Journal of Nematology 36(3):241–248. 2004.

Microarray Analysis of Gene Expression in Soybean Roots Susceptible to the Soybean Cyst Nematode Two Days Post Invasion[1]

R. Khan,[2] N. Alkharouf,[2,3] H. Beard,[2] M. MacDonald,[2] I. Chouikha,[2,3] S. Meyer,[4] J. Grefenstette,[3] H. Knap,[5] and B. Matthews[2,3]

Abstract: Soybean root cells undergo dramatic morphological and biochemical changes during the establishment of a feeding site in a compatible interaction with the soybean cyst nematode (SCN). We constructed a cDNA microarray with approximately 1,300 cDNA inserts targeted to identify differentially expressed genes during the compatible interaction of SCN with soybean roots 2 days after infection. Three independent biological replicates were grown and inoculated with SCN, and 2 days later RNA was extracted for hybridization to microarrays and compared to noninoculated controls. Statistical analysis indicated that approximately 8% of the genes monitored were induced and more than 50% of these were genes of unknown function. Notable genes that were more highly expressed 2 days after inoculation with SCN as compared to noninoculated roots included the repetitive proline-rich glycoprotein, the stress-induced gene SAM22, β-1,3-endoglucanase, peroxidase, and those involved in carbohydrate metabolism, plant defense, and signaling.

Key words: Glycine max, Heterodera glycines, microarray, plant-pathogen interaction, SGMD, signaling pathway, soybean cyst nematode, soybean genomics microarray database, syncytium.

Plant-parasitic nematodes attack many agronomically important plants, resulting in substantial economic losses worldwide. The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is the major pest of soybean and is responsible for an estimated $1 billion in damage each year in the United States (Wrather et al., 2001). Crop rotation and planting soybean varieties resistant to various SCN races provide some protection; nevertheless, substantial economic losses are incurred.

When a sedentary endo-parasitic nematode enters the root and initiates feeding, remarkable physiological and morphological changes occur in the cells of susceptible plants coerced to accommodate the nematode with a feeding site. In the case of soybean roots invaded by the soybean cyst nematode, numerous histological studies have documented changes at the feeding initiation site to form a syncytium. Major changes include the dissolution of surrounding cell walls, enlargement of nucleoli, accumulation of endoplasmic reticulum, deposition of callose, increase in vacuoles, and appearance of nematode secretions near the stylet between 18 hours and 4 days post-invasion (Endo, 1964, 1965, 1991; Gipson et al., 1971; Kim et al., 1987; Mahalingam and Skorupska, 1996).

Although there are several studies on changes in gene expression of plants during invasion by nematodes, there are few studies that identify soybean genes with increased expression due to invasion by nematodes (Hermsmeier et al., 2000; Neibel et al., 1996; Potenza, et al., 2001; Puthoff et al., 2003) (for review see Gheysen and Fenoll, 2002). The isolation and characterization of genes with a role in establishing SCN infection has been the focus of several studies (Vaghchhipawala et al., 2001; Williamson, 1999). Hermsmeier et al. (1998), using differential display analysis, identified 26 bands representing genes expressed in the soybean root of susceptible plants invaded by SCN, of which 15 were recovered as cDNA clones. Northern blots of these clones indicated that 5 of these cDNAs increased in expression whereas 10 decreased. From the cDNAs with decreased expression five were sequenced, and they were identified as possibly encoding aldolase genes, whereas the others encoded a TFIIA transcription factor, a small GTP-binding protein, and ADR12, respectively. None of the genes that showed an increase in expression were identified.

Differential display and cDNA subtraction were used to identify genes that may be increased in expression as indicated by RT-PCR, including catalase, cyclin, β-1,3-endoglucanase hydroxymethylglutaryl coenzyme A reductase, and heat shock protein 70 (Vaghchhipawala et al., 2001), thus providing a glimpse of what may be occurring at the molecular level. Microarrays were used to identify 12 genes that are up or down regulated in *Arabidopsis* due to SCN invasion (Puthoff et al., 2003). These 12 genes, however, are not unique to SCN invasion because they were also differentially regulated by sugar beet cyst nematode infection. In contrast, 116 unique genes with altered expression levels were observed when *Arabidopsis* was inoculated with sugar beet cyst nematode (*Heterodera schachtii*).

Cytological studies on soybean-SCN interaction in an incompatible (resistant) and compatible (susceptible) interaction indicate that signs of a resistant or susceptible response are evident typically 2 days after penetration (Mahlingham and Skorupska, 1996; Mahlingham et al., 1998). Syncytia are formed in both resistant and

Received for publication 15 September 2003.

[1] This work was supported by the United Soybean Board under grant 3214 and USDA CSREES National Research Initiative grant 99-35302-8189. CREES/USDA project no. SC-1700102. Clemson University Experiment Station Technical Contribution No. 4992.

[2] USDA-ARS, Soybean Genomics and Improvement Laboratory, Beltsville, MD 20705. Senior author current address: University of Maryland University College, Adelphi, MD 20783.

[3] George Mason University, School of Computational Sciences, Fairfax, VA 22030.

[4] Nematology Laboratory, Beltsville, MD.

[5] Clemson University, Clemson, SC.

The authors thank Kristina Pilitt and Michael Yang for their expert technical assistance.

E-mail: matthewb@ba.ars.usda.gov

This paper was edited by P. A. Roberts.

susceptible hosts by 2 days after infection; however, the syncytia in the resistant cultivar are small and restricted to the pericyclic tissues, whereas in the susceptible cultivar the syncytia grow in size and facilitate the growth of the nematode by providing nutrients.

