF473830	AAO33477	DG pioneer protein 4G05	5E-100	0.0087	-2.34	-2.16	-5.05
		AAP30757	DG pioneer protein 30G12	3E-91				
HgAffx.3764.3.S1_s_at	CK350050	AAO33477	DG pioneer protein 4G05	2E-26	0.0042	-2.52	-3.39	-8.54
		AAP30757	DG pioneer protein 30G12	3E-39				
HgAffx.5088.1.S1_at	AF473826	AAP30756	SvG pioneer protein 10C02	8E-37	0.0018	-2.21	-1.85	-4.08
		AAO33473	SvG pioneer protein 4E02	8E-37				

upregulated from 2 to 5 dpi and then significantly downregulated from 5 to 10 dpi. Transcripts in cluster 7 exhibit the opposite pattern. Cluster 5 includes transcripts that exhibited significantly nonconstant expression across the three timepoints but with no significant changes in expression between adjacent time points. Thus, the transcripts in this cluster tend to increase or decrease more gradually over the time course than transcripts in either cluster 1 or 9.

Five differentially expressed genes were selected randomly for validation by qPCR. The transcript details and the primer sequences are given in Supplemental Table 2. For comparison, RNA from freshly hatched parasitic J2 also was included in qPCR studies. A SCN *GAPDH* (CA939315) gene was used as an internal control for the relative quantification of gene expression using the $\Delta\Delta C_T$ method (described below). This SCN *GAPDH* was used as one of the internal controls on the chip. Our microarray analysis showed that this gene was upregulated from 2 to 5 dpi and stayed constant between 5 and 10 dpi. However, our qPCR analysis using a known concentration of total RNA from different parasitic and parasitic stages of SCN indicated that the expression of SCN *GAPDH* was stable across all of the nematode developmental stages tested (data not shown). This discrepancy likely is due to cross hybridization of other *GAPDH* family transcripts in microarray analysis. The qPCR results were consistent with the microarray results for all five genes selected (Fig. 3). The transcript level for a gene encoding a putative cuticular collagen (HgAffx.13886.1.S1_at) was very low in parasitic J2s and went up with the development of parasitic stages, reaching a peak at 5 dpi. A similar expression pattern was observed for a gene encoding putative esophageal gland cell protein Hgg-20 (HgAffx.2890.2.S1_at); however, the transcript level peaked at 10 dpi. Transcript levels for genes encoding chorismate mutase (HgAffx.24067.1.S1_at) and a putative FMRFamide-related peptide (HgAffx.23446.1.S1_at) precursor were high in parasitic J2s but consistently went down with the development of the nematode. In contrast,

expression of a gene encoding a putative secreted glutathione peroxidase (HgAffx.22879.1.S1_at) was high in parasitic J2s, downregulated at 2 dpi, and then went up during development of the parasitic nematode, to reach a peak at 5 dpi.

DISCUSSION

The genome-wide gene expression analysis conducted in this study represents the first successful example of using a single microarray to simultaneously determine gene expression profiles in a nematode and plant over a time course of the infection process. The identification of genes regulated in both the host and pathogen provides novel insights into the complex nature of the SCN–soybean pathosystem, which includes a transit from migratory to sedentary life stages and the establishment of a unique feeding structure in host plant roots. Using Affymetrix GeneChip soybean genome arrays, we have identified the most comprehensive list of transcripts regulated in both soybean and SCN during parasitism to date. Our results provide insight into the complex changes in plant gene expression occurring globally in response to SCN parasitism, and have identified coordinated expression of nematode genes potentially involved in parasitism and specific stages of development.

Simultaneous analysis of changes in messenger (m)RNA levels of 35,611 soybean transcripts and 7,431 SCN transcripts identified 429 soybean genes with significant changes in expression between SCN-infected and mock-inoculated roots and 1,850 SCN genes with significant changes in expression during parasitism when controlling FDR at approximately 5% and using a 1.5-fold cutoff. A subset of the 429 differentially expressed soybean genes was validated by qPCR. Many of the soybean genes differentially regulated by SCN infection were involved in metabolism, cell wall modification, stress or defense responses, phytohormone response, and cellular signaling. It is difficult to make direct comparisons with earlier microarray studies of cyst nematode–plant interactions due to differences in pathosystems, microarray platforms, time point selection, tissue sampling methods, and analysis; however, we point out a few similarities with the studies here. Khan and associates (2004) identified the upregulation of 99 genes and downregulation of one gene at 2 dpi in nematode-infected roots of a susceptible soybean line using a cDNA array with approximately 1,300 probes. Our data was consistent with this study for genes encoding peroxidase and extensin. Similarly, our data is consistent with an earlier microarray study of the *Arabidopsis*–beet cyst nematode interaction at 3 dpi (Puthoff et al. 2003) for genes encoding PR1, heat shock proteins, carboxypeptidases, glutathione-S-transferases (GSTs), expansins, extensins, pectinesterases, hexose transporter, nodulin, an ERF, and a WRKY family transcription factor. Our data extend their observations by measuring thousands more soybean and nematode genes simultaneously.

