

RESEARCH PAPER

# GeneChip profiling of transcriptional responses to soybean cyst nematode, *Heterodera glycines*, colonization of soybean roots

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## Abstract

Soybean cyst nematode (SCN) is currently the most devastating pathogen of soybean. SCN penetrates the root and migrates toward the central vascular bundle where it establishes a complex multinucleated feeding structure that provides plant-derived nutrients to support the development and growth of the nematode. To identify host genes that play significant roles in SCN development in susceptible roots, RNA from SCN-inoculated and non-inoculated root pieces were hybridized to the Affymetrix soybean genome GeneChips. RNA was collected at 8, 12, and 16 d post-inoculation from root pieces that displayed multiple swollen female SCN and similar root pieces from non-inoculated roots. Branch roots and root tips were trimmed from the root pieces to minimize the amount of RNA contributed by these organs. Of the 35 593 transcripts represented on the GeneChip, approximately 26 500 were expressed in the SCN-colonized root pieces. ANOVA followed by False Discovery Rate analysis indicated that the expression levels of 4616 transcripts changed significantly ( $Q$ -value  $\leq 0.05$ ) in response to SCN. In this set of 4616 transcripts, 1404 transcripts increased >2-fold and 739 decreased >2-fold. Of the transcripts to which a function could be assigned, a large proportion was associated with cell wall structure. Other functional categories that included a large number of up-regulated transcripts were defence, metabolism, and histones, and a smaller group of transcripts associated with signal transduction and transcription.

Key words: Affymetrix, cell wall, cyst nematode, False Discovery Rate, GeneChip, *Glycine max*, *Heterodera glycines*, histones, plant defence, soybean.

## Introduction

Nematodes, specifically cyst nematodes, are the most damaging pest to US soybean production (Wrather and Koenning, 2006). With an increasing number of acres planted with soybean and new varieties being developed to geographically broaden profitable cultivation of soybean in the US and across the world, the continued study of this devastating pest is of the utmost importance. Soybean cyst nematodes (SCN) are obligate parasites that can alter root cell development to support its nutritional and reproductive needs (Williamson and Gleason, 2003; Davis *et al.*, 2004). The localized commandeering of plant developmental processes by the nematode requires interactions with the host that involve changes in host gene expression. A number of studies have demonstrated that both root knot and cyst nematodes may alter the balance of plant hormones to achieve the cellular conditions needed for development of the feeding structure. In particular, auxin and ethylene may play important roles in the formation of the nematode feeding structure (Goverse *et al.*, 2000; Wubben *et al.*, 2001). Alteration in the localized balance of these two hormones would be expected to have a marked impact on gene expression, irrespective of any other factors that might also affect gene expression.

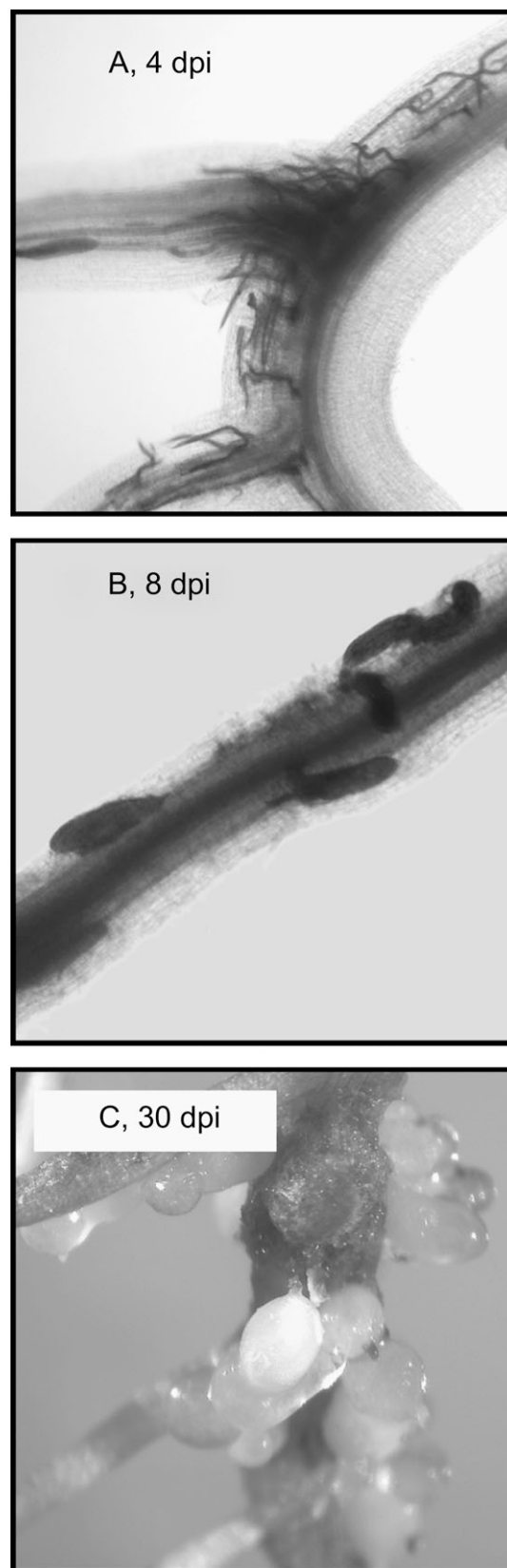
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How the nematode brings about changes in plant hormone levels and developmentally regulated processes in the host is of great interest. The nematode oesophageal glands are a potential source for pathogenicity factors secreted from the nematode. The oesophageal glands have been successfully targeted for preparation of organ-specific cDNA libraries and many transcripts identified have the potential to be pathogenicity factors (Gao *et al.*, 2003; Huang *et al.*, 2003). Currently a few of the oesophageal gland proteins have a demonstrated potential to alter plant development when expressed in transgenic plants (Doyle and Lambert, 2003; Wang *et al.*, 2005; Huang *et al.*, 2006).

In addition to the characterization of pathogenicity factors of nematode origin, cataloguing changes in susceptible host gene expression in response to SCN infection will identify developmental and mechanistic processes associated with SCN colonization of soybean roots and distinguish host targets for controlling nematode pathogenicity of soybean. To achieve this objective, soybean roots were inoculated with SCN and after 8, 12, and 16 d small pieces of the root that had multiple swollen female nematodes protruding from the root were dissected out. Elimination of root material not directly associated with actively growing nematodes greatly enhances our ability to detect changes in gene expression associated with SCN infection. The RNA from these root pieces was hybridized to Affymetrix soybean GeneChips and statistical analysis of the microarray hybridization signals identified 1404 transcripts that increase >2-fold in SCN-colonized root pieces and 739 that decreased >2-fold in the same root pieces.