Microarray technology facilitates the assay of changes in thousands of transcripts at one time in response to different signals. Several recent studies have used microarray analysis to look at plant defense responses in the presence of abiotic stresses such as drought and cold, diurnal cycling, salt, temperature, oxidative stress, small signaling molecules such as salicylic acid and methyl jasmonate, and biotic stresses (Kawasaki et al., 2001; Kiegle et al., 2000; Oztur et al., 2002; Reymond et al., 2000; Schaffer et al., 2001; Schenk et al., 2000; Seki et al., 2001).

Until now, microarrays have not been used to examine the expression of soybean genes during invasion by SCN; however, they have been used to compare gene expression in soybean root and shoots (Maguire et al., 2002) and compare transcript expression patterns during somatic embryogenesis (Thibaud-Nissen et al., 2003).

This paper provides an analysis of the gene expression in the SCN-susceptible soybean, cv. Kent, using microarrays containing over 1,300 cDNA inserts specifically isolated from soybean libraries infected with SCN population NL1-RHp, so genes relevant to the susceptible response would be detected on the microarray and genes important to the infection process could be identified.

Materials and Methods

Plant and nematode materials and RNA isolation: Kent (SCN-susceptible) seeds were surface-sterilized by soaking in 95% ethanol for 3 minutes, followed by a 10% sodium hypochlorite treatment for 10 minutes in a laminar flow hood using sterile culture techniques. The seeds were germinated on water agar for 3 days at room temperature in the dark. *Heterodera glycines* population NL1-RHp, which characterized as Race 3 in differential screening (data not shown), was collected on the Eastern Shore of Maryland by Robin Huettel. The population has been maintained for many years under sterile conditions in tissue culture by David Chitwood, Nematology Lab, USDA, Beltsville, Maryland. Eggs from SCN NL1-RHp females were incubated in sterile water at room temperature on a rotary shaker at 25 rpm to promote hatching. After 2 days the second-stage juveniles (J2) were collected and concentrated to around 3,000 J2/ml by centrifugation at 50*x* g for 3 minutes in 15 ml sterile disposable conical tubes. Using a method similar to Hermsmeier et al. (1998), 20 radicals were excised and placed in a pinwheel formation with the root tips pointing inward on a circle of sterile Whatman 3-mm paper on top of Gamborg's B5 media (GIBCOBRL, Gaithersburg, MD) in petri dishes. Two petri dishes containing 20 roots each were set up as two replicates. Each set of 20 roots was inoculated with 3,000 J2 in sterile water. The control replicates received the same volume of sterile water. A portion of the inoculated roots was stained, penetrating nematodes were counted, and progress of infection was monitored (Fig. 1). After 48 hours of exposure to the nematodes, the roots were rinsed in deionized water and flash-frozen in liquid nitrogen. The frozen root tissue was ground to a fine powder using mortar and pestle chilled in liquid nitrogen. Total RNA was extracted using the method of Mujer et al. (1996) for gene expression profiling. The yield was typically 300 µg of total RNA/g of wet tissue.

cDNA microarray clones: cDNA clones from three different libraries were used in assembling the microarray. The libraries were constructed from Peking and PI437654, soybean genotypes resistant to SCN population NL1-RHp. The Peking Lambda ZAP II cDNA library was made with mRNA from the roots and shoots of Peking plants, 48 hours after nematode infection, according to the manufacturer's instructions (Stratagene, Cedar Creek, TX). Two suppressive subtraction libraries prepared from roots of PI437654, 24 hours after nematode inoculation followed by a 10-hour infection period, were obtained from Clemson University Genomics Institute and were constructed as described in Tomkins et al. (1999). Briefly, mRNA was isolated from the SCN-infected and noninfected roots of PI437654. The cDNA from SCN-infected roots served as a tester, and the cDNA from noninfected roots served as a driver for forward subtraction. For reverse subtraction the tester and driver cDNAs were reversed. A total of 1,000 clones with an average insert size of 1,000 bp were picked from the Peking cDNA library into 96-well culture plates. Each clone was sequenced with a vector primer at the 5′-end of the insert, and the sequence was

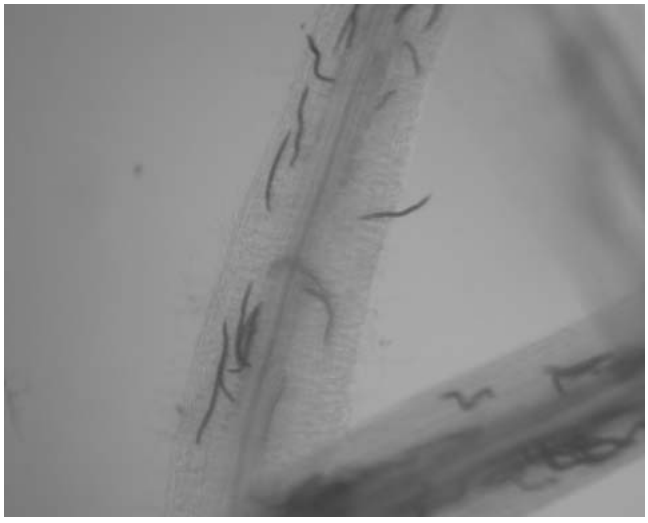

Fig. 1. Light micrograph of soybean roots, cv. Kent, infected with SCN NL1-RHp (race 3-type) for 2 days.