Changes in the expression of cell-wall-modifying proteins (CWMPs) have been shown to be important for cyst nematode–plant interactions. During penetration and intracellular migration through root tissues, cyst nematode juveniles secrete a mixture of CWMPs, including endoglucanases, pectate lyases, and expansins, to loosen cell walls (De Boer et al. 1999, 2002a; Goellner et al. 2000, 2001; Qin et al. 2004). Once a cyst nematode juvenile begins to feed from the initial syncytial cell, expression of nematode CWMPs declines (De Boer et al. 1999; Goellner et al. 2000, 2001) and the coordinated expression of plant CWMPs (Goellner et al. 2001) is activated for syncytium formation. This trend was reflected in our microarray study. We identified the differential regulation of nematode CWMPs, including β -1,4-endoglucanases, pec-

Fig. 3. Quantitative real-time reverse-transcription polymerase chain reaction (qPCR) confirmation of microarray analysis for soybean cyst nematode gene expression. Total RNA was isolated from roots harvested at 2, 5, and 10 days postinoculation (dpi). Total RNA from parasitic second-stage juveniles (J2) also was used for comparison. First-strand cDNA was synthesized and subjected to qPCR in triplicate. Values on the Y axis indicate log₁₀-fold change relative to the 2 dpi timepoint. The qPCR results are compared with microarray results at 2, 5, and 10 dpi.

tate lyase, and expansin, as well as soybean expansins, extensins, pectinesterases, and a xyloglucan endotransglycosylase during nematode parasitism of soybean. All of the nematode CWMPs, with the exception of a homolog of β -1,4-endoglucanase, fell into clusters 6, 8, and 9, which represent genes with a significantly decreasing pattern of expression over the course of infection, and this reflects their importance during the early stages of the nematode life cycle. In contrast, all CWMPs of plant origin, including extensins and expansins, exhibited a significantly increasing pattern of expression over time, with the exception of a xyloglucan endotransglycosylase homolog and two homologs of pectinesterase family proteins, which showed an opposite trend. Differential regulation of major CWMPs, including expansins, pectate lyases, xyloglucan endotransglycosylases, and polygalacturonases, also was observed in root-knot nematode-induced galls in *Arabidopsis* (Jammes et al. 2005).

Our study also suggests general activation of plant defense mechanisms in response to SCN infection. Defense-related genes, including PR proteins, stress-related transcription factors (e.g., ERFs), general stress-responsive genes (e.g., glutathione-S-transferases and osmotins), disease resistance responsive proteins, PGIPs, and homologs of a hypersensitive response (HR)-related gene, harpin-induced gene 1 (*Hin1*), were upregulated in nematode-infected roots. Upregulation of the *hin* family of genes has been observed in plants in response to bacterial (Gopalan et al. 1996) and fungal (Kim et al. 2000; Tian et al. 2003) pathogens as well as in response to symbionts (Le Quere et al. 2005). The general upregulation of plant defense responses in our study is opposite to the general suppression of plant defense mechanisms reported in the *Arabidopsis*-root knot nematode interaction (Jammes et al. 2005). This is not surprising considering that, unlike cyst nematodes which migrate intracellularly causing migration damage, root-knot nematodes migrate intercellularly and do not elicit a plant defense response along the path of migration. The earliest time point profiled by Jammes and associates (2005) was 7 dpi, at which time nematodes are sedentary within the developing gall. It is very possible that, in our study, suppression of general plant defense mechanisms at the nematode feeding site was masked by upregulation of defense-related genes due to wound-related responses in surrounding tissue caused by intracellular migration and, at later stages, as adult female nematodes expanded and eventually protruded from the root. This observation is consistent with our cluster analysis of differentially expressed plant genes, where all genes with an annotated function in the cluster combination mock cluster 5-infected cluster 2 appeared to be involved in plant defense responses. Genes in this cluster combination were significantly upregulated from 2 to 5 dpi but did not show any significant change between 5 and 10 dpi in nematode-infected roots. Expression of these genes did not change significantly between adjacent time points in the uninfected roots, consistent with the global activation of plant defense genes in response to nematode infection.