## Materials and methods

Soybean cyst nematodes (population NL1-RHg, HG-type 7, race 3) were reared and maintained in USDA greenhouses, Beltsville, MD, and infective juveniles (stage 2) prepared according to Matthews *et al.* (2003). Seeds of *Glycine max* cv. Williams were germinated in Perlite (Geiger, Harleysville, PA, USA) in the greenhouse and after 2 weeks seedlings were washed free of Perlite, combined into groups of six seedlings, and inoculated by pipetting 5000 2nd stage juveniles per seedling. The roots were then sprinkled with autoclaved moist Perlite and covered with a moist paper towel. The seedlings were kept in a moist environment in a growth chamber with 15 h of light and 9 h of dark. After the desired incubation interval, one of six seedlings in each group was collected for acid fuchsin staining to monitor nematode development and count the number of nematode infections (Byrd *et al.*, 1983). At 8, 12, and 16 d post-inoculation (dpi) the roots were examined under a stereomicroscope and root pieces colonized by SCN (SCN+) were dissected out (Fig. 1). Root pieces were collected from similar positions of non-inoculated aged roots to serve as control samples (SCN-). The lateral roots were trimmed and discarded from both SCN+ and SCN- root pieces. The trimmed root segments were frozen in liquid nitrogen and stored at -70 °C until RNA isolation. The entire experiment was repeated and separate tissue samples collected, processed, and hybridized as described below.



**Fig. 1.** Photographs of root segments colonized by SCN. The roots at 4 dpi and 8 dpi were stained with acid fuchsin, which preferentially stains the nematodes red.

Frozen roots were ground to a fine powder under liquid nitrogen, suspended in RNA extraction buffer using a polytron (Kinematica Polytron PT 1200, Brinkman Instruments, Westbury, NY, USA) and extracted as directed using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Total RNA was processed and Affymetrix GeneChip hybridization completed, and microarrays scanned at the GeneChip Facility at Iowa State University, Ames, IA. Probe synthesis and labelling were completed using the One-cycle Target Labelling and Control Reagent Package (Part# 900493) following the procedures described in the Affymetrix manual. A total of 15 µg of fragmented cRNA was used to make each hybridization cocktail containing 10% dimethyl sulphoxide, and an equivalent of 10 µg was hybridized to the Affymetrix Soybean GeneChip probe array. The Affymetrix GeneChip Operation Software version 1.4 was used to assign a hybridization signal strength for each probe set (PID) and calculate *P*-values used to indicate that the transcript hybridization signal was sufficiently above background ( $P \leq 0.05$ ) to be counted as being present in the RNA sample. GeneChip hybridizations were completed for 12 chips, i.e. replicate biological samples for three time points for SCN+ and SCN- root tissue. Given that half of the hybridizations include *H. glycines* RNA (SCN+) and the soybean GeneChip includes PIDs for 35 611 *Glycine max* transcripts and 7431 *Heterodera glycines* transcripts (Affymetrix GeneChip Soybean Genome Array datasheet), a typical normalization across the entire GeneChip would distort samples that include SCN RNA in comparison to samples that do not include SCN RNA (SCN-). Therefore, the hybridization signals were normalized using only the signals for the *Glycine max* PIDs.

Only PIDs for transcripts considered to be present ( $P \leq 0.05$ ) in the root pieces, 28 591 *G. max* PIDs, were included in the following statistical analysis. The hybridization signals for all 12 GeneChips were included in a two-way split-plot analysis of variance (ANOVA) using the SAS/STAT suite of programs, v.9.1 (SAS Institute, Cary, NC, USA). The raw *P*-values from the ANOVAs were converted to *Q*-values (Storey and Tibshirani, 2003) to identify probes exhibiting significant differential expression with a False Discovery Rate (FDR) no greater than 5% (i.e.  $Q \leq 0.05$ ). The *Q*-value for the expression of a PID is the PID's own measure of significance, relative to the chosen FDR. For example, a 5% FDR indicates that among all the genes identified as being differentially expressed (i.e. significant), 5% of them are truly not significant.

Selected changes in gene expression were clustered using *K*-means with 100 iterations. The software used for clustering was described by Chiang *et al.* (2001) and is available at <http://rana.lbl.gov/EisenSoftware.htm>.

## Results

### Global view of changes in gene expression

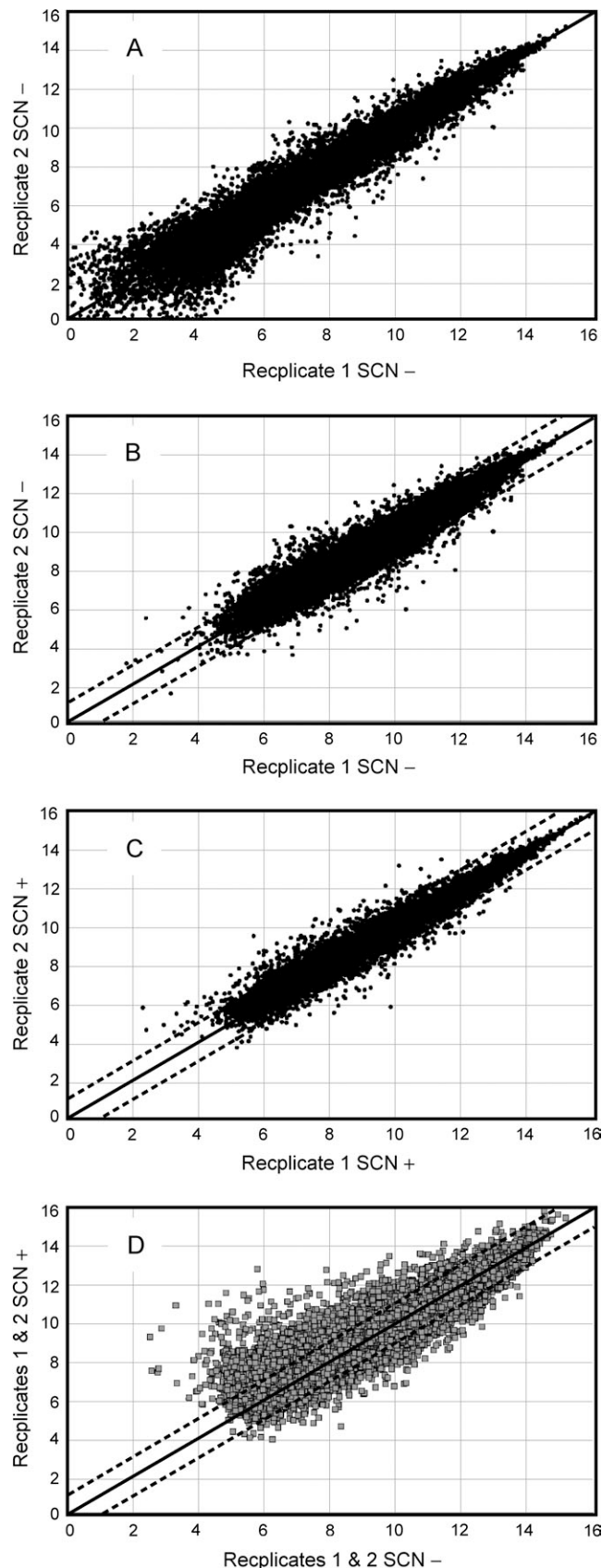
Excised and trimmed soybean root pieces densely colonized by SCN (SCN+) served as starting material to examine changes in gene expression occurring within and immediately surrounding developing syncytia (Fig. 1). In addition, trimmed root pieces from non-infected plants (SCN-), which were developmentally similar, served as the control and baseline for expression analysis. Tissues from infected and non-infected roots were collected from two biological replications conducted several months apart with independent GeneChip hybridizations completed for each replication and time point for a total of 12 hybrid-

izations. The Affymetrix soybean GeneChip includes >37 500 soybean PIDs representing an estimated 35 611 transcripts (Affymetrix GeneChip data sheet). Although not strictly true for all of the PIDs, herein changes in hybridization to an individual PID will be assumed to reflect the change in expression of a single gene.