detected on an ABI Prism 3100 sequencer. Nucleotide sequences and predicted amino acid sequences were compared against those in Genbank, EMBL, and Swiss Prot databases using BLAST tools (Altschul et al., 1997). Obviously redundant clones in the Peking cDNA library were identified by pairwise comparison of each clone against the EST database housed at the USDA-ARS-Beltsville soybean genome microarray database (SGMD) (http://psi081.ba.ars.usda.gov/SGMD/Default.htm) and then removed reducing the number of cDNAs to 481 clones. The 768 cDNA clones from the suppression subtractive libraries had an average insert size of 500 bp, as indicated on agarose electrophoretic gels, and were not screened for redundancy. The insert from each clone was amplified by polymerase chain reaction (PCR) in a 96-well microtiter plate in a PTC 225 thermocycler (MJ Research, Waltham, MA) using T3/T7 universal primers in a 100-µl reaction similar to the protocol of Hegde et al. (2000) with slight modification. Excess primers and unincorporated nucleotides were removed by ethanol precipitation in the presence of 2 M ammonium acetate. The amplified product from each clone was checked on a 1% agarose in 1× TBE gel for the presence of a single band and brought to a final volume of 5 µl in 50% DMSO 50% TE solution.

Microarray generation: The PCR products suspended in 50% DMSO/TE at a concentration of 0.4 µg/ml were arrayed from 96-well microtiter plates onto silylated Corning CMT-GAPs microscope slides. The PCR-amplified cDNA inserts were printed in quadruplicate (Biological Samples one and two) or in triplicate (Biological sample three), and standard deviation of printed spots within the slide was calculated to be < = 2 × the mean. Slide-to-slide variation was measured by comparing three identical slide hybridizations each for biological samples one and two, and two slide hybridizations for biological sample three. Slide-to-slide variation was calculated to be greater than spot-to-spot variation (Fig. 2). Microarrays were printed using a Cartesian robot model PixSys 5500 PA workstation with a telechem printing head and Stealth quill pins. A total of 1,305 soybean cDNA clones were arrayed. Three cDNAs from human, cow, and alfalfa weevil were printed at concentrations of 1 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/

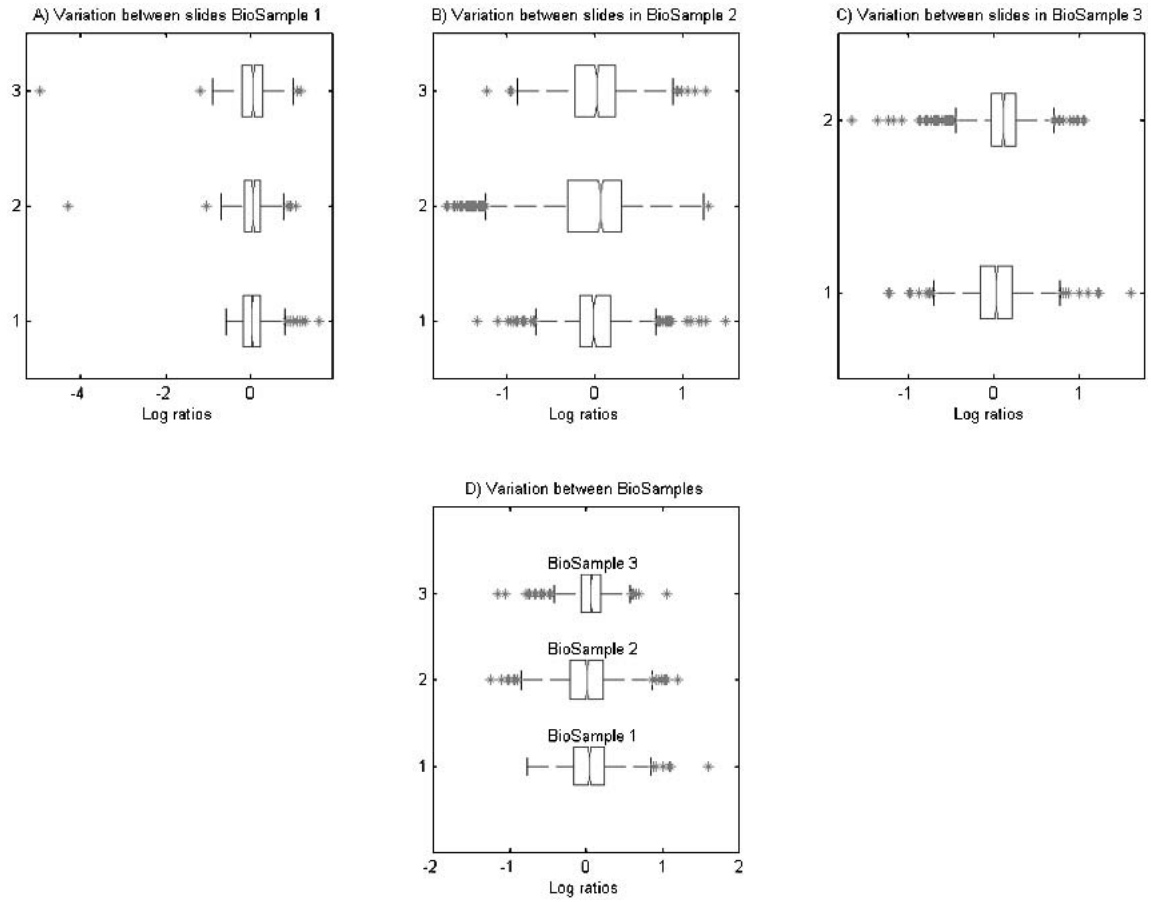

FIG. 2. Depictions of the variation in log ratios within slides and across biological samples. A,B,C) Box plots showing the $\log_2$ of the expression ratios from microarray replicates of three independent biological experiments. From each biological sample, total RNA from NL1-RHp-inoculated root was labeled with Cy3 and the noninoculated root with Cy5. For one of the slides the labels were switched. The labeled, pooled RNA was divided into three parts and each used for hybridization to a microarray. D) Box plots showing the $\log_2$ of the expression ratios across the three biological samples. The boxes have lines at the median and upper and lower quartile values. The whiskers show the extent of the rest of the data. The data with values beyond the end of the whiskers (outliers) are shown by red dots.

ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml to serve as internal controls and to assess for labeling and detection efficiencies. The printed arrays were crosslinked to the slide by UV irradiation at 250 mJ using UV Stratalinker 2400 (Stratagene, Cedar Creek, TX). The slides were stored in a humidity chamber at 25 °C.

Fluorescent probe preparation: Total RNA from each sample was labeled as described by Hegde et al. (2000) with few modifications. Briefly, the reverse transcription reaction was carried out in a 90-µl volume containing 30 µg of total RNA; 6 µg oligo (dT) 12-18 mer (Amersham Pharmacia, Arlington Heights, IL); 500 µM each of dATP, dCTP, dGTP, 100 µM dTTP, 100 µM of Cy3 dUTP, or Cy5 dUTP (NEN, Life Science Products Inc. Boston, MA); 500 µM DTT (Life Technologies, Carlsbad, CA); and 1,200 units of SuperScript II reverse transcriptase (Life Technologies, Carlsbad, CA) in 1× SuperScript first-strand buffer. Three specific control Poly $(A)^+$ RNAs (pλ11-3; pml-72; Sp3) corresponding to coding sequences from alfalfa weevil, cow, and human DNA, respectively, were synthesized by in vitro transcription using AmpliScribe™ transcription kit (Epicenter, Madison, WI) followed by oligotex-dT resin (Qiagen, Valencia, CA) purification. To serve as quantitative controls, the control RNAs were added to the reverse transcription reaction at a mass ratio of 1:1000, 1:10,000, and 1:100,000 (wt/wt), respectively. After incubation for 2 hours at 42 °C, the 90-µl reactions were stopped by addition of EDTA to a concentration of 1 mM. The RNA was degraded by adding 4.5 µl of 0.5N NaOH followed by a 10-minute incubation at 70 °C. The samples were neutralized by addition of 4.5 µl of 0.5N HCl. The probes were purified using a G-50 micro column (Amersham Pharmacia, Arlington Heights, IL) followed by two sequential ethanol precipitations in the presence of 2.5 M ammonium acetate. Pellets were washed once with 70% ethanol, dried, and resuspended in 30 µl filter-sterilized TE (pH 8.0). Ten microliters each of the Cy3 and Cy5 labeled samples were combined, and 20 µg each of human Cot-1 DNA (Life Technologies, Carlsbad, CA) and Poly (dA) (Amersham Pharmacia, Arlington Heights, IL) were added to it. The mixture was denatured by heating at 95 °C for 3 minutes and held at 37 °C until hybridized to the microarray slides. Gene expression patterns of gene transcripts were determined using microarrays hybridized with dual, fluorescently labeled cDNA probes. RNA from uninoculated roots was labeled with Cy3 and RNA from inoculated roots with Cy5, with the two mixed together and used for hybridization. In each experiment, for one of the slides the dye used for labeling each RNA was switched so the RNA from uninoculated roots was labeled with Cy5 and RNA from inoculated roots with Cy3. This switch in labeling was to account for any artifacts generated by differences between Cy3 and Cy 5, such as potential differences in dye incorporation.

Microarray hybridization: The arrays were prehybridized in 5× SSC, 1% BSA, and 0.1% SDS at 42 °C for 40 to 60 minutes. An equal volume of 2× hybridization buffer (50% formamide, 10× SSC, 0.2% SDS) prewarmed to 42 °C was added to the denatured probe. The mix was then applied onto the microarray slide surface and covered with cover slips (Grace Biolabs, Bend, OR). Arrays were transferred to a hybridization chamber and incubated for 18 to 20 hours at 42 °C in a water bath to maintain high humidity. Arrays were washed for 4 minutes at room temperature in slide racks placed inside Coplin jars in each of the following buffers: a low stringency wash buffer (1× SSC, 0.2% SDS) at 42 °C, a high stringency wash buffer (0.1× SSC, 0.2% SDS), and 0.1× SSC at room temperature. They were dried by centrifugation at 400 rpm for 1 minute and scanned for each fluor at a 10-micron resolution and a PMT and laser power set to 70, using ScanArray 4000 (GSI Lumonics, Meriden, CT).

Microarray data analysis: Table 1 lists the slides and their respective probe combination and probe order for each of the three biological samples. Fluorescence intensities were extracted using the image analysis package SPOT (available at http://www.cmis.csiro.au/iap/spot.htm). For each biological sample, one slide was hybridized with probes where the fluorescent dye labels were reversed (Table 1).

The elements with either printing or hybridization artifacts were flagged and discarded before analysis. Only spots with an intensity of at least two times above the local background in both channels were used for subsequent analysis. This filtering process usually removed 5% to 15% of the elements, the percentage of which varied from slide to slide. The extracted data from each slide were then log transformed (using log base two) and normalized using the Lowess print-tip group normalization method (Yang et al., 2002). Four replicates for each clone were printed side by side on each slide, and each slide was replicated three times by hybridizing it with the same probe pair. Generally low variability was found within microarray slides, with the 4 replicates for each clone having low standard deviations. Variability from slide to slide was greater, but only

TABLE 1. Probe labeling combinations for each microarray slide.

