Developmental Transcript Profiling of Cyst Nematode Feeding Cells in Soybean Roots

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Cyst nematodes of the genus *Heterodera* are obligate, sedentary endoparasites that have developed highly evolved relationships with specific host plant species. Successful parasitism involves significant physiological and morphological changes to plant root cells for the formation of specialized feeding cells called syncytia. To better understand the molecular mechanisms that lead to the development of nematode feeding cells, transcript profiling was conducted on developing syncytia induced by the soybean cyst nematode *Heterodera glycines* in soybean roots by coupling laser capture microdissection with high-density oligonucleotide microarray analysis. This approach has identified pathways that may play intrinsic roles in syncytium induction, formation, and function. Our data suggest interplay among phytohormones that likely regulates synchronized changes in the expression of genes encoding cell-wall-modifying proteins. This process appears to be tightly controlled and coordinately regulated with cell wall rigidification processes that may involve lignification of feeding cell walls. Our data also show local downregulation of jasmonic acid biosynthesis and responses in developing syncytia, which suggest a local suppression of plant defense mechanisms. Moreover, we identified genes encoding putative transcription factors and components of signal transduction pathways that may be important in the regulatory processes governing syncytium formation and function. Our analysis provides a broad mechanistic picture that forms the basis for future hypothesis-driven research to understand cyst nematode parasitism and to develop effective management tools against these pathogens.

Additional keywords: cytokinin, expansin, gibberellin, *Glycine max*, LCM, Xet.

Sedentary endoparasitic phytonematodes establish intimate associations with host plant roots by manipulating fundamental aspects of plant cell biology. Also, these nematodes are the most economically important group of plant-parasitic nematodes and cause extensive damage to a wide range of crop plants. Cyst nematodes (*Heterodera* and *Globodera* spp.) belong to this group of nematodes. Infective cyst nematode juveniles penetrate through the root epidermal cell layer

into the cortex and migrate intracellularly to the root vasculature, where they select individual cells to initiate the formation of fused feeding cells, referred to as “syncytia”. A syncytium sustains the host–parasite interaction by providing the nutrients required for the growth and development of a sedentary nematode. Syncytium formation is a complex plant response that appears to involve both indirect and direct modulation of plant cell gene expression by nematode secretions (Davis et al. 2004). As a result, plant cells are reprogrammed to develop into a cell type that is both unique and essential to the plant–nematode interaction. Although the nature and origin of the stimuli required for the formation of syncytia have not been resolved, nematode secretions originating from a set of three esophageal gland cells and injected through the nematode stylet (mouth spear) into host plant cells are believed to contain the inductive signals necessary for syncytium formation and maintenance (Davis and Mitchum 2005; Williamson and Hussey 1996). The syncytium forms from an initial syncytial cell by incorporating adjacent cells through progressive cell wall dissolution (Endo 1964); a process that utilizes cell-wall-modifying proteins (CWMPs) of plant origin (Goellner et al. 2001). The fusion of protoplasm among cells incorporated into the syncytium results in a multinucleate complex that continues to expand along the length of the vascular cylinder throughout the nematode life cycle. Feeding cell nuclei enlarge, develop an amoeboid appearance, have a prominent nucleolus, and are polyploid. The syncytium is metabolically highly active, there is an associated increase in cytoplasmic density, the large central vacuole is reduced to several smaller vacuoles, organelles proliferate, cell walls thicken, and finger-like protuberances (ingrowths) form along walls adjacent to the xylem, typical of transfer cells, to increase membrane surface area for solute uptake (Hussey and Grundler 1998).

Little is known about the molecular mechanisms that guide the development and determine the properties of a syncytium. Determining the expression profiles of syncytially expressed genes promises to significantly advance our current understanding. Moreover, determining how a nematode selects and modifies root cells to undergo pronounced changes in development to form a syncytium will contribute to our knowledge of basic plant cell biology. Several molecular approaches have been exploited to study changes in plant gene expression in response to cyst nematode parasitism, including differential screening and cDNA subtraction, differential display, promoter– β -glucuronidase fusions, in situ hybridizations, reverse-transcription polymerase chain reaction (RT-PCR), and microarrays (Gheysen and Fenoll 2002). Using a combination of these approaches, a relatively small panel of genes has been shown

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to actually be expressed within syncytia to date. The difficulty in studying plant gene expression changes within syncytia stems from the fact that these cyst nematode-induced feeding cells make up only a minute fraction of the root cell population. Thus, high-throughput functional genomic studies utilizing microarrays have been limited by their inability to obtain syncytial cell contents apart from surrounding cell types for analysis (Alkarouf et al. 2006; Ithal et al. 2007; Khan et al. 2004; Puthoff et al. 2003).

