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ORIGINAL PAPER

Gene expression profiling of resistant and susceptible soybean lines infected with soybean cyst nematode

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Abstract Soybean cyst nematode (SCN) is the most devastating pathogen of soybean. Information about the molecular basis of soybean–SCN interactions is needed to assist future development of effective management tools against this pathogen. Toward this end, soybean transcript abundance was measured using the Affymetrix Soybean Genome Array in a susceptible and a resistant reaction of soybean to SCN infection. Two genetically related soybean sister lines TN02-226 and TN02-275, which are resistant and susceptible, respectively, to the SCN race 2 infection were utilized in these experiments. Pairwise comparisons followed by false discovery rate analysis indicated that the expression levels of 162 transcripts changed significantly in the resistant line, of which 84 increased while 78 decreased. However, in the susceptible line, 1,694 transcripts changed significantly, of which 674 increased while 1,020 decreased. Comparative analyses of these transcripts indicated that a total of 51 transcripts were in common between resistance and susceptible responses. In this set, 42 transcripts increased in the resistant line, but decreased in the susceptible line. Quantitative real-time reverse-transcription polymerase chain reaction confirmed the results of microarray analysis. Of the transcripts to which a function could be assigned, genes were associated with metabolism, cell wall modification, signal transduction, transcription, and defense. Microarray analyses examining two genetically related soybean lines against the same SCN population provided additional insights into the specific changes in gene expression of a susceptible and a resistant reaction beneficial for identification of genes involved in defense.

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Introduction

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is a sedentary biotrophic endoparasite of roots that causes extensive damage to soybean, *Glycine max* (L.) Merr., worldwide (Wrather and Koenning 2006). To complete the life cycle, infective second-stage juveniles (J2) enter host roots and migrate intracellularly within the cortical tissue to the vascular cylinder. The J2 then initiate formation of specialized feeding sites called syncytia, which function as metabolic sinks to nourish the nematodes (Jones 1981). In susceptible cultivars, nematodes depend entirely on functional syncytia to acquire nutrients to develop into reproductive adult males or females. J2 also penetrate roots of resistant cultivars and initiate syncytia. However, resistance soon manifests itself by a degeneration of the young syncytia and a failure of the nematode to develop further (Endo 1991; Sobczak and Golinowski 2009). Syncytium

formation and maintenance are mediated through nematode signaling and accompanied by changes in plant gene expression (reviewed by Jasmer et al. 2003; Davis et al. 2008; Gheysen and Mitchum 2009). Identification and characterization of host plant genes that change expression and, therefore, are potentially involved in the plant–nematode interaction might prove to be an important tool to aid in the understanding of the molecular mechanisms involved in this complex interaction. Ultimately, understanding the mechanisms underlying in the host plant and nematode interaction leads to the development of durable crop protection strategies (Fuller et al. 2008; Klink and Matthews 2009; Li et al. 2009; Mitchum and Baum 2008; Tomczak et al. 2009; Williamson and Kumar 2006).

The GeneChip microarray technology is an established platform for measuring gene transcript levels. The Affymetrix Soybean GeneChip contains 37,744 *G. max* probe sets allowing the analysis of gene expression on a large scale. The soybean GeneChip has been used in a number of studies involving soybean–SCN interaction. Several of them focused on the susceptible reaction of soybean to SCN infection (Alkharouf et al. 2006; Ithal et al. 2007a, b; Puthoff et al. 2007). There are also notable studies using the soybean array to study SCN infection in both susceptible and resistant reactions (Klink et al. 2007a, b; 2009; 2010). These studies focused on using two different nematode populations that yielded susceptible and resistant reactions in the same soybean cultivar.

In the present study, we used the Affymetrix Soybean GeneChip to study SCN infection. Our study centers on using two genetically related soybean lines TN02-226 and TN02-275, which are resistant and susceptible, respectively, to the SCN race 2 infection. This approach provides an opportunity to gain additional insights into the transcriptional profiling of a resistant and susceptible reaction to SCN infection. These comparative analyses revealed specific changes in gene expression occurring in the resistant and susceptible reactions providing further knowledge beneficial for identification of genes involved in defense.

Materials and methods

Plant source

Two genetically related soybean lines TN02-226 and TN02-275, which are resistant and susceptible, respectively, to the SCN race 2 (HG type 1.2.5.7) were used throughout this study. The two F_6-derived sister lines, TN02-226 and TN02-275 were developed from the cross of two SCN resistant parents 'Fowler' × 'Anand' following a pod descent modification of the single seed descent procedure (Sleper and Poehlman 2006). Both cultivars Fowler and Anand were derived from cultivar Hartwig for their resistance. The parent Fowler is the registered cultivar CV-421 (Young 2001) developed by the cross Hartwig × 'Holladay', where Hartwig is the registered cultivar CV-297 (Anand 1992) and Holladay is the registered cultivar CV-341 (Burton et al. 1996). The parent Anand is the registered cultivar CV-428 that was developed from the cross Hartwig × Holladay (Anand et al. 2001). Thus, TN02-226 and TN02-275 share the same parents as well as the same grandparents in their pedigrees. At the time that the cross was made, Hartwig, Fowler, and Anand were the only cultivars known to have resistance to SCN race 2 (HG type 1.2.5.7) and the level of resistance differed somewhat among the three resistant cultivars. The genetic control of SCN race 2 (HG type 1.2.5.7) resistance is complex, as evidenced by the scarcity of resistant cultivars, and by the difficulty in recovering resistant lines from among recombinant inbred lines from crosses where one or more parents exhibited resistance. Our initial goal was to develop earlier maturing lines with broad resistance and to generate materials that would further augment SCN resistance research. The development of the genetically related lines TN02-226 and TN02-275 provided an opportunity to gain further insight on the underlying genetic control of resistance.

TN02-226 was tested in the USDA Southern Regional Tests from 2004 through 2007. It was tested in the 2004 Uniform Preliminary IV-S and in the 2005, 2006, and 2007 Uniform IV-s. TN02-275 was tested in the USDA Southern Regional Tests from 2005 through 2007. It was tested in the 2005 Uniform Preliminary IV-S, the 2006 Uniform IV-s, and the 2007 Uniform Preliminary V (Paris and Shelton 2005, 2006; Gillen and Shelton 2007, 2008). In these tests, reaction of soybean lines TN02-226 and TN02-275 was evaluated against SCN race 2, 3, and 14 (HG type 1.2.5.7, 5.7, and 1.3.5.6.7, respectively) according to standard classification and rating system. Plants were rated based on the number of cysts on the roots as 1 = 0–5 cysts on the roots, 2 = 6–10 cysts on the roots, 3 = 11–20 cysts on the roots, 4 = 21–40 cysts on the roots, and 5 = >40 cysts on the roots. The mean rating reported for each SCN population was calculated as follows: mean rating = (rating category × # plants receiving rating)/total # of plants.