Genes encoding enzymes involved in secondary metabolism, specifically the phenylpropanoid and flavonoid pathways, including *4cl* (4-coumarate-CoA ligase), *Chs* (chalcone synthase), *Chi* (chalcone isomerase), *Dfr* (dihydroflavonol reductase), *Ifr* (isoflavone reductase), and a lignin biosynthetic pathway gene encoding quercetin 3-O-methyltransferase (OMT), were upregulated in nematode-infected roots. Upregulation of genes in the multibranched phenylpropanoid pathway leading to the accumulation of flavonoid or isoflavonoid phytoalexins, deposition of lignin, or cell wall bound phenolics is also a characteristic of the HR and systemic acquired resistance (SAR) in plant-pathogen interactions (Keen 1992). A study by Edens

and associates (1995) showed that the transcript abundance for genes encoding phenylalanine ammonia lyase (PAL) and 4-coumaroyl CoA ligase as well as the activity of these two enzymes increased in resistant but not susceptible soybean cultivars after SCN or root-knot nematode infection. Synthesis of enzymes later in the phenylpropanoid pathway leading to glyceollin production also increased in both resistant and susceptible soybean in response to nematode infection, with a greater increase observed in resistant cultivars. The role of these secondary metabolites in compatible plant-nematode interactions is not clear. However, alterations in auxin distribution have been observed in nematode-infected roots and it has been suggested that increased flavonoid levels in response to infection may be involved in the local accumulation of auxin at feeding sites by blocking auxin efflux carriers (Hutangura et al. 1999). Consistent with this observation we see upregulation of an auxin-induced soybean gene, *PIN2*, encoding an auxin transport protein, indicating possible accumulation of auxin in SCN-infected soybean roots. In tomato and *Arabidopsis*, auxin has been shown to mediate the induction and morphogenesis of cyst nematode syncytia, and a strong reduction in cyst nematode development was observed on *Arabidopsis pin2* mutants (Goverse et al. 2000).

The 1,850 SCN genes displaying statistically significant changes in expression over the time course of infection were grouped into nine clusters based on similarity of significant expression changes across adjacent time points. Significantly, the clustering analysis revealed coordinated expression of several classes of nematode genes. Our interest in SCN parasitism genes led us to further analyze the temporal expression patterns of nematode genes represented on the Affymetrix GeneChip soybean genome array that previously were confirmed to contain a signal peptide and shown to be expressed in the nematode esophageal gland cells (Table 2). Interestingly, the majority of previously identified SCN parasitism genes (both candidate genes and those shown to be secreted with known function) (Gao et al. 2001, 2003; Lambert et al. 1999; Smant et al. 1998; Wang et al. 2001) fell into clusters 6, 8, and 9, which include transcripts whose expression levels decrease significantly over the course of infection. In cluster 9, *ENG-4* (endoglucanase; Gao et al. 2004) and the pioneer genes *GSB24* (*Hgg-15*; Gao et al., 2001), *33E05*, *34B08*, *4D09*, and *30G12/4G05* and *10C02/4E02* gene families (Gao et al. 2003) displayed a high level of mRNA abundance at 2 dpi that declined at 5 dpi and again at 10 dpi, suggesting that these genes may play a role in the early stages of the parasitic interaction. Similarly, in cluster 8, cellulase (*26D05*), *PEL* (pectate lyase; De Boer et al. 2002a), a gene with similarity to *Globodera rostochiensis* expansin (Qin et al. 2004), and dorsal gland pioneer genes *34B08* (Gao et al. 2003), *SYV42* (Wang et al. 2001), and *SB89* (*Hgg-25*, Gao et al. 2001) were present. In cluster 8, expression levels dropped significantly from 2 to 5 dpi and then showed no significant change from 5 to 10 dpi. *ENG-2* (Gao et al. 2004); *CHT* (chitinase; Gao et al. 2002); a putative annexin (*ANN*; *4F01*); *CM* (chorismate mutase; *45D07*; Gao et al. 2003); *CLAVATA3/ESR-like* (*CLE*; *4G12* [Gao et al. 2003] and *SYV46/2B10* [Gao et al. 2003; Wang et al. 2001]); *VAP* (venom-allergen protein; Gao et al. 2001); ubiquitin extension protein (*4G06*); *SKP1-like* (*8H07*, *28B03*, *29F07*, *GSB3*, *SB26*); pioneer genes *19C07*, *30C02*, *15A10*, and *32E03*; and *16B09/30E03/22C12*, *27D09/20G04/10A07*, and *30G12/4G05/25A01* gene families fell into cluster 6 (Gao et al. 2003). *SB89* and *34B08* also fell into cluster 6. Transcripts in cluster 6 showed no significant change in expression between 2 and 5 dpi but then dropped significantly from 5 to 10 dpi. The temporal expression profile suggests that these genes also play an important role in the earlier stages of the plant-nematode