Until recently, the specific isolation of nematode feeding cells has been limited to manual dissection or microaspiration of root-knot nematode (*Meloidogyne* spp.)-induced giant cells and cyst nematode-induced syncytial cells for cDNA library construction, differential display, and RT-PCR analysis (Bird and Wilson 1994; Juergensen et al. 2003; Wang et al. 2003). Although an effective means for identifying and characterizing the genes involved in cell-specific biological processes, these approaches were limited in that only mature feeding cells could be isolated. The powerful approach of laser capture microdissection (LCM) originally was developed as an effective tool for the isolation of individual cells in mammalian systems (Bonner et al. 1997; Emmert-Buck et al. 1996) and has been shown to be effective for gene expression analysis of microdissected plant cells as well (Asano et al. 2002). This technique also was employed recently for capturing giant cells and syncytia for RT-PCR and cDNA library construction, respectively (Klink et al. 2005; Ramsay et al. 2004). LCM allows for the isolation and cloning of mRNA populations from individual target cells within complex tissues, leading to the identification of plant genes involved in cell-specific biological processes. LCM, therefore, is superbly suited to study the developmental processes of syncytium induction, formation, and function.

In this study, we couple LCM technology with the latest soybean microarray platform available, representing 35,611 soybean genes, to provide a comprehensive gene expression profile of developing cyst nematode feeding cells in a compatible plant–nematode interaction. Our analysis of gene expression changes in developing syncytia ranging from 0 to 10 days postinoculation (dpi) identified (i) 1,765 soybean genes that change expression within the first 2 days after syncytium induction and (ii) a group of syncytium-expressed genes that change mRNA expression over time (i.e., cluster analysis on the 1,765 genes was used to determine expression changes from 2 to 5 dpi and 5 to 10 dpi). As a consequence, our approach provided the most comprehensive picture currently attainable and identified metabolic and regulatory pathways that may play intrinsic roles in syncytium induction, formation, and function.

RESULTS

LCM of syncytia.

LCM was used to specifically isolate syncytial cells from sections of paraffin-embedded soybean roots infected with soybean cyst nematodes (SCN). Cells were captured onto CapSure HS LCM caps as described below. The histological integrity of the root tissues remained intact in paraffin sections, providing enough detail for accurate identification of syncytial cells. The distinct morphology of syncytial cells, including enlarged nuclei and increased cytoplasmic density, made it possible to differentiate them from adjacent cell types for accurate harvesting. Using this approach, it was possible to specifically identify and capture feeding cells induced by SCN as early as 2 dpi. Upon penetration into the soybean root, SCN migrate toward the vasculature and start feeding from a cell within the cortex, endodermis, or pericycle (Endo 1964). In

our experimental system, we observed that the initial feeding cells were almost exclusively formed from cells in and around the pericycle region (Fig. 1A and C), and we chose only syncytia in this region for LCM. To estimate the basal level of gene expression in the cells of the pericycle region, we harvested these cells from noninfected root tissue at 4 days postgermination (Fig. 1B) (i.e., the equivalent timepoint to the age of the roots from which we captured syncytia at 2 dpi). Furthermore, we captured syncytia at 2, 5, and 10 dpi in order to identify those genes that change expression within syncytia over time. Sections are shown through SCN-infected roots at 2, 5, and 10 dpi before LCM (Fig. 1C, E, and G) and the captured cells on the LCM cap (Fig. 1D, F, and H). LCM enabled the capture of SCN feeding cells at all developmental stages, no adjacent cells were removed, and the captured cells remained intact after the capture.