Slide	Probe combination	Probe order
	Biological sample 1	
Slide 1 (s4)	K+/K–	Cy3/Cy5
Slide 2 (s14)	K+/K–	Cy3/Cy5
Slide 3 (s15)	K+/K–	Cy5/Cy3
	Biological sample 3	
Slide 1 (s35)	K+/K–	Cy3/Cy5
Slide 2 (s36)	K+/K–	Cy3/Cy5
Slide 3 (s37)	K+/K–	Cy5/Cy3
	Biological sample 3	
Slide 1 (s53)	K+/K–	Cy5/Cy3
Slide 2 (s54)	K+/K–	Cy3/Cy5

clones that exhibited similar expression patterns across all slide replications were selected for further analysis.

One-way analysis of variance (ANOVA) was used as a tool to detect similar expression levels across replicated slides. ANOVA tests were conducted to compare the mean expression level of clones across replicated slides (Bilban et al., 2002). Only clones with an F-value high enough to produce a probability of 0.1 or higher were selected as being similar across replicated slides. SQL procedural scripts were written to conduct such computationally intense calculations for every clone post filtration and normalization. A Web-based interface also was created for this purpose and can be accessed from the URL given below.

Significance analysis of microarrays (SAM) (Tusher et al., 2001) was used to determine statistically induced/suppressed genes across the three biological samples. The input to SAM is gene expression measurements from a set of microarray experiments, as well as a response variable from each experiment. SAM computes a statistic d_i for each gene i, measuring the strength of the relationship between gene expression and the response variable. SAM uses repeated permutations of the data to determine if the expression of any gene is significantly related to the response (Tusher et al., 2001). We used the one class response format in SAM to test whether the mean gene expression from the three biological samples differed from zero. The cutoff for significance in SAM is determined by a tuning parameter delta, chosen by the user based on the false positive rate. We chose a delta value of 0.29, which resulted in a 10.12 false positive rate. SAM found 100 significant genes, 99 of them induced and 1 suppressed.

A relational database was constructed using SQLServer2000 for storage, analysis, and comparison of microarray data for this experiment. SQL procedures and other in-house tools were developed to perform the statistical analysis without resorting to third-party software.

The database along with the tools that were developed to analyze and compare the data is available at http://psi081.ba.ars.usda.gov/SGMD/default.htm. Also available are the raw and normalized data for each slide that was printed.

RNA blot analysis: Ten μg of total RNA from samples that were also used for probing microarrays was electrophoresed in a 1.2% agarose gel containing formaldehyde in FA (MOPS, sodium acetate, EDTA) gel buffer and then transferred to a nylon membrane (Schleicher and Schuell, Keene, NH). The probes were prepared by PCR amplification of specific cDNA clones using vector-specific primers and a Strip-EZ™PCR kit from Ambion (Austin, TX). The unincorporated nucleotides were removed by passing through a G50 spin column (Amersham Pharmacia, Arlington Heights, IL). The blots were hybridized at 50 °C in 50% formamide, 5× SSPE, 5× Denhardt's reagent, 1 % SDS, and 100 μg/ml denatured salmon sperm DNA. The filter membranes were washed at 50 °C for 30 minutes in each of the following buffers: 2× SSPE/0.1% SDS, 1× SSPE/0.1% SDS and 0.1× SSPE/0.1% SDS. The membrane was against a Biomax X-ray film, and the film was exposed at −80 °C for approximately 18 hours.

Results

ESTs used for microarray printing: Microarrays were prepared using cDNA inserts from 1,305 soybean ESTs. Functional classification of the ESTs comprising the microarray is given in Table 2. Almost half of the printed clones had either no sequence similarity to known genes (null) or encoded products with no known function (unknown). Approximately 14% of the printed genes encoded structural proteins with a putative role in cell wall maintenance. A similar number of clones are involved in metabolism. Genes that are induced in response to stress or may be components of signal transduction pathways made up about 5% each of the total printed elements. Less than 1% of the genes had a function in membrane transport or synthesis of secondary metabolites.

Statistical analysis and gene expression profiles: The reproducibility between distinct biological samples (separate experiments) and between different slides of the same biological sample (same experiment) was examined. In Figure 2, each box plot shows the median $\log_2$ expression ratio and the spread of the data from the median. The plots in Figures 2A,B,C compare the deviation from the mean of separate hybridizations with probe prepared from the same RNA sample. As observed from the values of the median expression ratio and the upper and lower quartile for both the probe pairs (inoculated Kent vs. noninoculated Kent), there was slightly more variation within replicates of biological sample 2 than within biological samples 1 and 3. The median and the upper and lower quartile values

Table 2. Functional categorization of soybean ESTs on the array for monitoring expression changes in response to nematode infection.[a]

Category	Total printed ESTs	Percentage of total printed ESTs
Stress induced	58	4.4
Cell wall maintenance/development	181	13.8
Synthesis of secondary metabolites	10	0.8
Metabolic pathways	169	13
Signal transduction	71	5.4
Membrane transport	5	0.4
Null	152	11.6
Unknown	659	50.5

[a] The second column gives the number distribution of clones in each category. The third column represents the number of clones in each category as a percent of total printed ESTs.

were similar for biological samples 1 and 2 (Fig. 2D); however there was less spread in biological sample 3. Only clones that showed similar expression levels in biological as well as experimental replicates were used in further analysis.