interaction, but remain expressed at higher levels longer than those present in clusters 8 and 9.

A small subset of candidate SCN parasitism genes fell into clusters other than 6, 8, and 9. Two SCN parasitism genes fell into cluster 3, including a cellulose-binding protein (*CBP*) and the pioneer protein gene family *6E07/27D09* (Gao et al. 2003). Genes in cluster 3 showed an increase in expression level from 2 to 5 dpi, but declined from 5 to 10 dpi. SCN parasitism genes falling into clusters 1 and 2 included *SB84* (*Hgg-20*; Gao et al. 2001), *CBP*, *17G01*, *2D01/11A06/24A12*, and *4D06/18H08/29D09* pioneer protein gene families (Gao et al. 2003); a gene similar to *Meloidogyne incognita* subventral gland pioneer protein gene *5G05* (Huang et al. 2003); and an *M. incognita* dorsal gland *14-3-3b* gene (Jaubert et al. 2004). Genes in clusters 1 and 2 showed an increase in expression from 2 to 5 dpi, but either increased from 5 to 10 dpi (cluster 1) or showed no significant change from 5 to 10 dpi (cluster 2). Nematode genes falling into clusters 1, 2, and 3 may play a more predominant role during later stages of the plant–nematode interaction. *SB84* (*Hgg-20*), *17G01*, and a gene similar to *M. incognita* *14-3-3b* also fell into cluster 4. Additional candidate SCN parasitism genes in cluster 4 included the pioneer gene family *30D08/16A01/21E12* (Gao et al. 2003). A gene similar to *M. incognita* calreticulin (Jaubert et al. 2005) fell into cluster 5. The genes in this cluster did not show any significant changes between adjacent time points. Other genes in this cluster include *ENG 1* (Gao et al. 2004; Smant et al. 1998), dorsal gland pioneer genes *SB84* (*Hgg-20*), *32E03*, and the *30D08/21E12/16A01* family.

The expression profiles of the putative SCN parasitism genes *SYV42*, *SYV46*, and *SYV52* (now *Eng-1*) (Wang et al. 2001) previously were determined by nematode cDNA microarrays (De Boer et al. 2002b) and the overall expression profiles are consistent with our study. In addition, expression profiles of candidate parasitism genes *SYS7*, *SYS79*, *SYS86*, and *SYV55* identified from microaspirated gland cell contents and screened by signal peptide selection in yeast (Wang et al. 2001) were profiled by De Boer and associates (2002b). All four genes were detected in our microarray study here, but are excluded from our list of SCN parasitism genes because, although they contain a signal peptide, they have never been confirmed to be expressed in the esophageal gland cells. These genes fell into clusters 3, 8, 2, and 2. *SY20*, *SYS16*, and *SYV80* were not found in our list of 1,871 genes at 5% FDR to be differentially expressed across the time points used here.

The fact that the majority of previously identified putative parasitism genes fell into three clusters with similar profiles (i.e., expression level is high at 2 dpi compared with 10 dpi) allows us to speculate that other members of these clusters with unknown functions are likely candidates for novel parasitism genes involved in the cyst nematode–plant interaction. However, considering the fact that transition from the motile preparasitic stage to sedentary parasitic stages also occurs during early stages of parasitism, some of the transcripts in these clusters also may be involved in that developmental transition. For example, two putative cuticular collagen genes fell into clusters 8 and 9 with the majority of putative parasitism genes. Nevertheless, our observations warrant further studies to isolate full-length clones of corresponding unknown ESTs identified in these clusters. Bioinformatic analysis of sequences for the presence of signal peptides and in situ hybridization to detect gland-specific expression likely will lead to the identification of additional novel candidate cyst nematode parasitism genes.