Following capture, total RNA was isolated from syncytial cells. LCM of only a few syncytial cells yields picogram or nanogram amounts of total RNA, requiring high-efficiency linear amplification to produce a sufficient quantity of RNA for downstream applications, including cDNA library construction, quantitative real-time PCR, and microarray analysis. T7-RNA polymerase-based RNA amplification has been shown to be a highly reproducible method with no systematic bias for generating a sufficient quantity of RNA from a limited cell population for microarray studies (Schneider et al. 2004). Total RNA isolated from captured syncytia was subjected to two rounds of linear amplification using a T7-RNA polymerase-based RNA amplification approach to generate microgram quantities of cRNA.

Developmental transcript profiling of syncytia.

The amplified cRNAs generated from LCM tissues were used for hybridization to Affymetrix GeneChip Soybean Genome Arrays. Three independent biological replicates were obtained for each of our four treatments (noninfected and syncytia at 2, 5, and 10 dpi). We performed analysis of variance on the expression data from each gene. The *P* values from the contrast between the noninfected and 2-dpi syncytia were converted to *q* values (Storey and Tibshirani 2003) to identify differentially expressed genes while controlling the false discovery rate at approximately 0.5% and applying a fold-change cutoff of 1.5.

This analysis resulted in the identification of 1,765 genes displaying significant differential transcript abundance between 2-dpi syncytia and the cells from noninfected root tissue. Of these 1,765 genes, 1,116 were upregulated at 2 dpi whereas 649 were downregulated in syncytia at 2 dpi when compared with the cells from noninfected root tissue. In order to characterize the expression patterns of these genes from 2 to 10 dpi, we performed a cluster analysis for up- and downregulated genes. The genes were grouped into nine clusters based on changes in their expression across 2, 5, and 10 dpi. The clusters for upregulated and downregulated genes are shown in Figure 2A and B, respectively. The first cluster (labeled as $2 \approx 5 \approx 10$) includes transcripts that exhibited no significant changes in expression between adjacent timepoints, meaning that the observed expression changes were not statistically significant between 2 and 5 dpi and between 5 and 10 dpi. The remaining clusters contain transcripts that exhibited at least one significant expression change from 2 to 5 dpi or from 5 to 10 dpi, as indicated in the figure labels. For example, the cluster labeled $2 > 5 \approx 10$ includes transcripts exhibiting significantly greater expression at 2 than at 5 dpi but no significant change from 5 to 10 dpi. For the purposes of clustering genes with similar patterns, we used a relatively liberal 5% *P* value threshold for significance when assigning genes to clusters.

Fig. 1. Identification and laser capture microdissection of soybean cyst nematode (SCN)-induced syncytia from sections of soybean roots. **A**, Light micrograph of acid fuchsin stained SCN-infected soybean roots at 2 days postinfection (dpi) showing nematodes feeding from cells close to the vasculature. **B**, Longitudinal section of an uninfected soybean root; boxed area indicates the region within the pericycle from which the cells were captured to estimate the basal levels of gene expression. Longitudinal sections of an SCN-infected soybean root showing syncytia at **C**, 2- and **E**, 5 dpi. Syncytium captured on a laser capture microdissection (LCM) cap from **D**, 2- and **F**, 5-dpi sections. Longitudinal section of an SCN-infected soybean root at 10 dpi **G**, before and **H**, after LCM. Inset in **G** shows the 10-dpi syncytium at a higher magnification and inset in **H** is the syncytium captured on a LCM cap. N = nematode, Syn = syncytium, Lr = lateral root. Bars = 50 μ m.

Validation of plant gene expression in syncytia.