In these reaction tests, TN02-226 scored superior for SCN race 2 resistance than did TN02-275. As such TN02-226 scored a mean of '1', '1', and '1' whereas TN02-275 scored a mean of '4', '4', and '1' for SCN race 2, 3, 14, respectively (Paris and Shelton 2005, 2006; Gillen and Shelton 2007, 2008).

Nematode source

A SCN race 2 (HG Type 1.2.5.7) was originally collected from soybean fields in Beaufort County, NC, USA. It was

cultured in the greenhouse under controlled conditions of temperature and light with limited inbreeding and maintained on the roots of cv. Pickett-71 (Hartwig et al. 1971) for multiple generations before it was used for inoculum preparation (Arelli et al. 2000).

Determination of soybean reaction to SCN

A bioassay using the current HG Type classification system (Niblack et al. 2002) was used to evaluate the soybean lines TN02-226 and TN02-275 under our experimental conditions. The HG Type of classification system includes seven indicator lines and a susceptible line for determination of soybean reaction to SCN. The indicator lines include PI 548402 (Peking), PI 88788, PI 90763, PI 437654, PI 209332, PI 89772, PI 548316 (Cloud), and a standard susceptible control includes cv. Lee-74 (PI 548658) (Caviness et al. 1975).

The method for the SCN bioassay performed in the greenhouse followed established protocols (Arelli et al. 2000) with the modifications described in Arelli and Wang (2008). Each plant was grown in a 7-cm in diameter clay pot filled with steam sterilized soil on a greenhouse bench top with an evaporative cooling and under bench heating system. A computerized system with regulated duration of light, heating, and cooling systems was used in the greenhouse during the bioassay for proper growth of soybean seedlings and nematodes. Ten seedlings for each of the two soybean lines, indicator lines and a standard susceptible control were included in the experiment and maintained at 27°C (±2°C). The seedlings were grown for 4–5 days prior to their inoculation with SCN race 2 (HG Type 1.2.5.7) eggs. Each seedling was inoculated with 5 ml of inoculum consisting approximately 1,500 eggs. The eggs were suspended in de-ionized water and a pippettor dispensed the inoculum closer to the roots of the seedlings. Approximately 30 days after inoculation, plant roots were individually washed with a strong jet of water to dislodge the females and cysts. These were counted under a stereomicroscope, and a female index (FI) was calculated for the number of females developing on each soybean plant (Golden et al. 1970). Female index is the number of SCN females occurring on a soybean plant expressed as the percentage of mean number of females on susceptible Lee-74. Ratings of resistant (FI = 0–9%), moderately resistant (FI = 10–30%), moderately susceptible (FI = 31–60%), and susceptible (FI = >60%) were used to classify the reaction of soybean plants based on Schmitt and Shannon (1992).

Plant inoculation and tissue harvesting for microarrays

For microarray experiments, sterile soybean plants were established by germination of surface-sterilized seeds. Seeds were surface-sterilized by immersion in 70% ethanol for 5 min and in 2.1% sodium hypochlorite for 12 min and then rinsed three times in sterile distilled water for 10 min. Seeds were germinated in the dark at 26°C on 1% water agar for 2 days, transferred to magenta boxes containing sterile sand supplemented with Hoagland growth solution (Sigma, St. Louis, MO, USA), and grown for a week in a growth chamber at 26°C with a 16-h photoperiod of approximately 2,400 Lux provided by fluorescent light bulbs. The seedlings were then gently removed from the sand, rinsed with sterile water, and combined into groups of ten seedlings. Mature female nematodes were harvested and eggs were prepared 3 days previously according to standard recommended method (Niblack et al. 2002) with few modifications. Nematode population (race 2 = HG Type 1.2.5.7) was increased on susceptible soybean cultivar Hutcheson (Buss et al. 1988) in the greenhouse and cysts were harvested for preparation. In brief, the cysts were ground on the surface of a 100 μ sieve nested over 200 and 500. Released eggs and fine debris collected from the 500 sieve were centrifuged. The egg suspension and the fine debris were re-suspended and layered on the surface of a 30–40 ml sugar water mixture (45.5%) in a 50 ml centrifuge tube. The tube was centrifuged again at 2,000 rpm for 4 min. The resulting band of eggs was transferred to the 500 μ sieve and rinsed with distilled water. The eggs and water was reduced to a volume of 25 ml and treated with 20 ml stock solution of fungicide Hibitane, stirred and rinsed with distilled water. The egg density was determined by counting the number of eggs in an appropriate volume of suspension. Preparation of the infective J2s was performed essentially as described by Klink et al. (2007a) with some modifications. The eggs were placed in a small plastic tray with 1 cm of water. The tray was covered with plastic wrap and placed on a rotary shaker at 25 rpm. After 3 days, J2s were then separated from unhatched eggs by running them through a 41 μm mesh cloth. The J2s were concentrated by centrifugation for 1 min at 1,500 rpm to 2,000 J2/ml. This represented the inoculum. Each group of ten seedlings was placed on moistened germination paper and inoculated by pipetting 2,000 J2 per seedling directly on the roots. Control mock-inoculated replicates received the same amount of water. The roots were then sprinkled with sterile sand, covered with a moistened sheet of germination paper, placed in a plastic tray with a 1 cm of water in the bottom to add humidity, and kept in the growth chamber mentioned above.

Infection was synchronized by washing the infected roots (infected and mock-infected control) with water exactly 24 h after inoculation to remove the extraneous nematodes that had not yet penetrated the root. This prevented additional nematodes from entering the root. Samples were collected at 3, 6, and 9 days post inoculation

(dpi). For each time point, one of ten seedlings in each group was collected for acid fuchsin staining to determine the number and life stage of the juveniles (Hussey 1990). A total of three independent biological replicates were obtained. The experiment followed a randomized complete block design with the three replicates as blocks and with a full factorial treatment structure with three treatment factors. The three treatment factors were the time (3 levels), genotype (resistant or susceptible), and infection type (SCN or mock).

For total RNA isolation, excised root tissue from six seedlings in each group were pooled at each time point and flash frozen in liquid nitrogen. The tissue was ground to a fine powder under liquid nitrogen and stored at −80°C.

RNA extraction

Total RNA was extracted from excised root tissues harvested at time points 3, 6, and 9 dpi using the RNeasy Plant mini kit (Qiagen, Valencia, CA, USA), and DNA contamination was removed with an on-column DNase treatment (Qiagen) following the manufacturer's instructions. High quality RNA samples were confirmed by agarose gel electrophoresis and with a bioanalyzer.

Microarray analyses

Total RNA samples of time point 3 dpi were used in the GeneChip soybean genome array (Affymetrix). This oligonucleotide array contains over 37,500 probes sets representing 35,611 soybean transcripts. A description of the GeneChip® soybean genome array is available at the Affymetrix® website (http://affymetrix.com/index.affx). A total of 12 chips were purchased from Affymetrix (Santa Clara, CA, USA), i.e. replicate biological samples for the time point 3 dpi for SCN+ and SCN− root tissue of susceptible line (TN02-275) and resistant line (TN02-226).