Significance analysis of microarrays revealed that 99 of the 1,305 ESTs were induced in NL1-RHp inoculated Kent relative to the noninoculated control (K^+/K^-) (Table 3) and included proline-rich proteins and hydroxyproline-rich glycoproteins that are involved in cell wall synthesis and maintenance. In addition, there were genes with a putative role in the general plant-defense response—for example, peroxidase, copper containing amine oxidase, lipoxygenase, stress-induced gene (SAM22), and β-1,3-endoglucanase. All of the clones induced ($P = 0.05$) in the roots of Kent upon nematode infection were classified into different functional categories (Fig. 3). The ESTs involved in cell division/growth/maintenance, protein synthesis, and stress comprised approximately one-tenth of the induced clones in Kent. Around 15% of the induced clones are involved in metabolism, and more than half encode genes with as-yet undetermined function. A complete list of the induced genes ($P = 0.05$) can be found at our Web site http://psi081.ba.ars.usda.gov/SGMD/MicroarrayExps/2D_KentPaper.htm. DNA sequences of genes with low similarity to those in the soybean EST database were compared to those in the SCN EST database to identify genes derived from SCN. None of those genes had any significant correspondence with ESTs in SCN EST database. Therefore, the origin of ESTs that had no similarity to genes in either database is unclear.

TABLE 3. Soybean cDNA clones that were significantly induced in response to SCN invasion in the susceptible soybean cultivar Kent (K^+/K^-).[a]

Putative clone ID	Genbank accession #	e-value[b]	Fold induction[c]
Repetitive proline-rich protein—extensin	BM139486	6.0E-99	1.67+/−0.33
Copper containing amine oxidase	BM139859	4.0E-55	1.65+/−0.32
Peroxidase	Null	3.4E-23	1.64+/−0.42
Chloroplast nucleoid DNA binding			
Aspartic protease	BM139823	9.0E-06	1.43+/−0.52
Stress induced gene	BM139740	1.0E-105	1.43+/−0.70
Z. mays ribosomal protein S8 mRNA	BM139873	5.0E-08	1.40+/−0.16
P. sativum fructose-1, 6-biphosphate aldolase	BM140112	1.0E-17	1.36+/−0.15
Basic chitinase class 3	BM139485	5.0E-46	1.36+/−0.16
G. max beta-1,3-endoglucanase mRNA	BM139513	0	1.36+/−0.20
G. max sucrose synthase (SS) mRNA	BM140019	3.0E-39	1.36+/−0.18
A. thaliana cda1 gene	BM139535	3E-09	1.36+/−0.22

[a] The average $\log_2$ expression ratio of nematode infected vs. noninfected control from each of three biological samples; the fold-induction of each clone and the standard deviation are given.

[b] The e-value as predicted from the BlastN search of the clone sequence against the GenBank nr database.

[c] Mean of three biological replicates ± standard deviation.

Validation of microarray data by northern blot analysis: To confirm the expression observed from microarray analysis, the expression pattern of a stress-induced gene, a β1,3-endoglucanase, and dihydrodiadzein reductase was determined by northern blot analysis (Fig. 4). The expression levels of the genes were similar when measured with either technique. Both methods showed that expression of the stress-induced gene and β-1,3-endoglucanase were induced. Our microarray data indicated that there was no change in dihydrodiadzein reductase expression; this was confirmed by northern blot analysis.

DISCUSSION

This is the first study to examine the expression pattern of soybean genes in a susceptible soybean genotype in response to invasion by a host-specific pathogen at the genomic level using microarrays. Of the approximately 1,300 genes present on the array, 8% were induced in the presence of the nematodes. It is possible that the number of differentially regulated genes would be higher if it were not for the variation observed between the experiments in the infected vs. noninfected Kent (K^+/K^-). However, this result underscores the importance of performing at least two, if not more, biological replications. In our experiments the roots from different biological replicates were infected with similar numbers of nematodes as determined by examination of stained roots, but there still remain other factors such as different batches of hatched juveniles, light, humidity, and temperature post-inoculation—conditions that are not completely replicable from one experimental run to another that may contribute to variability between experiments. Among the genes with increased expression in response to nematode invasion were those encoding peroxidase, a stress-induced gene, structural cell wall proteins, and β-1,3-endoglucanase (Goellner et al., 2001; Kombrink et al., 1988; Vercauteren et al., 2001).

Approximately one-tenth of the induced genes have been shown to have a role in cell wall strengthening and development, which is an early defense response. This is consistent with an earlier study (Neibel et al., 1993) looking at the induction patterns of cell wall structural proteins, primarily extensins, in tobacco during a compatible interaction with cyst nematode. Extensin gene expression was found to be induced slightly and transiently, most likely due to wounding caused by penetration of the nematodes at an early stage after infection. Similar to the function of extensins in a compatible interaction of root-knot nematodes, these structural proteins may be strengthening the syncytium walls to protect the nutrient contents within, during a com-

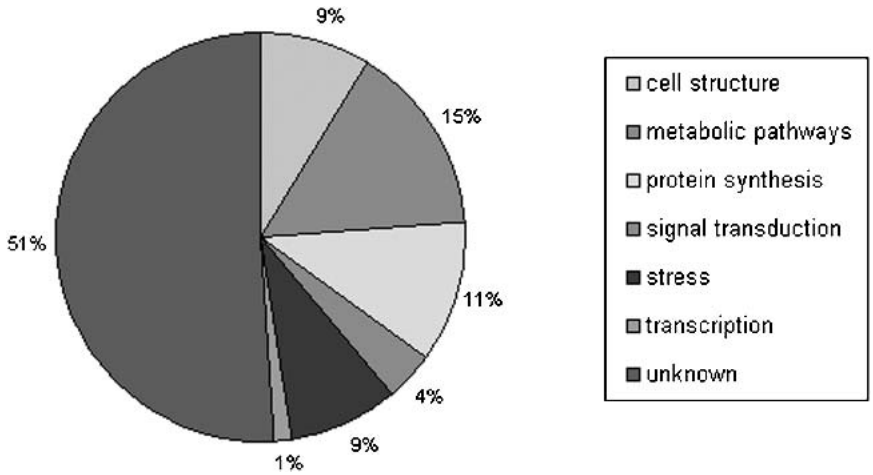

FIG. 3. Functional classification of induced genes. Genes induced specifically in nematode-infected Kent (K^+/K^-) were classified into different tentative functional categories based upon the homology search results.