Quantitative real-time RT-PCR (qRT-PCR) for 25 genes and in situ hybridization for 3 genes were conducted to validate the microarray results. Genes encoding enzymes of the lignin and jasmonic acid pathways were chosen for qRT-PCR validation. In addition, nine other genes were randomly selected for validation. The list of genes selected for qRT-PCR validation and in situ hybridization with the sequences of primers used in qRT-PCR and cloning of in situ probes are given in Supplemental Table 3. The quantification of gene expression was performed using a relative quantification ($\Delta\Delta C_T$) method by comparing the data with an internal control as described below. The fold-change values were calculated by comparing the transcript abundance in syncytia at each timepoint (2, 5, and 10 dpi) with the transcript level in cells captured from noninfected root tissues at 4 days postgermination. Similarly, fold-change values were calculated from microarray data for these genes and compared with qRT-PCR results (Table 1; Figs. 3 and 4). Overwhelmingly, the

microarray and qRT-PCR results were in agreement, because only 4 of 25 assayed genes showed partially differing results, as follows. (i) A gene encoding a putative glycosyl transferase family which was identified as downregulated at 10 dpi by microarray analysis was shown to be upregulated by qRT-PCR analysis at both 5 and 10 dpi (Table 1). (ii) Two genes, glucose-6-phosphate isomerase and a pectinesterase family protein, were upregulated in the microarray analysis at 10 dpi but not in qRT-PCR analysis (Table 1). (iii) An unknown gene was upregulated at 10 dpi in qRT-PCR analysis but not in the microarray analysis. The qRT-PCR results for all other genes selected were qualitatively in agreement with microarray hybridization results. The discrepancy between the microarray results and qRT-PCR might be explained by cross-hybridization of gene family members in the microarray analysis or a greater specificity of qRT-PCR primers for individual gene family members.

For in situ localization of transcripts in SCN-infected roots, we selected three genes, two encoding putative CWMPs (ex-

Fig. 2. Cluster analysis of soybean genes based on their expression pattern during syncytium development for genes **A**, upregulated and **B**, downregulated in syncytia at 2 days postinfection (dpi) relative to noninfected root tissue. Cluster labels indicate the nature of expression changes across adjacent timepoints. For example, the cluster labeled 2 > 5 ≈ 10 contains genes exhibiting significantly greater expression at 2 than at 5 dpi but no significant change from 5 to 10 dpi. For visualization purposes, each gene's estimated mean log-scale expression profile was standardized to have mean 0 and variance 1 prior to plotting.

Table 1. Validation of microarray results by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Affymetrix probeset ID	GenBank ^b	Putative function	Fold-change compared with PC ^a					
			2 dpi		5 dpi		10 dpi	
			MA	PCR	MA	PCR	MA	PCR
GmaAffx.28397.1.S1_at	BM568229	Xyloglucan endotransglycosylase	3.60	28.58	31.16	615.08	22.97	361.34
Gma.10241.1.S1_at	BF069083	Serine carboxypeptidase S10 family protein	1.19	-1.29	-4.00	-6.89	-9.09	-17.54
Gma.11191.2.S1_at	BQ786168	Glycosyl transferase family 2 protein	1.04	1.04	-1.14	1.52	-1.78	1.61
Gma.13190.1.A1_at	CD391247	Glucose-6-phosphate isomerase	7.5	1.95	3.4	3.89	5.2	1.47
Gma.17727.3.A1_at	CD407603	Unknown	1.69	2.63	1.93	7.57	1.01	2.05
Gma.2262.1.S1_at	BE821923	Pectinesterase family protein	8.40	4.48	4.10	4.19	4.10	1.33
Gma.4401.2.S1_a_at	BI970725	Germin-like protein	2.40	4.13	3.30	6.89	7.50	6.15
Gma.6682.1.A1_at	CD417217	Expansin	2.40	8.02	9.90	352.59	14.82	237.44
Gma.8518.1.S1_at	AF243362	Glutathione S-transferase	11.5	28.88	6.60	11.39	6.60	11.87

^a PC = expression level in cells in and around the pericycle region of noninfected root tissue equivalent in development to the root tissues from which syncytia were captured 2 days postinoculation (dpi). MA = microarray and PCR = qRT-PCR. Dark gray boxes indicate genes upregulated by at least 1.5-fold, light gray boxes indicate genes downregulated by at least 1.5-fold, and white boxes indicate genes with changes less than 1.5-fold between two timepoints.

^b GenBank accession numbers.

pansin [EXP] and xyloglucan endotransglycosylase [XET]) and a third gene annotated as a putative pathogen-responsive protein transporter. Our microarray analyses predicted transcripts of these three genes to be upregulated in syncytia compared with uninfected roots. In situ hybridization confirmed accumulation of transcripts for all three genes in syncytia at 5 dpi (Fig. 5). Little or no staining was observed in tissue sections probed with sense probes.