The GeneChips were processed at the University of Tennessee Knoxville Affymetrix Core Facility where cDNA and cRNA preparation, fragmentation, hybridization, staining, and scanning steps were performed according to the manufacturer's recommended protocols. As described by Panthee et al. (2009), the Affymetrix protocol for one-cycle eukaryotic target preparation was followed. First, 1 μg of total RNA was converted into single stranded cDNA using SuperScript II and a T7-Oligo (dT) primer (Affymetrix). Second strand cDNA was synthesized using dNTPs, second strand reaction buffer, *E. coli* DNA ligase, *E. coli* DNA polymerase I, and *E. coli* RNase H (all reagents from Affymetrix). The one cycle cDNA synthesis was followed by cleanup using Affymetrix GeneChip sample cleanup modules. Biotin-labeled cRNA was prepared using an Affymetrix IVT labeling kit. After cleanup of the in vitro transcription products, the purified cRNA was fragmented to a size range from 35 to 200 bases using fragmentation buffer at 94°C for 35 min. Fifteen micrograms of the fragmented cRNA was mixed into a hybridization cocktail containing hybridization buffer, B2 oligo control RNA (Affymetrix), herring sperm DNA, and BSA (both from Invitrogen). The solution was hybridized to a GeneChip at 45°C for 16 h at a setting of 60 rpm. After hybridization, the cocktail was removed from the GeneChip and stored for potential future analyses. Using the Affymetrics fluidics 450 wash station (Affymetrix fluidics protocol EUKGE_WS2v5), the GeneChip was washed and stained with streptavidine-phycoerythrin (Molecular Probes), followed by a wash with biotinylated antibody goat IgG and another staining with streptavidine phycoerythrin. The GeneChips were immediately scanned with a GeneChip 7G high-resolution scanner. The individual GeneChip scans were quality checked for the presence of control genes and background signal values.

The MAS5.0 Algorithm in Affymetrix's GCOS was utilized to scale and normalize the data to provide signal value intensities. This data was imported into Partek Genomics Suite (Partek, St. Louis, MO, USA) and logarithm base-2 transformed. Pairwise comparisons were performed between the groups (the SCN inoculated sample was compared to the control mock sample) and genes with at least twofold differential expression and a P value of less than 0.05 were selected for processing with a forward step-wise false discovery rate (FDR) method. Genes passing the P value selected after FDR were categorized as statistically significant differentially expressed genes.

The differentially expressed genes were annotated using the Affymetrix GeneChip Soybean Genome Array annotation page developed as part of SoyBase and The Soybean Breeder's Toolbox (http://soybase.org/AffyChip/). The website allows users to upload a file of probe set identifiers and download the corresponding available annotation data. The provided annotation data was generated by comparing the Soybean Genome Array consensus sequences, from which the probe sets were designed, with the UniProt protein database, the Pfam protein database, and the predicted coding sequences from the *Arabidopsis thaliana* genome [The Arabidopsis Information Resource (TAIR), using BLASTX Altschul et al. 1997].

Quantitative real-time reverse transcription-polymerase chain reaction

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) 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,

USA). These primers amplified a single product for each corresponding gene (Supplementary Table S1), as confirmed by the melting temperature of the amplicons and gel electrophoresis. One microgram of the total RNA from the three biological replicates was synthesized into first strand cDNA in a 20 µl reaction using the high capacity cDNA reverse transcription kit (Applied Biosystems). Total RNA was divided evenly into two reverse-transcription reactions. One of these reactions was used as the reverse transcription (RT) control (no reverse transcriptase was added), and the other reaction was used for later qRT-PCR. In the RT control reaction, water was substituted for reverse transcriptase. qRT-PCR was conducted in triplicate in 20 µl using Power SYBR Green PCR master mix (Applied Biosystems). PCR conditions used were 50°C for 2 min and 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Soybean ubiquitin-3 gene whose expression remains constant under different treatment conditions (Mazarei et al. 2007) was used as an internal control. We further determined by qRT-PCR that expression of this gene is stable across the treatment groups in the present study. The quantification of gene expression was performed using the relative ΔΔCT method by comparing the data with the internal gene (Bustin 2002).

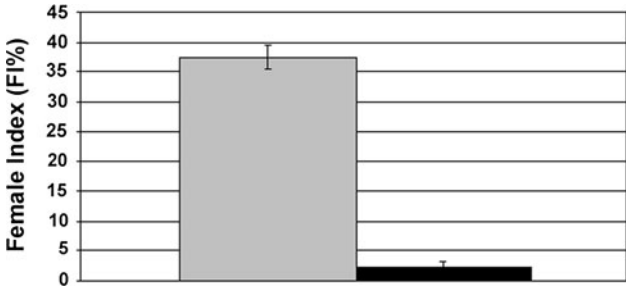

Fig. 1 Reaction assay of soybean lines TN02-275 (susceptible) and TN02-226 (resistant) after inoculation with soybean cyst nematode race 2 (HG type 1.2.5.7). Female index (FI) was used to differentiate susceptible and resistant reaction based on the standard classification system described in "Materials and methods". Each bar, TN02-275 (*gray*) and TN02-226 (*black*), represents the mean of three independent experiments with the standard errors of the mean noted

Table 1 Number of soybean cyst nematode race 2 (HG type 1.2.5.7) juveniles in roots of the soybean lines TN02-275 (susceptible) and TN02-226 (resistant) at 3, 6, and 9 days post inoculation (dpi)

Soybean line	3 dpi	6 dpi	9 dpi
TN02-275	193 (±2.65)[a]	203 (±3.53)	208 (±1.53)
TN02-226	185 (±2.65)	191 (±4.36)	215 (±3.22)

[a] Each value represents the mean of three replicates ± standard error. Statistical analyses using a two-sample *t* test showed no significant difference between the number of nematodes in roots of the two soybean lines at each time point

Results

Soybean reaction to SCN

Details of resistant and susceptible disease tests for soybean lines TN02-226 and TN02-275 in response to various races of SCN have been published previously (Paris and Shelton 2005, 2006; Gillen and Shelton 2007, 2008). These tests demonstrated that TN02-226 is resistant to SCN race 2 (HG Type 1.2.5.7) whereas TN02-275 is susceptible to the SCN race 2. We further examined reaction of these soybean lines to the SCN race 2 infection under our experimental conditions. A total of three independent SCN bioassay experiments were performed in the greenhouse and FI was used to differentiate resistant and susceptible individuals based on the standard classification system. Our results indicated that soybean TN02-226 is a highly resistant line and TN02-275 is a susceptible line with a tenfold difference in reaction response to the SCN infection (Fig. 1). We used simple sequence repeat (SSR) markers to examine the genetic differences between these soybean lines (Kazi et al. 2010). The markers included were Satt632 (LG A2) associated with *Rhg4*, Satt309 (LG G) associated with *rhg1*, and Satt574 (LG D2) associated with *Rhg3*. The resistant line TN02-226, inherited three resistant alleles from its Hartwig ancestry for markers Satt632, Satt309, and Satt574. The susceptible line TN02-275 did not inherit the Hartwig source of resistance from the allele Satt632 but did receive the other resistant alleles.