A comparison of the cluster analysis for differentially expressed plant and nematode genes from this study also provides novel insights into the plant–nematode interaction. For

example, nematode cluster 2 is the largest cluster, with 729 genes. The genes in this cluster show significant upregulation from 2 to 5 dpi but nonsignificant changes between 5 and 10 dpi. Many of the genes in this cluster are involved in metabolism and included ribosomal proteins, ATP synthase, pyruvate dehydrogenase, alcohol dehydrogenase, and so on. Similarly, soybean-infected cluster 2, which exhibits a similar expression pattern as that of nematode cluster 2, represents 68 genes, most of them involved in plant defense responses and cell wall modification. The members of this cluster included PR proteins, GSTs, osmotin-like proteins, extensins, expansins, and germin-like proteins. Increased feeding activity and nematode body expansion within the root may be inducing plant defense and wound responses at this stage. Despite these trends, the large majority of differentially expressed nematode and soybean genes identified in this study are of unknown function. In the near future, availability of the complete soybean and nematode genome sequences with more accurate annotations will help us integrate our observations of the differential expression of both plant and nematode genes from this study.

Cross hybridization among related transcripts from a gene family, possible redundancy of transcripts on the chip, poor annotation, and limited functional genomics resources for soybean are some of the limitations that need to be considered when drawing conclusions from this study. Interestingly, this study identified only 429 differentially expressed genes (approximately 1.2%) of the total 35,611 soybean transcripts measured, in contrast to 1,850 differentially expressed genes (approximately 24%) of the total 7,431 nematode transcripts. This may be due to dilution of nematode responsive cells in the excised whole root tissues used in this study. We applied stringent experimental, biological, and statistical measures to make the results meaningful and reliable. Inherent biological variation in the system was reduced to a minimum by bulking samples, controlling the number of nematodes at each inoculation, and synchronizing the infection. Furthermore, we used three biological replicates for microarray analysis. The microarray results and the sequence identity for selected targets were validated by qPCR analysis. In addition, our microarray data is in agreement with results from previous studies that employed microarrays as well as those from more traditional methods of expression analysis. These comparisons provide additional verification of our results, giving a high degree of confidence in the data.

In summary, our analysis using the latest microarray technology available provides a global profile of changes in soybean and SCN gene expression during different stages of nematode parasitism. We have identified several novel genes, both in soybean and SCN, whose expression level changes during nematode infection of roots. More detailed biological analysis of the genes identified in this study will provide a better understanding of SCN parasitism of soybean and help to identify potential targets for genetic improvement of crop plants for enhanced SCN resistance. Ultimately, identifying key biological processes involved in cyst nematode feeding-cell formation will require a combined approach of cell-type-specific isolation (Klink et al. 2005; Ramsay et al. 2004) and microarray profiling over a time course of syncytium development, and these experiments are underway.

MATERIALS AND METHODS

Plant material and nematodes.

Soybean (*G. max* (L.) Merr.) cv. Williams 82 was used in this study. Soybean cyst nematode (*H. glycines* Ichinohe) inbred line PA3 (HG type 0, race 3) was maintained by mass selection on susceptible cv. Williams 82 in greenhouse cultures.

Plant infections.