Identification of key biological processes altered during syncytium development.

To assess the biological processes altered in syncytia based on the observed transcript abundance changes over a time-course of their development, we annotated the differentially expressed soybean transcripts by finding homologs in *Arabidopsis*, as described below. Using this approach, we were able to assign putative functions for 1,282 (833 upregulated and

Fig. 3. A, Lignin biosynthetic pathway (modified from Campbell and Sederoff 1996) and **B**, quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis of expression of lignin biosynthetic pathway genes during syncytium development. Amplified RNA was prepared from laser capture microdissection (LCM) syncytia at 2 (b), 5 (c), and 10 (d) days postinfection. Amplified RNA prepared from LCM of cells within the pericycle (a) was used for comparison. First-strand cDNA was synthesized from amplified RNA and subjected to qRT-PCR in triplicate. Values on the Y axis indicate $\log_{10}$ -fold change relative to expression within the pericycle region of noninfected root tissue. The qRT-PCR results are compared with the microarray results. PAL = phenylalanine ammonia-lyase, TAL = tyrosine ammonia-lyase, C4H = cinnamate 4-hydroxylase, C3H = 4-hydroxycinnamate 3-hydroxylase, COMT = caffeic acid 3-O-methyltransferase, F5H = ferulate 5-hydroxylase, 4CL = 4-coumarate:CoA ligase, CCoA-3H = coumaroyl-coenzyme A 3-hydroxylase, CCoA-OMT = caffeoyl-coenzyme A O-methyltransferase, CCR = cinnamoyl-CoA reductase, and CAD = cinnamyl alcohol dehydrogenase.

449 downregulated) of the 1,765 differentially expressed soybean genes. The list of 1,116 upregulated and 649 downregulated genes with their classification based on predicted function is available in Supplemental Tables 1 and 2, respectively. Because the putative functions are based solely on nucleotide and amino acid similarity and not experimental evidence, we cannot directly infer functions of any given protein in the SCN-soybean interaction. However, we can discern strong trends and, for the first time, formulate powerful hypotheses based on these predicted protein functions, allowing us to select with high confidence candidate genes of interest for further work to obtain biologically relevant functional data. For example, we were able to identify a general upregulation of primary and secondary metabolism as well as the differential regulation of individual gene family members belonging to several different classes of CWMPs (Table 2; Fig. 5A through D), many of which represent new genes encoding proteins potentially involved in the cell wall architectural modifications during syncytium development. Consistent with changes in primary metabolism, we identified genes encoding proteins with possible roles in sugar or carbohydrate, metal ion, and amino acid transport that were differentially regulated within developing syncytia. We also confirmed the spatial expression pattern of the putative soybean ortholog of an *Arabidopsis* pathogen-responsive protein transporter within developing syncytia by in situ mRNA hybridization (Fig. 5E and F). Transcript abundance changes related to secondary metabolism included genes encoding enzymes that catalyze multiple steps of the phenylpropanoid pathway and its branches, leading to lignin and suberin biosynthesis (Table 3; Fig. 3). The majority of genes encoding key enzymes involved in lignin biosynthesis were determined to be upregulated within syncytia in microarray analyses and confirmed by qRT-PCR (Fig. 3B).

This study provides additional insight into the details of phytohormone synthesis and signaling in the developing

syncytium. Although our data are consistent with previous studies in *Arabidopsis* that implicate an important role for auxin and ethylene in syncytium development, we have identified both up- and downregulation of genes encoding components of auxin and ethylene signaling pathways not previously shown within syncytia (Table 4). Moreover, we identified the differential regulation of soybean genes potentially involved in other phytohormone biosynthetic and signaling pathways, including gibberellin (GA), jasmonic acid (JA), and cytokinin (Table 4). For example, the majority of soybean homologs of genes encoding JA biosynthetic enzymes, including lipoxygenase (LOX1), allene oxide synthase (AOS), and 12-oxophytodienoate reductase (OPR1/2), with the exception of a homolog of 12-oxophytodienoate reductase (*OPR3*), were downregulated within developing syncytia (Table 4; Fig. 4A). Further analysis of our data with slightly less stringent criteria (q value < 0.05) also revealed downregulation of a homolog of *AOC* (encoding allene oxide cyclase) as well as a jasmonate-responsive gene encoding a vegetative storage protein (VSP) in the syncytia (Table 4). The observed patterns of differential expression of JA biosynthetic pathway genes in developing syncytia were further confirmed by qRT-PCR (Fig. 4B).