Gene expression profiling

The Affymetrix Soybean GeneChip was used to examine transcript accumulation in the soybean lines TN02-226 (resistant) and TN02-275 (susceptible) following SCN race 2 infection. Samples were collected at 3, 6, and 9 dpi. Nematode penetration into soybean roots was equally effective in both the susceptible and resistant soybean lines, since equal numbers of nematodes were observed inside the inoculated roots (Table 1). Nematode growth was evident by the increase in nematode-diameter in the roots of the susceptible line (Fig. 2a–c) but not in the roots of the resistant line (Fig. 2d–f). Assessment of nematode development in roots of the two soybean lines up to 28 dpi showed that juveniles in the roots of the resistant failed to mature. On the other hand, nematodes that infected susceptible roots developed into adult males and females, indicating a normal progression of the SCN life cycle (data not shown). These results demonstrated that, under our experimental conditions, the soybean lines TN02-226 and TN02-275 are resistant and susceptible, respectively, to the SCN race 2 population used in this study.

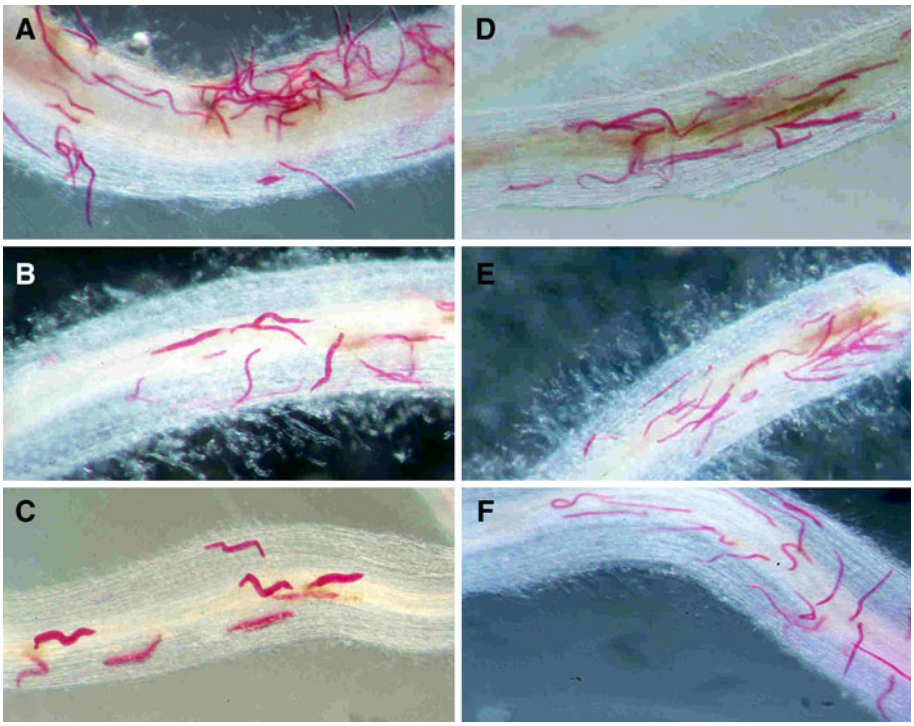

Fig. 2 Developmental stages of soybean cyst nematode race 2 (HG type 1.2.5.7) in soybean roots of TN02-275 (susceptible) and TN02-226 (resistant) lines. Roots were harvested at 3, 6, and 9 days post inoculation (dpi) and stained with acid fuchsin, which stains the nematodes *red*. **a–c** TN02-275 line infected with nematodes at 3, 6, 9 dpi, respectively. **d–f** TN02-226 line infected with nematodes at 3, 6, 9 dpi, respectively

High quality RNA was isolated from root samples. RNA samples of 3 dpi were used in microarrays, whereas RNA samples of 6 and 9 dpi were considered for subsequent analysis in qRT-PCR assays. We selected 3 dpi as an initial time point to avoid the gene expression changes associated with the early migratory phase of infection process and to regard the time point that nematodes have typically initiated feeding (Endo 1991; Alkharouf et al. 2006; Klink et al. 2007a, b).

Our analyses focus on two types of differentially expressed probe sets: unique and common. Unique probe sets are defined as those that are differentially expressed and found in only resistant or susceptible reactions. Common probe sets are defined as those that are differentially expressed and overlap between resistant and susceptible reactions. We first examined the number of transcripts exhibiting differential accumulation between SCN- and mock-inoculated plants for each soybean line. Pairwise comparisons were performed and transcripts with fold regulation cut-off of >2.0 and P value < 0.05 were selected for FDR processing. We detected 162 transcripts that were differentially expressed between mock and SCN treatments in the resistant line. This set of transcripts was associated with FDR of P value < 0.005, of which 84 transcripts increased while 78 transcripts decreased (Fig. 3a). We detected 1,694 differentially expressed transcripts in susceptible line TN02-275 with FDR of P value < 0.017, of which 674 transcripts increased while 1,020 transcripts decreased (Fig. 3a). Largely, probe sets measuring differential transcript levels were the most prevalent in the susceptible line with approximately tenfold more probe sets as compared to the resistant line (Fig. 3a). We also detected 51 transcripts that were differentially expressed in both the resistant and susceptible lines (Fig. 3a).

Microarray analysis identified 111 unique transcripts exhibiting differential accumulation in the resistant line. It identified 41 probe sets (of the 111) measuring induced transcript levels that were unique to the resistant line (Fig. 3b). These probe sets represented disease resistance protein KR3, systemic acquired resistance (SAR) regulator protein NIMIN1, salicylic acid methyl transferase protein (SABATH2), pathogenesis-related (PR)10 proteins, polygalacturonase inhibiting proteins (PGIP), stress-induced proteins (SAM22), peroxidases, cytochrome P450 proteins, components of jasmonic acid and gibberellin biosynthetic pathways, and bHLH and AS2 transcription factors (Supplementary Table S2). Microarray analysis identified 1,643 unique transcripts exhibiting differential accumulation in the susceptible line. It identified 668 probe sets (of the 1,643) measuring induced transcript levels that were unique to the susceptible line (Fig. 3b). These probe sets corresponded to genes encoding many cell-wall modifying enzymes including pectinesterase, expansin, extensin, and xyloglucan endotransglucosylase, many auxin-induced proteins, cell cycle proteins, secondary metabolic proteins involved in phenylpropanoid and flavonoid pathways, ethylene-related proteins, many peroxidases, PR thaumatin family proteins, harpin-induced family protein, major latex protein (MLP), various families of transcription factors including AUX-IAA, WRKY, bZIP, and MYB