Soybean cv. Williams 82 seed were soaked in running water for 30 min and surface sterilized in 10% bleach for 10 min followed by rinsing in running water for 30 min. Seed were germinated for 2 days in ragdolls at 28°C in dark. Two-day-old seedlings with a 2- to 3-cm-long uniform root were inoculated with freshly hatched nematodes according to Mahalingam and associates (1998), with the following modifications. Cysts of SCN were extracted from infested soil by flotation in water and collected on a 250- μ m (no. 60) sieve. Harvested cysts were crushed gently using a drill press and the eggs collected on a 25- μ m (no. 500) sieve (Faghihi and Ferris 2000) and further purified on a sucrose density gradient. The eggs were sterilized in 0.02% sodium azide for 20 min and washed extensively in sterile water to remove any traces of sodium azide. Preparasitic J2s were isolated by hatching eggs in the presence of gentamycin at 1.5 mg/ml and nystatin at 0.05mg/ml at 28°C on a modified Baermann pan for 2 days. Hatched juveniles were collected, rinsed with sterile water, and suspended in 0.1% sterile agarose. Then, 100 μ l of 0.1% agarose containing 300 ± 25 preparasitic J2s or agarose alone (mock) was applied approximately 1 cm above the root tip of each soybean root which was marked with a dot using a black Sharpie pen. Infection was synchronized by washing the infected roots with water exactly 24 h after inoculation. Seedlings were rolled into ragdolls, placed in Hoagland's nutrient solution (1/10 dilution), and allowed to grow in a plant growth chamber at 26°C with a photoperiod of 16 h of light and 8 h of dark with constant aeration. Samples were collected at 2, 5, and 10 dpi. For total RNA isolation, root pieces of approximately 2 cm in length were excised using a scalpel at the zone of maximum infection, which could be located by the black dot on the root made with a Sharpie pen. At later time points, lateral roots were removed from excised root pieces. Excised root pieces from 30 different plants were pooled at each time point and flash frozen in liquid nitrogen. All experiments were repeated two more times for a total of three independent biological replicates.

For each time point, a random sample of 10 roots were stained to determine the number and life stage of the juveniles, as described by Dropkin and associates (1969). Briefly, roots were washed in 10% household bleach for 7 min and then rinsed in tap water for 20 min. Acid fuchsin stain (1 ml of 0.35 g of acid fuchsin in 25 ml of glacial acetic acid) was added to a beaker containing 50 ml of boiling water. Roots were boiled in the solution for 1 min, then allowed to cool at room temperature for 30 min. Roots were rinsed in water and transferred to a beaker containing warm glycerol and incubated at 65°C for several hours. The stained roots were squashed between two glass slides, and juveniles were counted and photographed under a stereomicroscope. For light microscopy of feeding cells, infected root pieces were fixed in 4% paraformaldehyde and embedded in paraffin, and 5- μ m sections were stained with toluidine blue stain.

RNA extraction.

Total RNA was extracted from excised whole root tissues using the RNeasy Plant mini kit (Qiagen, Valencia, CA, U.S.A.) and treated with RNase-free DNase (Qiagen) following the manufacturer's instructions. Total RNA samples were sent to the Iowa State University GeneChip microarray core facility where cDNA and cRNA preparation, fragmentation, hybridization, staining, and scanning steps were performed according to the manufacturer's recommended protocols (Affymetrix, Santa Clara, CA, U.S.A.).

Freshly hatched preparasitic J2s of SCN PA3 were collected and washed in sterile water. A 100- μ l nematode pellet was flash

frozen in liquid nitrogen. The pellet was ground using a 200- μ l Kontes Dual tissue grinder (Fisher Scientific, Pittsburgh, PA, U.S.A.) in the presence of PureScript cell lysis buffer (Gentra Systems, Minneapolis, MN, U.S.A.). Total RNA was extracted using the PureScript RNA isolation kit (Gentra Systems), and on-column DNase I treatment (Qiagen) was performed following the manufacturer's recommendations.

Microarray chip description.

The GeneChip soybean genome array (Affymetrix) was used in study. This oligonucleotide array contains 37,593 probe-sets representing 35,611 soybean transcripts, 7,530 probesets representing 7,431 soybean cyst nematode transcripts, and more than 15,800 probesets representing 15,421 *Phytophthora sojae* transcripts. A description of the GeneChip soybean genome array is available at the manufacturer's website.

Data analysis.

GeneChip Operating Software version 1.0 (GCOS v. 1.0) signal data were natural-log transformed and normalized by centering the log-scale median for each chip at 0. Mixed linear model analyses using the normalized data were conducted separately for each gene. When analyzing soybean genes, each mixed linear model included fixed effects for treatments (infected or uninfected), times (2, 5, or 10 dpi), and treatment-time interactions, as well as random effects for replications and replication-time interactions. This is a standard mixed model for a split-plot experimental design (Kuehl, 2000) with time as the whole-plot factor and treatment as the split-plot factor. *F* tests, resulting in *P* values, were performed to test for the significance of each factor, to test for treatment effects separately at each time point, and to test for changes of expression across adjacent time points within each treatment. The *P* values for each test were converted to *q* values using the method described by Storey and Tibshirani (2003). For each test of interest, genes with *q* values less than 0.05 were declared statistically significant to produce a list of genes with an estimated FDR of 5%. This gene list was further filtered by applying a cutoff of a 1.5-fold change between mock and treatment group.