Of significant interest was the identification of a large panel of genes coding for putative transcription factors and components of signal transduction pathways that now are potential candidates with roles in the regulatory processes governing syncytium formation and function. In addition, 483 genes with no significant homology to any known genes or proteins also were differentially expressed. These genes represent strong candidates for future studies to elucidate additional novel aspects of syncytium development.

In an earlier microarray study using excised root tissue segments containing syncytia, we identified 618 differentially expressed genes at a 5% false discovery rate using the same microarray platform, soybean cultivar, and SCN population (Ithal et

Fig. 4. A, Jasmonic acid biosynthetic pathway and **B,** quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis of expression of jasmonic acid biosynthetic pathway genes during syncytium development. Amplified RNA was prepared from laser capture microdissection (LCM) syncytia at 2 (b), 5 (c), and 10 (d) days postinfection. Amplified RNA prepared from LCM of cells within the pericycle (a) was used for comparison. First-strand cDNA was synthesized from amplified RNA and subjected to qRT-PCR in triplicate. Values on the Y axis indicate log₁₀-fold change relative to the expression within the pericycle region from noninfected root tissue. The qRT-PCR results are compared with the microarray results.

al. 2007). A comparison of the results from these two experiments identified 85 soybean genes in common (Table 5). Of the 85 genes in common, 81 genes showed similar trends of expression in both experiments. For the majority of the genes in common, the magnitude of change was, on average, 26-fold higher (ranging from 1.7- to 598-fold) in this study compared with the excised root tissue experiment (Table 5). Four genes (Gma.8370.1.S1_at, GmaAffx.62364.1.S1_at, Gma.3893.3.S1_at, and Gma.11026.2.S1_s_at) showed opposing trends. These four genes were downregulated in the LCM experiment but were upregulated at 5 dpi in the excised root tissue experiment (discussed below).

DISCUSSION

Despite the use of LCM for cell-type-specific analysis in human and animal pathology and its recent applications for cDNA library construction and transcript profiling of specific plant cell types (Asano et al. 2002; Casson et al. 2005; Kerk et al. 2003; Nakazono et al. 2003), LCM has not yet been coupled with microarray profiling to facilitate cellular studies at

the plant–nematode interface. However, its utility for capturing giant cells and syncytia for RT-PCR and cDNA library construction, respectively, recently was demonstrated (Klink et al. 2005; Ramsay et al. 2004). By combining these two powerful technologies, we have determined the transcript abundance changes occurring specifically within developing feeding cells induced in soybean roots by parasitic cyst nematodes. Our approach led to the identification of 1,765 transcripts enriched or suppressed in syncytia at an early developmental timepoint (2 dpi) compared with cells from a corresponding region of non-infected root tissues. Transcript abundance changes reflected alterations in cellular processes related to metabolism, defense, cell signaling, transport, and cell wall architecture, providing new insights into the complex molecular mechanisms underlying syncytium formation.

Extensive modification to the cell wall architecture is one of the hallmarks of developing syncytia (Jones 1981). Recent molecular studies have shown that genes encoding CWMPs of plant origin are differentially expressed in response to cyst nematodes (Goellner et al. 2001; Ithal et al. 2007; Khan et al. 2004; Puthoff et al. 2003; Vercauteren et al. 2002; Wieczorek

Fig. 5. In situ localization of transcripts for soybean homologs of **A** and **B**, an expansin, **C** and **D**, xyloglucan endotransglycosylase, and **E** and **F**, putative protein transporter in syncytia. Root sections from soybean cyst nematode-infected soybean roots at 5 days postinfection were hybridized either with **A**, **C**, and **E**, sense probe or **B**, **D**, and **F**, antisense probe. **N** = nematode and **Syn** = syncytium. Bars = 50 μ m.