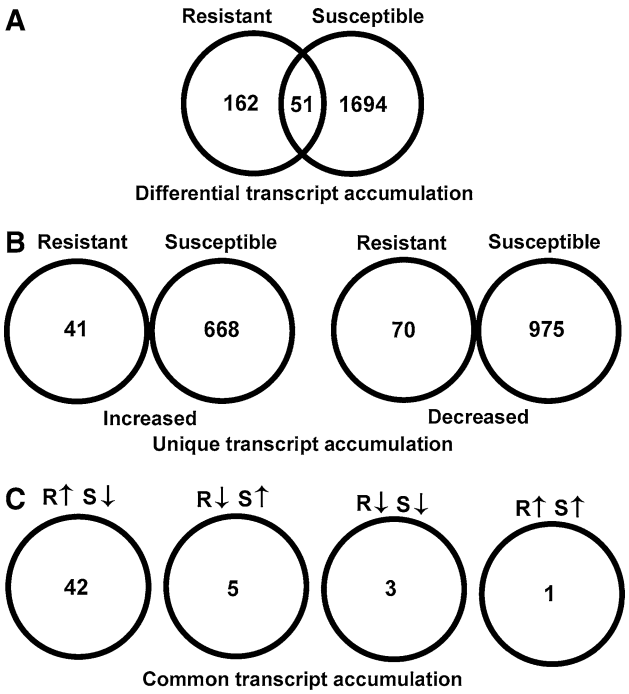

Fig. 3 *Venn diagrams* showing the number of gene transcripts in the TN02-226 (resistant) and TN02-275 (susceptible) soybean lines after soybean cyst nematode inoculation compared with mock water-inoculation. **a** Transcripts that displayed differential accumulation. **b** Transcripts that displayed increased or decreased accumulation uniquely in resistant and susceptible line. **c** Transcripts that overlapped between resistant and susceptible lines exhibiting increased (↑) or decreased (↓) accumulation

(Supplementary Table S3). Probe sets measuring unique suppressed transcript levels was identified as $N = 70$ in the resistant and $N = 975$ in the susceptible line (Fig. 3b). Notably, many probe sets corresponding to disease resistance-responsive family protein and leucine-rich repeat (LRR) receptor-protein kinases were suppressed in the susceptible line that were not present in the suppressed probe sets of the resistant line (Supplementary Tables S4, S5).

Microarray analysis identified 51 probe sets that were overlapping between the resistant and susceptible lines (Fig. 3c). Of these 51 common transcripts, markedly, 42 transcripts were increased in the resistant line while decreased in the susceptible line, which include CC-NBS-LRR protein kinase, Kunitz trypsin inhibitor (KTI) proteins, peroxidase, polyphenol oxidase, UDP-xylose phenolic glycosyltransferase, and extracellular dermal glycoprotein (EDGP) (Supplementary Table S6).

Gene expression grouped by function

We further grouped the genes into functional categories. This adds further insights into classes of proteins that play a role in nematode infection. Annotation of the *G. max* probe sets into 16 functional categories (http://seedgenenetwork.net/annotate) was used for our analysis. Our functional categorizations are complementary to Fig. 3 and reveal the genes undergoing differential expression as a consequence of a resistant or susceptible reaction. These analyses revealed differences between the types of genes expressed in resistant or susceptible line as reflected by percentage of transcript levels altered following SCN infection.

We observed sizeable differences between the numbers of probe sets measuring unique induced transcript abundance in the resistant line as compared to the susceptible line (Fig. 4a). This induced transcript abundance was noticeable for the following categories—Disease & Defense (19.5% in resistant vs. 6.4% in susceptible) and Secondary Metabolism (12.2% in resistant vs. 3.3% in susceptible) (Fig. 4a). On the contrary, more probe sets corresponding to categories Cell Structure, Metabolism, and Transporter were induced in susceptible line (Fig. 4a). Also, no induced probe sets corresponding to categories Cell Growth and Division, Energy, Intracellular Traffic, Post Transcription, Protein Synthesis, and Transposon were detected in resistant line (Fig. 4a). Differences in the quantities of various groups were also noted in probe sets measuring suppressed transcript abundance between the resistant and susceptible line (Fig. 4b). Although the majority of the functional categories did not show marked differences, two functional grouping differed noticeably—Post Transcription and Protein Synthesis whose corresponding probe sets was suppressed approximately 12-fold more in the resistant line as compared to the susceptible line (Fig. 4b). Accordingly, no probe sets corresponding to these two categories had induced transcript abundance in the resistant line (Fig. 4a).

We also observed quantitative differences in probe sets overlapping between the resistant and susceptible lines. All the probe sets corresponding to the category Disease & Defense with the majority of the probe sets belonging to unknown proteins were identified as induced transcript levels in the resistant but suppressed in the susceptible line (Table 2).

qRT-PCR

We conducted qRT-PCR to validate the microarray results (RNA samples of time point 3 dpi) and to analyze the gene expression changes of selected genes in the RNA samples of time points 6 and 9 dpi. We selected genes from various functional categories corresponding to probe sets measuring unique induced or suppressed transcript levels in resistant and/or susceptible line as well as probe sets overlapping between the resistant and susceptible lines. Total RNA of 3, 6, and 9 dpi from the same biological replicates

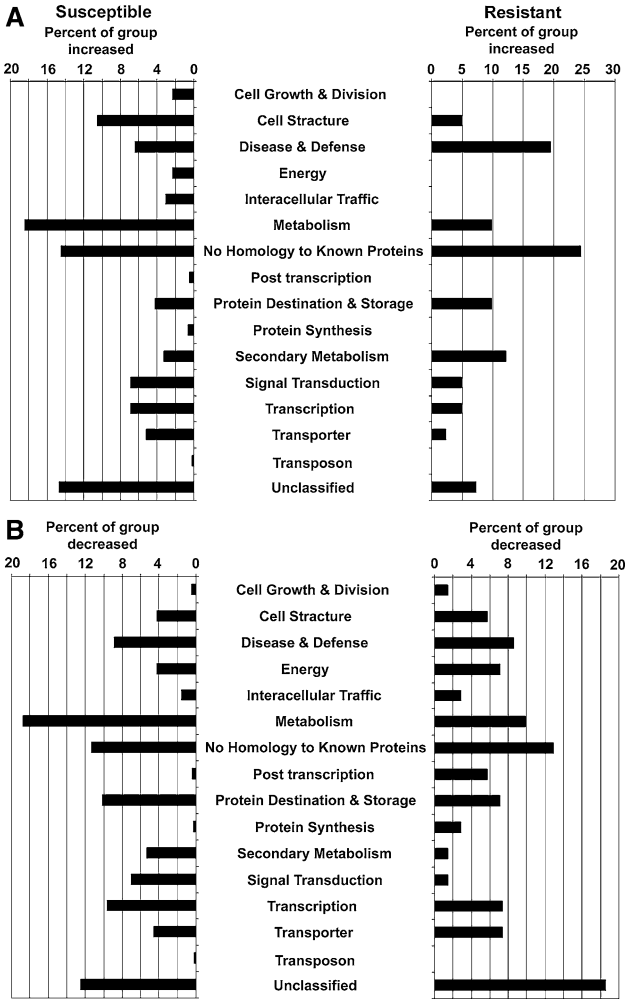

Fig. 4 Functional categorization of the probe sets that displayed differential accumulation uniquely in the TN02-226 (resistant) and TN02-275 (susceptible) soybean lines after soybean cyst nematode inoculation compared with mock water-inoculation. Percentage of probe sets within each functional category exhibiting increased (**a**) or decreased (**b**) accumulation