To gain a perspective on the variability associated with gene expression in the two biological replications, the hybridization signals for the SCN- root pieces were averaged separately for each biological replication across all of the time points (8, 12, and 16 dpi) and the log<sub>2</sub> transformed values were plotted (Fig. 2A, B). The same calculations were completed for the SCN+ root pieces and the log<sub>2</sub> transformed data were plotted (Fig. 2C). The overall extent of changes in gene expression associated with SCN colonization can then be viewed by comparing the averaged hybridization signals for SCN+ root pieces in both replicate experiments to the averaged signals for SCN- root pieces (Fig. 2D). The global change in expression level is noted by the large divergence of the data points away from the diagonal line and outside of the dashed lines (Fig. 2D).

### Statistical analysis of GeneChip hybridization results

Of the 37 593 soybean PIDs on the GeneChip, ~75% (28 591) supported hybridization signals that were significantly above background and the corresponding genes counted as being expressed in the root pieces (Table 1). ANOVA followed by FDR analysis indicated that none of the gene expression results exhibited a significant SCN treatment (trt)×time interaction effect (i.e. all the *Q*-values were >0.24). This indicated that any differences in the level of expression between SCN- and SCN+ was consistent at all three time points or that more replications were needed to detect a trt×time interaction effect at a 5% FDR. Subsequently, to improve the statistical test for significance of a treatment and time main-effect, the variability associated with the non-significant trt×time interaction effect was pooled with the ANOVA residual term by removing trt×time from the model. The ANOVA identified 7082 genes with expression levels that were significantly different ( $P \leq 0.05$ ) between the SCN- and SCN+ treatments. Subsequent FDR analysis of the SCN- compared with SCN+ results narrowed this to 4608 genes with a 5% FDR ( $Q \leq 0.05$ ). In addition to the SCN effect, 1637 genes exhibited statistical significance ( $Q \leq 0.05$ ) among at least two of the three times observed. Use of the FDR (*Q*-values) provides a practical balance between being over-protective (i.e. adjusting the *P*-values to protect against inflation of the rate of false positives; hence, identifying too few genes with significant changes in gene expression) and being too liberal (i.e. accepting all significant *P*-values without any adjustment; hence, identifying too many genes with significant change in expression).



To better display the change in expression level in SCN+ roots compared with SCN- roots, the hybridization signals for the two replicate experiments were averaged at each time point and the ratio of SCN+ to SCN- was calculated and  $\log_2$  transformed. The number of individual PIDs that changed >2-fold ( $\log_2 \geq 1$  or  $\leq -1$ ) and had associated with them a  $Q$ -value of  $\leq 0.05$  are tabulated in Table 1. The percentage of genes whose expression levels changed >2-fold in response to SCN colonization was small being <8% relative to the total number of genes expressed in the root pieces (Table 1). If the cut-off for SCN-induced change is increased to 8-fold, the percentage of genes with  $\log_2$  ratios  $\geq 3$  is 0.6% and those  $\leq -3$  is 0.03% of the total number of root-expressed genes (Table 1). As expected, there are many fewer genes that decrease >8-fold than increase >8-fold. This is because transcripts that are newly evoked by SCN infection, even in a select few cells within the root piece (e.g. confined to the syncytial cells and not surrounding cells), produce a large relative fold increase; whereas to observe a similar fold decrease, transcript suppression throughout the entire root segment would be required, which is highly unlikely.

#### Gene expression grouped by function

Additional functional annotation was applied to each of the PIDs (genes) using the annotation assigned to the most similar *Arabidopsis* or rice gene (B Le, A Bui, and B Goldberg, University of California at Los Angeles, personal communication). Grouping the genes into 18 functional categories adds further insight into classes of proteins that play a role in nematode infection. Although the majority of the functional categories did not undergo marked changes as reflected by the small percentage of transcript levels altered by SCN, three functional groupings changed markedly—cell wall proteins, histone and histone-associated proteins, and disease- and defence-related proteins (Fig. 3). Conversely, gene expression in the category for protein synthesis was unchanged by the SCN treatment (Fig. 3).

Selected genes were further subgrouped and the expression results plotted as volcano plots to reveal changes in particular cellular mechanisms or enzyme activities (Fig. 4). The volcano plots include the statistical significance

**Fig. 2.** Scatter plots for the  $\log_2$  transformed hybridization signals averaged across 8, 12, and 16 dpi. (A) Average of the signals for the non-infected root pieces (SCN-) comparing biological replicates 1 and 2. (B) Same as (A) except gene transcripts that were determined to be absent from the RNA samples were excluded. The continuous diagonal line represents equal signal in both samples and the dashed lines represent  $\pm 2\sigma$  and enclose approximately 95% of the data points. (C) Same as (B) except hybridization signals for the biological replicates of SCN-infected root pieces (SCN+). Dashed lines were calculated as described above. (D) Average hybridization signals across time and biological replicates of transcripts present in SCN+ root pieces compared with SCN- root pieces. Dashed lines shown in (D) are those from (C) above.

**Table 1.** Tabulation of the number and percentage of soybean PIDs (genes) that fulfil selected criteria

Except for the category for total soybean PIDs, only hybridization signals that were counted as being significantly above background ( $P \leq 0.05$ ) in both samples at each of 8, 12, and 16 dpi were included in the calculations. In other words the transcript must be present in all the root pieces infected with SCN or in all the non-inoculated root pieces in order to be included in the tabulations. An ANOVA and then FDR analysis was performed on all signal data that were counted as significantly above background using the criteria described above. Only PIDs with a  $Q$ -value  $\leq 0.05$  for the SCN treatment were included in the tabulations. SCN+ includes hybridizations for 8, 12, and 16 dpi root pieces and, SCN- hybridizations for 8, 12, and 16 d non-inoculated root pieces. The sum listed for the  $\log_2$  ratios includes the gene in the sum if the  $\log_2$  ratio at any of the three time points was above or below the limit indicated.