A different analysis strategy was necessary for analyzing nematode genes because there were no nematode transcripts present in mock-infected tissues. Thus, the data from the nematode genes were analyzed as a completely randomized design with time as the only factor. Analysis again was performed on a gene-by-gene basis to obtain *F* tests for changes over time. The *P* values from these tests were converted to *q* values, and the genes with *q* values less than 0.05 were declared statistically significant as described for the soybean genes. The genes exhibiting more than a 1.5-fold change between at least one of the three time combinations (2 to 5, 5 to 10, and 2 to 10 dpi) were considered biologically significant in this analysis.

The 1,850 nematode genes that exhibited significant (*q* value <0.05 and fold change >1.5) nonconstant expression across levels of dpi were separated into clusters according to expression patterns. Estimated means of adjacent levels of dpi were compared using *t* tests. The *P* values from these *t* tests were used to group genes into clusters. Specifically, the genes were categorized by whether the mean expression at 2 dpi was significantly greater than, less than, or not significantly different from 5 dpi and whether the mean expression at 5 dpi was significantly greater than, less than, or not significantly different from 10 dpi. In each case, significance was defined by *P* value < 0.05. This testing for differences in expression across adjacent time points created nine clusters, including a cluster with genes that exhibited significant nonconstant expression over

time despite nonsignificant changes from 2 to 5 dpi and from 5 to 10 dpi. Some of these genes gradually increased over the time course whereas others gradually decreased, which explains the “X” shape of this small cluster.

For comparison with the nematode expression patterns, the infected and uninfected expression profiles of the 429 soybean genes with significant treatment effects were clustered using the clustering strategy employed for the nematode genes. The *q* values from the mixed-linear-model tests for changes of expression across adjacent time points within each treatment were used to place each profile into one of the nine clusters.

The nucleotide sequences of the ESTs corresponding to the differentially expressed probeset identified from microarray analysis were obtained from the Affymetrix NetAffix analysis center. Soybean EST sequences were used as a query to search for *Arabidopsis* orthologs in the TAIR database using WU-BLAST2 search. The soybean genes were annotated based on the *Arabidopsis* top hit with an *e* value < 10⁻³. Nematode genes were annotated by BLASTX searches against the nonredundant protein sequence database at the National Center for Biotechnology Information. SEQtools 8.2 software was used for batch analysis of sequences. The microarray data has been deposited in the ArrayExpress database at the European Bioinformatics Institute (EBI) under accession number E-MEXP-808.

qPCR analysis.

qPCR was carried out using the Applied Biosystems 7500 Real-Time PCR system. Specific primers for each gene selected were designed using Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.). For quantifications, a cDNA template prepared from the total RNA from the same biological replicates used for microarray analysis was used. qPCR was conducted in triplicate in 20 µl using SYBR Green PCR master mix (Applied Biosystems). The qPCR reactions were conducted in triplicate for each biological replicate. PCR conditions used were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A soybean gene encoding glutathione-S-transferase (*GSTa*-accession number AF048978) was used as an internal control for soybean. This is one of seven soybean internal control genes represented on the Genechip soybean genome array. We determined by qPCR that the expression of this *GST* remained relatively constant across treatments compared with the other internal controls and it was chosen for validation studies. However, additional probesets for glutathione-S-transferase family members are printed on the chip and several of these displayed differential expression across treatments. An SCN *GAPDH* (accession number CA939315) was used as an internal control for SCN qPCR. Nematode *GAPDH* and actin were used as control genes on the GeneChip soybean genome array. The expression of *GAPDH* and actin was tested by qPCR and we determined that SCN *GAPDH* remained relatively constant across nematode developmental stages compared with actin. The RNA samples from inoculated soybean roots were used for SCN qPCR. The RNA extracted from freshly hatched parasitic J2s also was included for comparison in qPCR analysis of SCN gene expression. The quantification of gene expression was performed using the relative $\Delta\Delta C_T$ method by comparing the data with internal control genes whose expression was determined to remain constant under different treatment conditions.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ArrayExpress database: www.ebi.ac.uk/arrayexpress/
 GeneChip: www.affymetrix.com/products/arrays/specific/soybean.affx
 NetAffix analysis center: www.affymetrix.com/analysis/netaffix/index.affx
 SEQttools 8.2: www.seqtools.dk