Table 2 Functional categorization of the 51 probe sets that overlapped between soybean lines TN02-226 (resistant) and TN02-275 (susceptible) after soybean cyst nematode infection

Functional category	Probe set[a]	Resistant	Susceptible
Cell Growth & Division	0		
Cell Structure	2	+	–
Disease & Defense	9	+	–
Energy	2	+	–
Intracellular Traffic	1	+	–
Metabolism	5	+	–
	2	–	+
	1	–	–
No Homology to Known Proteins	7	+	–
	2	–	+
	1	–	–
Post Transcription	2	+	–
Plant Destination & Storage	3	+	–
Protein synthesis	1	–	–
Secondary Metabolism	2	+	–
	1	+	+
Signal Transduction	1	+	–
Transcription	3	+	–
Transporter	3	+	–
Transposon	0		
Unclassified	2	+	–
	1	–	+

[a] Number of probe sets within each functional category

+, probe sets that displayed increase transcript accumulation; –, probe sets that displayed decrease transcript accumulation

used for microarray analysis or considered for subsequent analysis was used for qRT-PCR.

Collectively, the microarray and qRT-PCR data correlated very closely. Although the magnitude of expression was different in microarray and qRT-PCR, the direction of expression was the same (Table 3). This agreement between the quantitative PCR results and microarray results further promise the precision of our gene expression data of the time points 6 and 9 dpi obtained by qRT-PCR. As shown in Table 3, expression analysis of our selected genes during the time course of SCN infection indicated that the disease resistance protein KR3, SAR regulator protein NIMIN1, PGIP, SAM22, and SABATH2 were consistently induced at all three time points in the resistant line, with the exception of the KR3 whose expression was induced at 3 and 6 dpi but was unchanged at 9 dpi. Expression of these genes remained unchanged at all three time points in the susceptible line, with the exception of the SAR regulator protein NIMIN1 whose expression was unchanged at 3 and 9 dpi but was suppressed at 6 dpi. Cell-wall modifying expansin, xyloglucan endotransglucosylase and pectate lyase, and peroxidase were consistently induced over time in the susceptible line scoring more than twofold induction at each of the time points, whereas they were unchanged in the resistant line with less than twofold change at all the time points. Chalcone isomerase and chalcone synthase (CHS) related to biosynthesis of flavonoids, and MLP were induced or suppressed at 3 dpi but suppressed at 6 dpi and unchanged at 9 dpi in the susceptible line. The defense-related secondary metabolite phenylalanine ammonia lyase (PAL) was consistently suppressed over time in the susceptible line while was induced at all three time points in the resistant line. For the genes overlapping between the resistant and susceptible lines, several such as disease resistance protein MsR1, EREBP transcription factor, EDGP, and cytochrome P450 were both induced and suppressed over time in the resistant or susceptible line, except for KTI and

Table 3 Fold change of selected probe sets as determined by microarray analysis and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Probe ID, description	By[a]	Fold change[b]					
		Resistant			Susceptible		
		3 dpi[c]	6 dpi	9 dpi	3 dpi	6 dpi	9 dpi
Gma.8456.1.S1_a_at	q	+5.83	+6.48	−1.09	+1.03	−0.88	+1.15
Disease resistance protein KR3	M	+4.14			NP[d]		
Gma.12976.1.S1_at	q	+3.18	+3.26	+2.88	−1.34	−2.38	+1.25
Systemic acquired resistance (SAR) regulator protein NIMIN-1	M	+2.06			NP		
GmaAffx.91749.1.S1_s_at	q	+2.89	+2.27	+2.98	−1.04	−1.18	+1.74
Polygalacturonase inhibiting protein (PGIP)	M	+2.52			NP		
Gma.6999.1.S1_s_at	q	+2.19	+3.04	+2.53	−1.11	+0.98	+0.55
Stress-induced protein SAM22	M	+2.01			NP		
Gma.12911.1.A1_s_at	q	+8.22	+8.04	+3.53	+0.87	+1.98	+1.45
Salicylic acid methyl transferase (SABATH2)	M	+6.59			NP		
GmaAffx.671.1.S1_at	q	+1.19	+1.06	+0.93	+4.11	+3.18	+3.55
Expansin A4	M	NP			+2.08		
GmaAffx.12832.1.S1_at	q	+1.48	−1.86	+1.03	+5.08	+2.98	+4.15
Xyloglucan endotransglucosylase33	M	NP			+2.16		
Gma.11298.3.S1_a_at	q	+1.19	+1.06	+1.33	+4.91	+2.09	+3.85
Pectate lyase family protein	M	NP			+2.55		
Gma.4829.1.S1_at	q	+0.79	+1.86	+1.53	+5.18	+4.29	+4.85
Peroxidase	M	NP			+3.14		
Gma.2826.1.S1_at	q	+1.49	+1.26	+0.53	+3.26	−2.17	−1.38
Chalcone isomerase	M	NP			+2.30		
Gma.5579.1.S1_at	q	+1.19	+2.08	+1.43	−6.18	−3.08	−1.35
Chalcone synthase (CHS)	M	NP			−4.04		
GmaAffx.75675.1.A1_at	q	+2.69	+4.58	+2.13	−5.11	−2.05	−2.55
Phenylalanine ammonia-lyase (PAL)	M	NP			−3.42		
Gma.8957.1.A1_at	q	+1.87	+1.38	−1.55	+1.89	−3.98	+1.09
Major latex protein (MLP)	M	NP			+3.23		
GmaAffx.41564.1.S1_s_at	q	+4.99	+3.28	+3.13	−6.18	−5.07	−3.24
Kunitz trypsin inhibitor (KTI)	M	+2.13			−4.92		
Gma.1622.1.A1_s_at	q	+8.09	+4.28	+1.10	−3.25	−4.07	−1.12
Disease resistance protein MsR1	M	+2.43			−5.94		
Gma.4239.2.S1_at	q	+10.67	+5.08	+3.10	−13.95	−11.43	−2.92
UDP-xylose phenolic glycosyltransferase	M	+7.41			−29.08		
GmaAffx.69792.1.S1_s_at	q	+1.95	+2.54	+3.76	−9.18	−4.43	+2.12
EREBP transcription factor	M	+2.32			−7.42		
GmaAffx.90785.1.S1_s_at	q	+2.89	+1.76	+2.65	−9.98	−2.18	+1.12
Extracellular dermal glycoprotein (EDGP)	M	+2.14			−11.21		
GmaAffx.92620.1.S1_s_at	q	+5.01	+3.47	−1.55	−3.18	−3.56	−3.95
Cytochrome P450	M	+2.30			−2.04		