Criteria	Sum of PIDs	Percentage
Total soybean PIDs	37 593	100.00
PIDs expressed in SCN-	27 566	73.33
PIDs expressed in SCN+	26 474	70.42
PIDs expressed in SCN+ or -	28 591	76.05
PIDs with $Q \leq 0.05$ for SCN+/-	4 608	16.12
PIDs with $\log_2$ ratio $\geq 5.0$ SCN+/-	37	0.13
PIDs with $\log_2$ ratio $\geq 4.0$ SCN+/-	68	0.24
PIDs with $\log_2$ ratio $\geq 3.0$ SCN+/-	176	0.62
PIDs with $\log_2$ ratio $\geq 2.0$ SCN+/-	515	1.80
PIDs with $\log_2$ ratio $\geq 1.0$ SCN+/-	1 404	4.91
PIDs with $\log_2$ ratio $\leq -1.0$ SCN+/-	739	2.58
PIDs with $\log_2$ ratio $\leq -2.0$ SCN+/-	98	0.34
PIDs with $\log_2$ ratio $\leq -3.0$ SCN+/-	8	0.03

( $-\log_{10}$  of  $Q$ -values) and the  $\log_2$  ratio of the SCN+/- hybridization signals averaged over 8, 12, and 16 dpi. Several subgroups were chosen for display, either because they included putative functions previously reported to respond to nematode infection, or to serve as reference groups for visual comparisons. A set of 50 and 600 genes was randomly selected from the 28 591 genes expressed in the root pieces and plotted to demonstrate how the gene expression data would appear if not grouped for a particular function (Fig. 4A, B). In addition, for comparison, the protein synthesis elongation factors 1 alpha and 1 beta, which are often used as constitutive controls in gene expression experiments, are relatively unchanged by the SCN treatment as indicated by  $\log_2$  ratios of nearly zero and relatively small  $-\log_{10}$   $Q$ -values (Fig. 4C). Other functional groupings in Fig. 4 have more significant changes in gene expression and will be discussed below with regard to their putative role in SCN colonization of roots.

#### Gene expression grouped by change from 8 dpi to 16 dpi

In order to analyse the expression data better and get a better understanding of the biological roles of genes with altered mRNA levels, the 4608 genes that had a significant SCN treatment effect were sorted into two groups,  $\log_2$  ratios  $>0.0$  and  $<0.0$  (i.e. up-regulated and down-regulated genes). The two groups were then clustered separately based on increasing, decreasing, or relatively constant expression

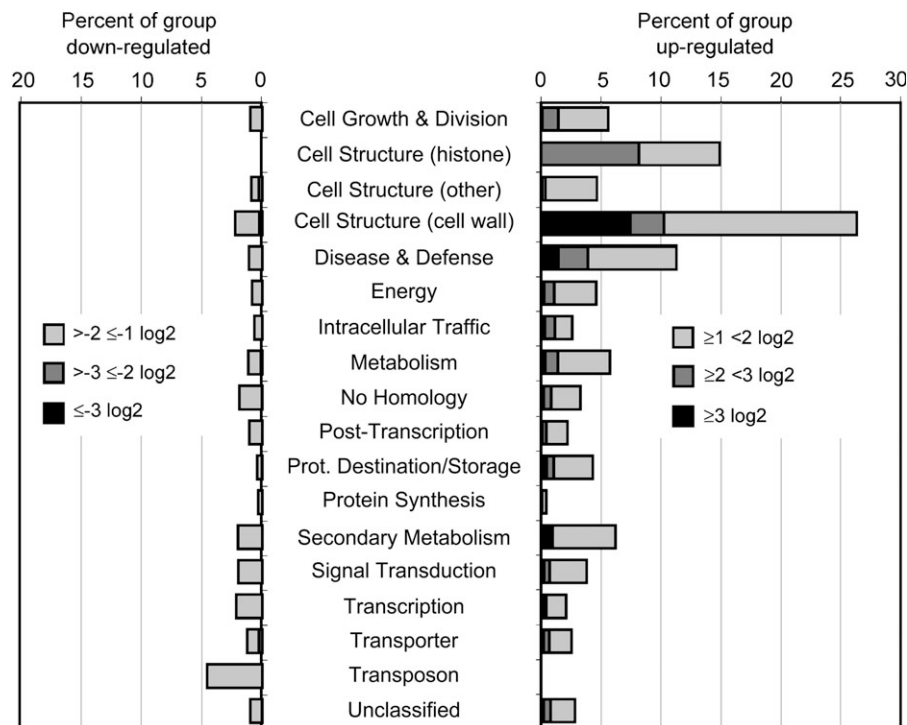
between 8, 12, and 16 dpi (see Supplementary Table S1 at JXB online). Most of the 18 functional categories included one or more genes in each of the six clusters (see Supplementary Table S1 at JXB online). However, histones, which showed an overall increase in expression in SCN+ roots (Fig. 4F), were particularly noteworthy because they clustered primarily in the group with decreasing expression from 8 dpi to 16 dpi (e.g. several of the histone genes showed relatively large  $\geq 3 \log_2$  ratios at 8 dpi that fell sharply at 12 dpi and 16 dpi; Fig. 5).

#### Gene expression grouped by magnitude of change

Above the statistical analyses of the present gene expression data have been described and selected groupings of genes plotted; however, the change in expression of a single gene in a gene family or functional group can be very important to SCN pathogenicity. It is not possible here to evaluate the importance of the many changes in individual genes; nevertheless, Table 2 lists all of the genes with a statistically significant increase in expression of  $>32$ -fold or a decrease of  $>8$ -fold in response to SCN colonization. In addition, Supplementary Table S1 (at JXB online) includes information on all 4608 genes that had a significant SCN treatment effect and the entire dataset can be found at <http://bldg6.arsusda.gov/mtucker/Public/Tucker.html>.

#### Comparison to other GeneChip results for SCN

In a recent publication, Ithal *et al.* (2007a) describe experiments very similar to those described here in which they collected SCN-infected soybean root tissue at 2, 5, and 10 dpi. In their GeneChip hybridizations they identified 618 genes that showed statistically significant differential expression between SCN-infected and non-infected root tissues. Of the 618 genes, 429 showed a greater than 1.5-fold change in expression. The fold differences reported by Ithal *et al.* (2007a) were converted to  $\log_2$  ratios and all 618 ratios were plotted against the  $\log_2$  ratios from the present results for the same 618 genes (Fig. 6A). It is worth noting that 40 of the 618 genes reported on by Ithal *et al.* (2007a) were below the statistically significant threshold of detection (absent) in the hybridizations used in our study. Nevertheless, plots for all pairwise combinations of their 2, 5, and 10 dpi results and our 8, 12, 16 dpi results showed roughly correlated ratios, i.e. ascending regression lines with correlation coefficients ( $R^2$ ) between 0.10 and 0.34. Two example comparisons are shown in Fig. 6A and B. To provide further perspective on this sort of comparison, our 8 dpi  $\log_2$  ratios (SCN+/-) were plotted against our 12 dpi  $\log_2$  ratios (SCN+/-) for the same 618 genes, including the ratios for the 40 genes that were considered to be absent from our RNA samples (Fig. 6C). The correlation coefficients for the regression lines comparing their 10 dpi and 5 dpi data to our 8 dpi data are less than that for the regression line for our data (Fig. 6). The comparison of



**Fig. 3.** Percentage of  $\log_2$  transformed hybridization signals within each functional group averaged across time and biological replicates. Genes in the left panel decrease in the SCN+ root pieces compared with SCN- roots and in the right panel increase in response to SCN infection.

graphs indicates, nevertheless, that many of the genes identified as differentially expressed in our experiments are the same as those in the experiments of Ithal *et al.* (2007a).