patible cyst nematode interaction. The functional composition of the statistically induced genes indicates that 2 days after challenge with the nematode there is increased activity of cell wall reinforcing genes, stress related genes, and genes involved in protein synthesis and metabolism. This is consistent with published reports that the genes induced early in nematode infection in the susceptible cultivar upon invasion of alfalfa roots by *Meloidogyne incognita* are related to metabolic pathways or to stress/defense response (Potenza et al., 2001).

An important aspect of the microarray technology is to identify unknown genes and assign function to them. It is quite likely that the induced genes with unknown functions, once characterized, may provide useful information regarding the signaling pathways used by the susceptible soybean in response to nematode invasion. Many of these genes may not play a role in susceptibility but may be responding to wounding occurring during nematode invasion.

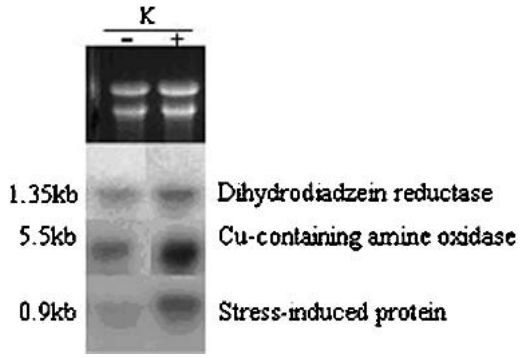

FIG. 4. Validation of microarray data by gel blot analysis. Northern blot analysis was performed on a gene encoding a stress-induced protein, a copper containing amine oxidase gene, and dihydrodiadzein reductase. Roots of soybean plants (Kent) were either infected with SCN for 48 hours or were noninfected, and total RNA was isolated for northern blot analysis. The expression level was normalized to the total RNA in the ethidium bromide stained gel. The names of the genes and the transcript sizes are indicated on the side.

A limitation of our experiments is the small size of the array. The cDNA clones used for generation of the microarray used in this study were prepared from whole plants and whole roots of soybean plants displaying a resistance response when infected with SCN. A larger number of genes represented on the array, including genes derived specifically from libraries constructed from RNA extracted from susceptible soybean roots after SCN invasion, might capture other genes important to the soybean response to SCN. As such, many of the clones may represent genes that are involved in a systemic response and not a localized response associated with the infection site or formation of feeding structure. As the soybean EST database expands, more clones representing genes involved in resistance can be added and redundancy of clone representation decreased. A more comprehensive picture of putative signaling pathways in the soybean-SCN interaction will emerge from looking at the gene expression patterns over time with a larger soybean array and with clones derived from cDNA libraries prepared from tissue immediately surrounding and including the nematode feeding structure.

LITERATURE CITED

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research 25:3389–3402.

Bilban, M., L. K. Buehler, S. Head, G. Desoye, and V. Quaranta. 2002. Normalizing DNA microarray data. Current Issues in Molecular Biology 4:57–64.

Endo, B. Y. 1964. Penetration and development of *Heterodera glycines* in soybean roots and related anatomical changes. Phytopathology 54:79–88.

Endo, B. Y. 1965. Histological responses of resistant and susceptible soybean varieties, and backcross progeny to entry and development of *Heterodera glycines*. Phytopathology 55:375–381.

Endo, B. Y. 1991. Ultrastructure of initial responses of susceptible and resistant soybean roots to infection by *Heterodera glycines*. Revue de Nématologie 14:73–94.

Gheysen, G., and C. Fenoll. 2002. Gene expression in nematode feeding sites. Annual Review of Phytopathology 40:191–219.

Gipson, I., K. S. Kim, and R. D. Riggs. 1971. An ultrastructural study of syncytium development in soybean roots infected with *Heterodera glycines*. Phytopathology 61:347–353.

Goellner, M., X. Wang, and E. L. Davis. 2001. Endo-beta-1,4-glucanase expression in compatible plant-nematode interactions. Plant Cell 13:2241–2255.

Hegde, P., R. Qi, K. Abernathy, C. Gay, S. Dharap, R. Gaspard, J. E. Hughes, E. Snesrud, N. Lee, and J. Quackenbush. 2000. A concise guide to cDNA microarray analysis. Biotechniques 29:548–562.

Hermsmeier, D., J. K. Hart, M. Byzova, S. R. Rodermal, and T. J. Baum. 2000. Changes in mRNA abundance within *Heterodera schachtii*-infected roots of *Arabidopsis thaliana*. Molecular Plant Microbe Interactions 13:309–331.

Hermsmeier, D., M. Mazarei, and T. J. Baum. 1998. Differential display analysis of the early compatible interaction between soybean and soybean cyst nematode. Molecular Plant Microbe Interactions 11:1258–1263.

Kawasaki, S., C. Borchert, M. Deyholos, H. Wang, S. Brazille, K. Kawai, D. Galbraith, and H. J. Bohnert. 2001. Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 13:889–905.