[a] Method by which the fold change was derived: *M* microarray experiment and *q* qRT-PCR

[b] Fold changes for each probe set at each time point in soybean cyst nematode (SCN)-infected roots were determined by comparison to corresponding mock sample. Positive values indicate that the probe set displayed increase transcript accumulation and negative values indicate that the probe set displayed decrease transcript accumulation

[c] Days post inoculation

[d] Probe set that did not pass the twofold cutoff at $P < 0.05$ level and the false discovery rate (FDR) at approximately 1%

UDP-xylose phenolic glycosyltransferase whose expressions were consistently induced over time in the resistant line while suppressed at all three time points in the susceptible line.

Discussion

The soybean–SCN interaction is an excellent model because it is possible to compare gene expression occurring in resistant and susceptible reactions. The resistant and susceptible reactions can be obtained in the same soybean genotype which is resistant to one SCN population and susceptible to another. Similarly, the same nematode population can successfully infect one soybean genotype, yet cannot develop to maturity in another genotype. Gene expression profiling of such soybean–SCN interaction would illuminate distinct differences in gene expression between the resistant and susceptible reactions. Affymetrix soybean microarray to study plant gene expression during SCN infection has been used in susceptible reactions (Alkharouf et al. 2006; Ithal et al. 2007a, b; Puthoff et al. 2007). There are also notable reports of using Affymetrix soybean microarray to study SCN infection in both susceptible and resistant reactions (Klink et al. 2007a, b; 2009; 2010). These studies used two different nematode populations that yielded susceptible and resistant reactions in the same soybean cultivar. In the present report, we used the Affymetrix soybean microarray to study SCN infection in two genetically related soybean lines, which are susceptible and resistant to the same nematode population. This approach provides an opportunity to gain additional insights into the specific differences in gene expression between the susceptible and resistant reaction to SCN infection.

We used a twofold regulation level as a cutoff point at $P < 0.05$ level determined by pairwise comparisons to identify soybean genes with significant changes in expression between SCN-infected and mock-inoculated roots for each susceptible or resistant soybean line. To make the selection more stringent, we used FDR. Genes passing the FDR at approximately 1% were categorized as statistically significant differentially expressed genes. The majority of the previous studies of Affymetrix soybean microarray associated with SCN infection have used a 1.5-fold cutoff (Alkharouf et al. 2006; Ithal et al. 2007a, b; Klink et al. 2007a, b; 2009; 2010). Thus, our approach of using a twofold cutoff and controlling FDR might elucidate a reduced number of differentially expressed genes, however, these genes were selected with extra stringency. Our analysis of changes in transcript levels of 37,744 soybean probe sets identified 1,694 soybean genes in the susceptible and 162 soybean genes in the resistant line with significant changes in expression. A subset of these differentially expressed genes was validated by qRT-PCR. Interestingly, probe sets measuring differential transcript levels were approximately tenfold more in the susceptible line compared to the resistant line. This may be due to the small quantity of the probe sets that belong to the disease and defense group that makes up only 3% of the total probe sets present on the microarray (http://seedgenenetwork.net/annotate).

We sought to determine if there were genes expressed uniquely in the resistant or susceptible line following SCN infection. Our gene classifications allowed the identification of quantitative differences in probe sets between resistant or susceptible lines. Of particular interest is the category Disease & Defense—with 19.5% induced transcript abundance in the resistant versus 6.4% in the susceptible line (Fig. 4a). The disease and defense-related probe sets that were induced uniquely in resistant line include disease resistance protein KR3: TIR-NBS-LRR class identified in soybean (Wang et al. 2004); NIMIN1: SAR regulator protein known to interact with NPR1 (nonexpresser of PR1) which regulate SAR in soybean (Weigel et al. 2005; Sandhu et al. 2009); SABATH2: secondary metabolite involved in defense responses mediated by salicylic acid (Ross et al. 1999; Loake and Grant 2007), and PGIP: extracellular protein involved in plant defense against fungi infection (Federici et al. 2006). Several PR10 and SAM proteins were also induced uniquely in the resistant line. The disease and defense probe sets that induced uniquely in resistant reaction (Supplementary Table S2) are likely relevant to resistance because they are not present in a susceptible reaction. These genes regulated during a resistant reaction define candidates that may play a role in the resistance to SCN infection.

On the contrary, more probe sets corresponding to category Cell Structure were induced uniquely in the susceptible line as compared to the resistant line—10.5% in the susceptible versus 4.9% in the resistant line (Fig. 4a). The majority of these cell structure probe sets that were induced in the susceptible line represent cell-wall modifying enzymes including pectinesterase, expansin, extensin, β-1,4-endoglucanases, cellulose synthase, glycoside hydrolase, and xyloglucan endotransglucosylase (Supplementary Table S3). Cell-wall modifying proteins have been shown to play imperative roles in cyst nematode–plant interactions. Nematode secretion and a coordinated expression of plant cell-wall modifying enzymes mediate the formation of nematode induced-feeding structures (De Boer et al. 1999, 2002; Goellner et al. 2000, 2001; Qin et al. 2004; Kudla et al. 2007; Vanholme et al. 2007; Wieczorek et al. 2006; Hewezi et al. 2008). Also, many probe sets corresponding to components of auxin and ethylene signaling pathways were induced uniquely in the susceptible line (Supplementary Table S3), however, several of them, in

particular the EREBP family of transcription factors, were also suppressed uniquely in the susceptible line (Supplementary Table S4). Several studies have shown that auxin signaling is essential for feeding site formation by nematodes (reviewed by Grunewald et al. 2009b). Nematodes induce a local accumulation of auxin in feeding structures by perturbing polar auxin transport through inhibition of auxin efflux and increasing auxin influx carriers (Hutangura et al. 1999; Goverse et al. 2000; Grunewald et al. 2009a; Lee et al. 2011; Mazarei et al. 2003). Similarly, it has been shown that increased ethylene signaling leads to increased susceptibility to cyst nematode (Goverse et al. 2000; Wubben et al. 2001, 2004). Nevertheless, a number of probe sets corresponding to the category Disease & Defense were also induced uniquely in the susceptible line. These probe sets include disease resistance protein RPM1, PR thaumatin family protein, harpin-induced family protein, MLP, phytoalexin-deficient4 protein, chitinase, and peroxidases (Supplementary Table S3). These observations may imply general activation of plant defense mechanisms in response to SCN infection reported in other Affymetrix soybean microarray studies of susceptible interaction (Alkharouf et al. 2006; Ithal et al. 2007a, b; Klink et al. 2007a, b; Puthoff et al. 2007). Yet, many probe sets corresponding to disease resistance-responsive family protein and LRR receptor-protein kinases as well as those corresponding to mitogen-activated protein kinase had suppressed transcript levels in the susceptible line. Also, several probe sets corresponding to defense-related secondary metabolites including PAL and CHS were suppressed in the susceptible line (Supplementary Table S4). On the other hand, these observations may imply general suppression of plant defense mechanisms, perhaps as a part of SCN infection strategy.