## DISCUSSION

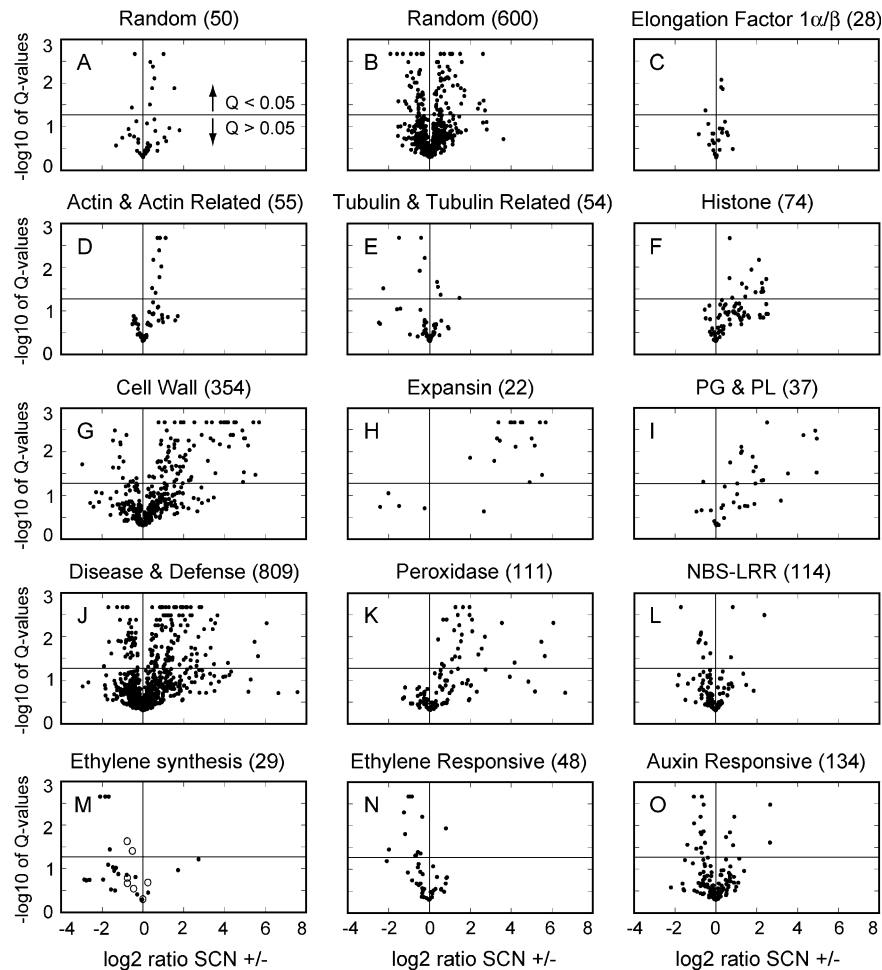
While studies on model plant species are tremendously useful, knowledge of nematode interaction with specific crop species is valuable information and may differ from model plants. Here, changes in gene expression occurring during infection and colonization of soybean roots by SCN have been assessed. It was decided to excise small segments of roots (1–3 mm) that were colonized by multiple SCN because collection of RNA from whole roots is greatly diluted with RNA from non-infected tissues. Moreover, although changes in gene expression may be identified in whole-root collections, a whole-root approach would favour the identification of systemic changes in gene expression and may primarily reflect defence responses. Also, by waiting until 8 dpi and later it was easy to distinguish, under a stereomicroscope, swollen female nematodes protruding from the root and thereby be certain of collecting root tissue that contained the nematode feeding structure. Using this approach 1404 soybean genes were identified up-regulated >2-fold and 739 down-regulated >2-fold in SCN+ roots at 8, 12, or 16

dpi. This is a large number of genes. The information on each of these genes that changed significantly in response to SCN is provided in Supplementary Table S1 at *JXB* online and data for all soybean PIDs can be obtained at <http://bldg6.arsusda.gov/mtucker/Public/Tucker.html>.

Here the focus is on demonstrating that the changes in gene expression that were observed fit with the known biology of nematode development in roots and compare favourably with previous gene expression studies on a smaller number of genes and in model systems.

Puthoff *et al.* (2003) used *Arabidopsis* GeneChip arrays to identify changes in gene expression associated with cyst nematode infection of whole roots with either *Heterodera schachtii* (BCN) or *Heterodera glycines* (SCN). They identified 128 and 12 genes with significantly altered steady-state mRNA levels following BCN infection and SCN infection, respectively. These include the induction of genes for trypsin inhibitor, polygalacturonase, cytochrome P450, and annexin, which were also found to increase in our soybean GeneChip hybridizations (Supplementary Table S1 at *JXB* online).

Recently, much as in the present experiments, Ithal *et al.* (2007a) used the Affymetrix GeneChip to examine SCN-induced changes in gene expression in soybean roots. A comparison of the gene expression results for the two studies indicated many similarities in the transcriptional profiles; however, there were a great many differences (Fig 6). Ithal *et al.* (2007a) used an HG-type

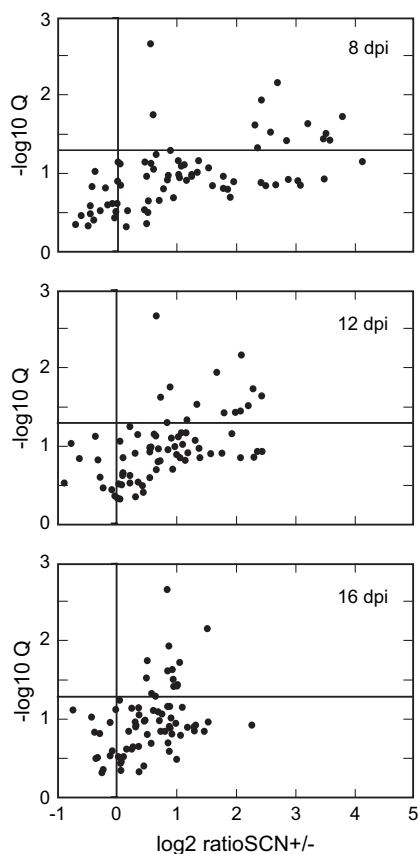


**Fig. 4.** Volcano plots of  $-\log_{10}$ -transformed  $Q$ -values relative to  $\log_2$  ratios for SCN+/- hybridization signals averaged over time and biological replicates. The horizontal line in each panel marks the  $-\log_{10}$  transformation of a  $Q$ -value equal to 0.05, which is the cut-off used for significant differential expression for the SCN treatment, i.e. points above the line are significantly different for the SCN treatment. The soybean genes were selected and grouped based on the cellular or biochemical function given for the most similar *Arabidopsis* gene. The number in parenthesis indicates the total number of genes included in the plot. The panels labelled as 'Random' were genes selected randomly from the 28 591 genes expressed in either SCN+ or SCN- root pieces. PG, Polygalacturonase; PL, pectate lyase. In the graph for ethylene synthesis genes (M) the open circles indicate ACC synthase genes and closed circles ACC oxidase.