Kiegle, E., C. A. Moore, J. Haseloff, M. A. Tester, and M. R. Knight. 2000. Cell-type-specific calcium responses to drought, salt, and cold in the *Arabidopsis* root. Plant Journal 23:267–278.

Kim, Y. H., R. D. Riggs, and K. S. Kim. 1987. Structural changes associated with resistance of soybean to *Heterodera glycines*. Journal of Nematology 19:177–187.

Kombrink, E., M. Schröeder, and K. Hahlbrock. 1988. Several pathogenesis-related proteins in potato are β-1,3-glucanases and chitinases. Proceedings of the National Academy of Sciences USA 85:782–786.

Maguire, T. L., S. Grimmond, A. Forrest, I. Iturbe-Ormaetxe, K. Meksem, and P. Gresshoff. 2002. Tissue-specific gene expression in soybean (*Glycine max*) detected by cDNA microarray analysis. Journal of Plant Physiology 159:1361–1374.

Mahalingam, R., H. T. Knap, and S. A. Lewis. 1998. Inoculation method for studying early responses of *Glycine max* to *Heterodera glycines*. Journal of Nematology 30:237–240.

Mahalingam, R., and H. T. Skorupska. 1996. Cytological expression of early response to infection by *Heterodera glycines* Ichinoe in resistant PI437654 soybean. Genome 39:986–998.

Mujer, C. V., D. L. Andrews, J. R. Manhart, S. K. Pierce, and M. E. Rumpho. 1996. Chloroplast genes are expressed during intracellular symbiotic association of *Vaucheria litorea* plastids with the sea slug *Elysia chlorotica*. Proceedings of the National Academy of Sciences USA 93:12333–12338.

Neibel, A., J. de Almeida Engler, A. Hemerly, P. Ferreira, D. Inze, M. Van Montagu, and G. Gheysen. 1996. Induction of cdc2a and cyc1At expression in *Arabidopsis thaliana* during early phases of nematode-induced feeding cell formation. Plant Journal 10:1037–1043.

Neibel A., J. de Almeida Engler, C. Tire, G. Engler, M. V. Montagu, and G. Gheysen. 1993. Induction patterns of an extensin gene in tobacco upon nematode infection. Plant Cell 5:1697–1710.

Oztur, Z. N., V. Talame, M. Deyholos, C. B. Michalowski, D. W. Gozukirmizi, R. Tuberosa, and H. J. Bohnert. 2002. Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Molecular Biology 48:551–573.

Potenza, C., S. H. Thomas, and C. Sengupta-Gopalan. 2001. Genes induced during early response to *Meloidogyne incognita* in roots of resistant and susceptible alfalfa cultivars. Plant Science 161:289–299.

Puthoff, D. P., D. Nettleton, S. R. Rodermel, and T. J. Baum. 2003. *Arabidopsis* gene expression changes during cyst nematode parasitism revealed by statistical analysis of microarray expression profiles. Plant Journal 33:911–921.

Reymond, P., H. Weber, M. Damond, and E. E. Farmer. 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell 12:707–719.

Schaffer, R., J. Landgraf, M. Acerbi, V. Simon, M. Larson, and E. Wisman. 2001. Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. Plant Cell 13:113–123.

Schenk, P. M., K. Kazan, I. Wilson, J. P. Anderson, T. Richmond, S. C. Somerville, and J. M. Manners. 2000. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proceedings of the National Academy of Sciences USA 97:11655–11660.

Seki, M., M. Narusaka, H. Abe, M. Kasuga, K. Yamaguchi-Shinozaki, P. Carcini, Y. Hayashizaki, and K. Shinozaki. 2001. Monitoring the expression pattern of 1,300 *Arabidopsis* genes under drought and cold stresses using a full-length cDNA microarray. Plant Cell 13:61–72.

Thibaud-Nissen, F., R. T. Shealy, A. Khanna, and L. O. Vodkin. 2003. Clustering of microarray data reveals transcript patterns associated with somatic embryogenesis in soybean. Plant Physiology 132: 118–136.

Tomkins, J. P., R. Mahalingam, H. Smith, J. L. Goicoechea, H. T. Knap, and R. A. Wing. 1999. A bacterial artificial chromosome library for soybean PI 437654 and identification of clones associated with cyst nematode resistance. Plant Molecular Biology 41:25–32.

Tusher, V., R. Tibshirani, and C. Chu. 2001. Significance analysis of microarrays applied to ionizing radiation response. Proceedings of the National Academy of Sciences USA 98:5116–5121.

Vaghchhipawala, Z., R. Bassuner, K. Clayton, K. Lewers, R. Shoemaker, and S. Mackenzie. 2001. Modulations in gene expression and mapping of genes associated with cyst nematode infection of soybean. Molecular Plant Microbe Interactions 14:42–54.

Vercauteren, I., E. V. D. Schuren, M. V. Montagu, and G. Gheysen. 2001. *Arabidopsis thaliana* genes expressed in the early compatible interaction with root-knot nematodes. Molecular Plant Microbe Interactions 14:288–299.

Williamson, V. M. 1999. Plant nematode resistance genes. Current Opinions in Plant Biology 2:327–331.

Wrather, J. A., Stienstra, W. C., and S. R. Koenning. 2001. Soybean disease loss estimates for the United States from 1996 to 1998. Canadian Journal of Plant Pathology 23:122–131.

Yang, Y. H., S. Dudoit, D. M. Lin, V. Peng, J. Ngai, and T. P. Speed. 2002. Normalization for cDNA microarray data: A robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Research 30:e15.