We also sought to determine if there were genes overlapping between resistant and susceptible reactions following SCN infection. Notably, a large number of the common genes exhibited an opposite trend—42 probe sets (of the 51) had induced transcript levels in the resistant while suppressed transcript levels in the susceptible line (Supplementary Table S6). This opposite expression trend suggests that complementary gene expression may be important during the course of SCN infection of resistance and susceptibility. Specifically, our functional categorizations indicated a noteworthy difference for the category Disease & Defense between resistant and susceptible lines. All the probe sets in this category had induced transcript levels in the resistant line but suppressed levels in the susceptible line (Table 2). These probe sets represented the disease resistance protein-like MsR1 (CC-NBS-LRR class), KTI proteins, and EDGP, which were induced in the resistant but suppressed in the susceptible line (Supplementary Table S6). Markedly, many KTI probe sets were also identified as measuring suppressed transcript levels (up to 38-fold) in the susceptible line (Supplementary Table S4), whereas there was no induced KTI in the susceptible line (Supplementary Table S3) or suppressed KTI in the resistant line (Supplementary Table S5). It was also noted that a number of EDGP probe sets were consistently suppressed (up to 18-fold) in the susceptible line (Supplementary Table S4), whereas there was no induced EDGP in the susceptible line (Supplementary Table S3) or suppressed EDGP in resistant line (Supplementary Table S5). Also, probe sets corresponding to UDP xylose phenolic glycosyltransferase were induced (up to sevenfold) in the resistant while always suppressed (up to 29-fold) in the susceptible line (Supplementary Table S6 and S4). It has been shown that UDP glycosyltransferase is also induced specifically in resistance response to viral and bacterial pathogens and its involvement in resistance was related to salicylic acid accumulation (Chong et al. 2002; Lee et al. 2009). It has been shown that expression of glycosyltransferases is necessary for resistance to bacterial pathogen and its role was related to cell-death and hypersensitive responses (Langlois-Meurinne et al. 2005). Moreover, glycosyltransferases confer increased resistance to fungal pathogen (Lorenc-Kukuła et al. 2009). Studies of susceptible and resistance interactions with root knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood, also identified an induced glycosyltransferase in the resistance response and revealed its role in *Mi*-mediated nematode resistance (Schaff et al. 2007). We also observed that polyphenol oxidase involved in bacterial disease resistance (Li and Steffens 2002) was induced in the resistant but consistently suppressed in the susceptible line. The EREBP and NAC families of transcription factors were also induced in the resistant while many of them were consistently suppressed in the susceptible line. There was only one NAC probe set induced in the susceptible line. Similarly, glutathione S-transferase (GST) family proteins induced in the resistant while consistently suppressed in the susceptible line and only one probe set corresponding to soybean GST21 was induced in the susceptible line. Peroxidase class III was also induced in the resistant line, however, many of these family proteins were both induced and suppressed in the susceptible line (Supplementary Tables S3, S4, and S6). Nonetheless, expression analysis of peroxidases class III in roots of resistant and susceptible wheat lines infected by nematode *Heterodera avenae* suggested that peroxidases play different roles in the defense response to nematode infection (Simonetti et al. 2009). Collectively, these observations indicate that soybean roots respond differently during the course of the resistant or susceptible interaction. Contrasting gene expression of defense-related probe sets where they were consistently induced in the resistant while suppressed in the susceptible reaction may, in fact, be related to the resistance to SCN infection.

It is difficult to make direct comparisons with the previous studies of Affymetrix soybean microarray of the SCN infection due to differences in experimental conditions and applied measures (Alkharouf et al. 2006; Ithal et al. 2007a, b; Klink et al. 2007a, b; 2009; 2010; Puthoff et al. 2007). However, our data show several similarities to these studies. The following are examples for the susceptible interaction: typical induction of cell-wall modifying enzymes and auxin- and/or ethylene-related proteins, and frequent induction of components of phenylpropanoid and flavonoid pathways, chitinases, harpin-induced family protein, and heat shock proteins. Similarly, probe sets corresponding to peroxidases, lipoxygenases, cytochrome P450 proteins, and various families of transcription factors including WRKY, bZIP, MYB, and EREBP were both induced and suppressed in the susceptible interaction. The complete list of the commonly found probe sets are presented in Supplementary Table S7. We also observed similarities to the previous studies of the resistance interaction (Klink et al. 2007a, b; 2009; 2010). For example, of the 41 probe sets measuring induced transcript levels unique to the resistant line (Supplementary Table S2), 19 exact probe sets were also induced at time points 3 and 8 dpi in the resistance interaction of those previous studies. Furthermore, 14 exact probe sets of the 42 common genes that were induced in the resistant but suppressed in the susceptible line (Supplementary Table S6), were also induced at time points 3 and 8 dpi in the resistance interaction of those previous studies. These probe sets included secondary metabolites SABATH2 and oxygenase family protein, disease resistance protein KR3, PGIP, KTI, and PR10. The complete list of the commonly found probe sets are presented in Supplementary Table S8. Likewise, our experiments involving the gene expression analysis of the selected genes at further time points showed the induction of these genes at time points 6 and/or 9 dpi (Table 3). Additionally, PAL and CHS were induced in the resistant line while consistently suppressed over time in the susceptible line (Table 3). Similarly, the NAC transcription factor probe sets were consistently induced in the resistant while suppressed in the susceptible interaction.

Collectively, the present study revealed specific changes in gene expression occurring in the susceptible and resistant reaction to SCN infection. It detected a number of gene transcripts exhibiting distinct differential accumulation between the susceptible and resistant responses in soybean. The genes identified here offer additional candidate genes that regulate or affect soybean defense mechanism or are involved in mediating the successful establishment of SCN in soybean. These results provide further insights into the molecular mechanism(s) involved in the complex soybean–SCN interaction and add to the knowledge base beneficial for identification of genes that play roles in resistant process. This comparative analysis of the susceptible and resistant response to SCN infection offer further understanding how soybean responds to infection, leading to identification of genes involved in defense.

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