0 strain of SCN while an HG-type 7 strain was used in our study; nevertheless, both HG-types are considered to be race 3-like and probably have a very similar genetic background. Moreover, closely related soybean varieties (Williams versus Williams 82) were used in both studies. The differences in gene expression levels between the two studies are most likely due to differences in the experimental conditions (e.g. age of the tissue inoculated with SCN) and the density of the nematode infections. Ithal *et al.* (2007a) inoculated root tips, marked the position of inoculation, and then harvested the marked tissue several days later. Instead, in our study, the entire root system of 14-d-old plants was inoculated and any part of the root that showed a high density of SCN colonization was harvested. In our experiments, the greatest density of infections does not occur near the root tip but in older root tissue most often near the base of a lateral root (Fig. 1).

The developmental age of the infected tissue may be an important factor in the differences between the two studies. In fact, in the present statistical analysis there was a significant time effect that was independent of the SCN treatment—1637 genes exhibited statistical significance ( $Q \leq 0.05$ ) among at least two of the three times observed. This supports our proposition that the age of the infected cells may influence the magnitude or even the direction of SCN-elicited changes in gene expression.

Although the gene expression was examined in a susceptible host, defence genes are nonetheless expressed in the SCN-infected roots (Figs 3, 5). Peroxidases, which are often associated with an  $H_2O_2$  defence response, have been observed to increase markedly in response to nematode infection (Uehara *et al.*, 2007), and are some of the most highly induced in the present samples (Fig. 4K). NBS-LRR (nucleotide-binding site containing leucine-rich



**Fig. 5.** Volcano plots for the histone genes at each collection time. The  $Q$ -values ( $Q$ ) are those calculated for the SCN treatment effect. The horizontal line indicates a  $Q$ -value equal to 0.05 (see Fig. 4).

repeat) proteins are often linked to specific defence responses and other signalling events (DeYoung and Innes, 2006). Several NBS-LRR genes change significantly during SCN infection. Moreover, with regard to other general stress responses, changes in gene expression associated with water stress or water movement have been reported for cyst nematodes (Klink *et al.*, 2005; Uehara *et al.*, 2007). Several genes annotated as being similar to osmotins or dehydration-responsive proteins were up-regulated in our system and two genes with similarity to PIP1 aquaporins were significantly up-regulated (see Supplementary Table S1 at *JXB* online).

In another related project, Ithal *et al.* (2007b) used laser capture micro-dissection to isolate syncytia from SCN-infected soybean roots. They noted an increase in the expression of genes in the phenylpropanoid pathway for lignin synthesis. The same set of genes is up-regulated in our experiments. However, in our case, lignin synthesis may be more closely associated with the repair of damaged cells caused by the migration of nematodes to the vascular bundle and in SCN feeding structures that terminate prematurely (Fig. 1A). Many SCN can infect a small segment of root and several of the SCN will begin

to swell (Fig. 1A), which indicates the onset of feeding (Jung and Wyss, 1999). However, many SCN will not survive to an adult stage and the damaged cells must be sealed off to prevent water loss and opportunistic infection by other pathogens. This healing process often includes synthesis of lignin and suberin-like materials (Dixon and Paiva, 1995).

By looking more closely at the biological significance of some of the changes in gene expression it is possible to establish further the significance of the differential expression results obtained in our GeneChip hybridizations. SCN establishes a feeding structure in soybean roots by inducing the formation of a syncytium (multinucleated cell; Jung and Wyss, 1999). The syncytium is formed by disassembling the cell walls and incorporation of as many as 200 cells into a single syncytium (Jung and Wyss, 1999). A change in the expression level of cell wall loosening and degrading enzymes is therefore expected. Some of the greatest changes in gene expression observed in *Arabidopsis* were associated with cell wall modifications (Vercauteren *et al.*, 2001; Puthoff *et al.*, 2003; Jammes *et al.*, 2005; Wiczorek *et al.*, 2006). Similarly, the greatest overall change in gene expression in the present GeneChip hybridization experiments was for cell wall-associated proteins (Figs 3, 4 see Supplementary Table S1 at *JXB* online). RT-PCR of transcripts for 32 cell wall-modifying proteins confirmed the altered expression profiles for several of the cell wall-associated genes included on the Affymetrix GeneChip (Tucker *et al.*, 2007).

Cell wall disassembly to form the syncytium is an easily observed morphological change associated with SCN colonization, but other notable changes also occur in the syncytia and surrounding cells. The nuclei of syncytia are greatly enlarged indicating an increase in polyploidy (de Almeida Engler *et al.*, 1999). Moreover, it has been suggested that cells surrounding the developing syncytium may divide and then become incorporated into the growing syncytium (Golinowski *et al.*, 1996). Histones and genes associated with DNA replication and cell division might therefore be expected to increase during SCN infection. Although histone genes are constitutively expressed in eukaryotic cells, a significant increase in histone transcripts was found in the RNA from the SCN+ root pieces (Fig. 4F). The increase in histone gene expression was greatest at 8 dpi and declined at 12 dpi and 16 dpi (Fig. 5). Similarly, a slight increase in gene expression for cell growth and cell division genes was observed (Fig. 3) and, furthermore, some of the genes linked to cell division grouped with the histone genes in the clustering analysis (see Supplementary Table S1 at *JXB* online). This suggests that DNA replication is primarily an early event in SCN colonization and may be ending by 8 dpi. This conclusion is supported by Golinowski *et al.* (1996) who measured the incorporation



**Table 2.** Soybean transcripts homologous to the Affymetrix target sequences (genes or PIDs) that increase  $\geq 32$ -fold or decrease  $\geq 8$ -fold in SCN-colonized root tissues

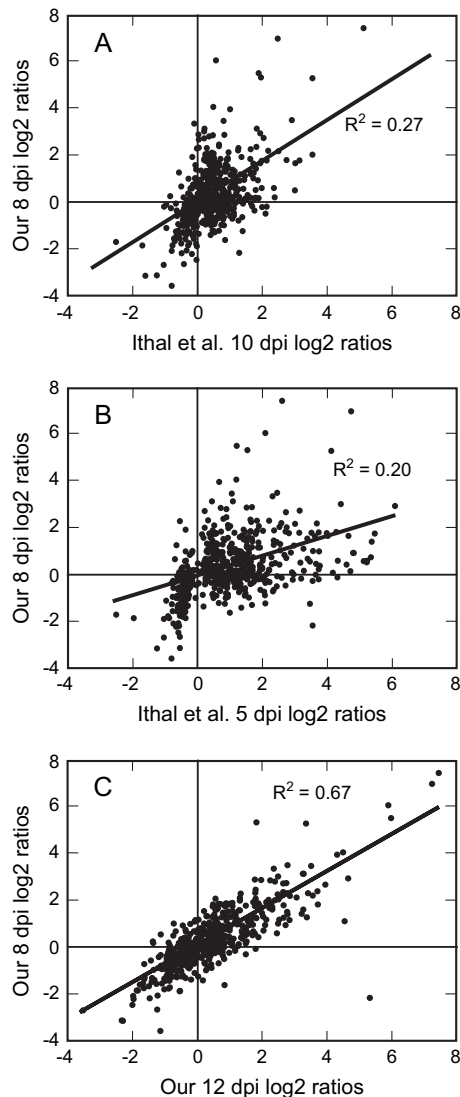
The  $\log_2$  ratios are for the ratios of hybridization signals for SCN+ root pieces relative to SCN- root pieces. The signals for replicate experiments were averaged at 8, 12, and 16 dpi. The  $Q$ -value is the false discovery rate (FDR) comparing all the hybridization signals in the SCN-colonized root pieces with non-colonized pieces (11 d.f.). The *Arabidopsis* gene annotation indicates the putative function of the *Arabidopsis* gene with the greatest similarity to the soybean GeneChip target sequence (B Le, A Bui, and B Goldberg, University of California at Los Angeles, personal communication).

PID	log <sub>2</sub> ratios			Q-value for SCN treatment	Arabidopsis gene annotation	Functional category
	8 dpi	12 dpi	16 dpi			
(A) Genes with SCN +/- log <sub>2</sub> ratios ≥5.0 at 6, 12, or 16 dpi						
Gma.33.1.S1_at	5.7	5.9	4.6	0.034	Expansin (EXBP2)	Cell structure (wall)
Gma.7784.1.A1_at	6.5	4.9	4.1	0.007	Expansin (EXP1)	Cell structure (wall)
GmaAffx.85557.1.S1_at	5.4	4.1	3.3	0.008	Expansin (EXP1)	Cell structure (wall)
Gma.13110.1.S1_at	5.2	4.4	3.9	0.002	Expansin (EXP1)	Cell structure (wall)
GmaAffx.42013.1.S1_at	2.7	4.8	5.5	0.002	Expansin (EXP1)	Cell structure (wall)
Gma.6682.1.A1_at	5.5	6.0	5.5	0.002	Expansin (EXP15)	Cell structure (wall)
GmaAffx.93176.1.S1_s_at	7.2	5.1	4.6	0.002	Expansin (EXP3)	Cell structure (wall)
GmaAffx.93176.1.S1_at	5.7	4.7	4.4	0.050	Expansin (EXP3)	Cell structure (wall)
Gma.7006.1.S1_at	4.8	5.6	3.4	0.002	Expansin (EXP4)	Cell structure (wall)
GmaAffx.90009.1.S1_s_at	5.4	5.3	4.2	0.005	Expansin (EXP8)	Cell structure (wall)
GmaAffx.12649.1.S1_s_at	5.3	4.9	4.3	0.005	Pectate lyase	Cell structure (wall)
Gma.1326.3.S1_at	5.3	4.3	4.9	0.030	Pectate lyase	Cell structure (wall)
GmaAffx.13717.1.S1_at	5.0	5.6	3.9	0.003	Pectate lyase	Cell structure (wall)
Gma.1326.1.S1_at	4.6	5.0	3.0	0.004	Pectate lyase	Cell structure (wall)
Gma.17993.1.S1_at	5.3	1.8	3.5	0.018	Osmotin-like (OSM34)	Disease and defence
Gma.4829.1.S1_at	7.2	5.3	5.4	0.005	Peroxidase	Disease and defence
GmaAffx.85202.1.S1_at	6.4	4.6	4.8	0.013	Peroxidase	Disease and defence
Gma.4919.1.S1_at	6.1	5.9	4.2	0.028	Peroxidase	Disease and defence
Gma.4471.1.A1_at	5.6	3.7	2.5	0.040	Peroxidase	Disease and defence
Gma.8520.1.S1_at	5.3	3.6	2.8	0.019	Acid phosphatase	Metabolism
GmaAffx.50670.1.A1_at	5.0	5.7	3.7	0.002	Aminotransferase	Metabolism
Gma.14299.1.S1_at	7.5	7.4	6.4	0.002	—	No homology
Gma.9875.1.S1_at	5.1	1.3	1.1	0.046	—	No homology
Gma.10606.1.S1_at	4.4	5.1	3.4	0.002	—	No homology
GmaAffx.79350.1.S1_s_at	3.9	6.0	3.6	0.003	—	No homology
GmaAffx.29818.1.A1_at	2.8	5.3	3.9	0.002	—	No homology
Gma.3473.1.S1_at	6.6	6.6	6.0	0.002	17.6 kDa heat shock protein	Destination/storage
GmaAffx.63611.1.S1_at	5.7	6.2	5.3	0.002	Chloroplast DNA-binding	Destination/storage
GmaAffx.72230.1.S1_at	7.3	6.6	5.9	0.003	Serine carboxypeptidase	Destination/storage
GmaAffx.3364.1.A1_at	7.0	7.2	6.7	0.002	Serine carboxypeptidase	Destination/storage
GmaAffx.67397.1.S1_at	1.0	1.6	6.5	0.035	Oligopeptide trans. (POT)	Transporter
GmaAffx.52963.1.S1_at	6.8	1.5	2.3	0.003	Auxin-responsive protein	Unclassified
Gma.11204.3.S1_a_at	1.1	4.6	5.1	0.035	Expressed protein	Unclassified
GmaAffx.82499.1.S1_at	3.8	3.9	5.7	0.040	Germination protein-related	Unclassified
Gma.1098.1.S1_at	5.5	2.7	0.9	0.026	Hydroxyproline-rich protein	Unclassified
Gma.12911.1.A1_s_at	5.6	4.5	4.3	0.002	SAM:carboxyl methyltrans.	Unclassified
Gma.14284.1.S1_at	5.3	5.7	8.7	0.012	Similar to CG10964-PA	Unclassified
(B) Genes with SCN +/- log <sub>2</sub> ratios ≤ −3.0 at 6, 12, or 16 dpi						
GmaAffx.26516.1.S1_at	−3.4	−3.1	−2.1	0.020	Xyloglucan trans. (XTR6)	Cell structure (wall)
Gma.1329.1.S1_at	−2.0	−3.3	−1.5	0.034	Thiazole biosyn. (ARA6)	Metabolism
Gma.13449.1.A1_at	−1.6	−2.2	−3.8	0.038	—	No homology
Gma.10760.1.S1_at	−2.6	−2.1	−3.0	0.002	ABC transporter	Transporter
GmaAffx.51710.1.A1_at	−3.9	−2.1	−1.6	0.048	Putative polypeptide	Transposon
GmaAffx.74681.1.S1_at	−3.1	−1.7	−2.5	0.019	Expressed protein	Unclassified
GmaAffx.6017.1.A1_at	−1.9	−3.5	−2.5	0.012	Expressed protein	Unclassified
GmaAffx.82387.1.A1_at	−0.5	−0.6	−3.4	0.047	Pentatricopeptide (PPR)	Unclassified

of [ $^3\text{H}$ ]thymidine into the DNA of syncytia in *Arabidopsis* infected with *Heterodera schachtii*, and found that the greatest amount of incorporation occurred at 5 dpi with much lower levels at 9 dpi and very low levels by 15 dpi.

In addition to hypertrophied nuclei and DNA replication, the fully developed syncytium is greatly enlarged

with a dense cytoplasm filled with many small vacuoles (de Almeida Engler *et al.*, 2004). Reporter gene expression studies indicated that actin microfilaments increased in syncytia with a peak at approximately 4–5 dpi that remained higher than in surrounding cells through 15–20 dpi (de Almeida Engler *et al.*, 2004). Tubulins, which are



**Fig. 6.** Scatter plot of  $\log_2$ -transformed expression ratios of the Ithal *et al.* (2007a) SCN-responsive genes compared with the ratios for the same genes in the present experiments. (A) The hybridization signal ratios of Ithal *et al.* (2007a) at 10 dpi for the 618 genes with significantly altered SCN affected expression ( $Q$ -values  $\leq 0.05$ ) are compared with the hybridization signal ratios at 8 dpi in the present experiments. (B) The same as (A) except that the hybridization signal ratios of Ithal *et al.* (2007a) at 5 dpi were used for comparison. (C) The same genes plotted in (A) and (B) but comparing the  $\log_2$  ratios for the 8 dpi results compared with the 12 dpi results. All of the genes with significant differential expression in the study of Ithal *et al.* (2007a) were included irrespective of whether or not the ratios were above an arbitrary cut-off. The diagonal lines are the regression lines calculated for the data plotted in that panel.

commonly associated with cell wall deposition and dividing cells, were high in syncytia and the dividing cells that surrounded the syncytia (de Almeida Engler *et al.*, 2004). In the present experiments, there were significant changes in both actin and tubulin gene expression; however, it was the actin-associated genes that showed the greatest overall increase in expression in response to SCN infection (Fig. 4D, E).

To form a syncytium, parasitic plant nematodes must co-opt and alter existing developmental programmes in the plant root to form the feeding structure. It has been demonstrated by several researchers that plant hormones are necessary for successful formation of the nematode feeding structure. In particular, ethylene and auxin appear to play essential roles in syncytium development (Glazer *et al.*, 1986; Goverse *et al.*, 2000; Wubben *et al.*, 2001; Bent *et al.*, 2006). Many genes on the GeneChip are annotated as being responsive or associated with auxin and ethylene. If all the genes annotated as being auxin responsive are plotted, it is seen that some are up-regulated while others are down-regulated (Fig. 4O). While the pattern of changes for auxin-linked genes was variable, changes in the expression of a single hormone-associated gene could be very important in SCN development. For example, the 8-fold up-regulation of the auxin-transport protein (EIR1), GmaAffx.47493.1.S1\_at (see Supplementary Table S1 at JXB online), could be critical to syncytium development.

Ethylene is an interesting enigma. Ethylene synthesis was demonstrated to increase during infection of tomato with root knot nematodes, and the addition of ethylene to the root environment increased the number of nematodes that matured on the root (Glazer *et al.*, 1985). Moreover, genetic mutants that overproduce ethylene were hyper-susceptible to cyst nematodes whereas mutants resistant to ethylene action had fewer cyst nematodes on the roots of *Arabidopsis* (Wubben *et al.*, 2001). The role of ethylene in nematode colonization of roots is in contrast to the more typical role of ethylene, which would be to mount a pathogen defence response (Broekaert *et al.*, 2006). Because of these earlier studies on ethylene, it was expected that an SCN elicited increase would be observed in gene expression for ethylene synthesis and ethylene-responsive genes. However, in general, the opposite was observed. The trend was more toward a downward regulation of the ethylene synthesis and responsive genes (Fig. 4M). There are several possible explanations for this and three are listed here. It is possible that the GeneChip is missing the ethylene synthesis genes that increase during SCN colonization and many ethylene-responsive genes are not annotated as ethylene responsive, or that the peak in ethylene synthesis occurred prior to 8 dpi and at 8 dpi there is already a feedback inhibition on the ethylene-associated gene expression. Alternatively, the nematode could be synthesizing ethylene itself in a very localized region of the root; however, there is no evidence in the literature for synthesis of ethylene by a plant parasitic nematode.

No matter how much or where the ethylene is synthesized, based on the evidence, it is clear that ethylene is very important to nematode development in roots (Wubben *et al.*, 2001). Unfortunately, there were only 48 genes on the GeneChip that were expressed in roots and annotated as being ethylene responsive. This is probably not an accurate number of all the ethylene-responsive

genes in soybean roots. It has been proposed that many of the cell wall-modifying proteins evoked during nematode infection might be regulated by ethylene (Goverse *et al.*, 2000). One notable developmental process linked to ethylene and cell wall dissolution in roots is the formation of aerenchyma, air channels used for movement of gases when oxygen diffusion is limited by flooding (Kozela and Regan, 2003). The early stages in the formation of the long hollow tubes necessary to make aerenchyma may share some of the same developmental signals used for formation of the nematode-induced syncytium, which includes cell wall dissolution. Aerenchyma development, however, culminates with programmed cell death to open the long tubes to gases (Drew *et al.*, 2000; Kozela and Regan, 2003). Programmed cell death clearly does not occur in the successful formation of a syncytium; however, a comparison of differential gene expression in SCN+ roots and roots induced to form aerenchyma might highlight shared gene expression and regulatory mechanisms. In addition to aerenchyma, adventitious and lateral root initiation is also linked to ethylene (Kemmerer and Tucker, 1994; Clark *et al.*, 1999; Aloni *et al.*, 2006). Comparison of gene expression profiles during lateral root initiation might also be interesting. Identifying and examining global changes in gene expression in developmental processes and the role of individual genes in the induction of networks of gene expression is a complex process. The present results for SCN-induced changes in gene expression add to the knowledge base needed to identify both the individual genes and genetic mechanisms that support development of the SCN feeding structure.

## Supplementary data

The following table is available at *JXB* online.

**Table S1.** Excel file for all of the genes with a significant SCN treatment effect ( $Q$ -value  $\leq 0.05$ ).

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