et al. 2006). In this study, we observed both up- and down-regulation of soybean genes encoding proteins with high similarities to different classes of plant CWMPs, including reversibly glycosylated polypeptides, different classes of glycosyl transferases, xyloglucan endotransglycosylases (XETs), and α -expansins (EXP) within developing syncytia. These observations were validated by confirming the spatial expression pattern of *XET* and *EXP* within syncytia by in situ mRNA hybridization. Significantly, this is the first report of *XET* expression specifically within cyst nematode-induced feeding cells. XETs are cell-wall-loosening enzymes involved in cell wall expansion (Darley et al. 2001; McQueen-Mason et al. 1992) and their upregulation suggests that they may play a role in maintaining the flexibility of a highly dynamic cell wall

during syncytium formation in conjunction with other classes of CWMPs.

Upregulation of genes in the multibranched phenylpropanoid pathway which gives rise to a wide array of soluble secondary metabolites in plants, including flavonoids, anthocyanins, and components of the secondary cell wall, lignins, and suberin, was observed in developing syncytia. The accumulation of flavonoid or isoflavonoid phytoalexins, deposition of lignin, or cell-wall-bound phenolics is a characteristic plant defense response in plants (Keen 1992). Our study suggests an upregulation of phenylpropanoid pathway genes specifically within the syncytia of a susceptible soybean line and is consistent with recent microarray studies using whole root tissues infected with nematodes (Alkarouf et al. 2006; Ithal et al. 2007). The role of

Table 2. Differential expression of cell wall-related genes in syncytia

Affymetrix probeset ID	Values		Fold change between dpi ^a				Numbers ^b			Annotation ^c
	<i>P</i>	<i>q</i>	PC to 2	2 to 5	5 to 10	2 to 10	Clus.	Acces.	AGI	
Upregulated										
Gma.2464.1.S1_a_at	5.77E-05	0.0015	5.87	1.08	-1.13	-1.04	1	BG839541	AT3G02230	RGP1
Gma.2464.1.S1_at	3.36E-07	0.0002	21.76	-1.16	-1.84	-2.14	8	BG839541	AT3G02230	RGP1
Gma.2464.1.S1_x_at	4.92E-07	0.0002	18.17	-1.15	-1.68	-1.93	8	BG839541	AT3G02230	RGP1
Gma.2464.2.S1_at	2.07E-05	0.001	9.03	-1.19	-1.52	-1.80	8	BM092071	AT3G02230	RGP1
Gma.2464.3.S1_s_at	4.75E-06	0.0005	14.88	-1.49	-1.88	-2.80	3	BQ740648	AT3G02230	RGP1
Gma.9323.1.S1_at	6.66E-05	0.0016	2.53	-1.20	1.36	1.14	9	CD409789	AT2G03220	FUT1
Gma.6081.1.S1_at	9.57E-05	0.002	2.89	1.57	1.88	2.94	7	BI967474	AT1G05850	Chitinase-like protein 1 (CTL1)
Gma.2262.1.S1_at	1.74E-05	0.0009	8.41	-2.03	1.01	-2.01	2	BE821923	AT3G14310	Pectinesterase family protein
Gma.7890.1.A1_at	3.99E-04	0.0041	343.78	-2.16	-1.73	-3.74	1	AW309342	AT3G43270	Pectinesterase family protein
Gma.15636.2.S1_x_at	1.22E-07	0.0001	54.05	1.11	1.22	1.35	1	BU577532	AT1G23720	Proline-rich EXT-like protein
GmaAffx.34785.20.S1_at	4.66E-04	0.0045	5.37	1.48	1.82	2.69	1	AW307368	AT1G23720	Proline-rich EXT-like protein
GmaAffx.50677.1.S1_s_at	4.99E-04	0.0047	60.95	1.45	-2.56	-1.77	1	BI971744	AT4G08400	Proline-rich EXT-like protein
GmaAffx.92021.1.S1_x_at	1.72E-07	0.0001	84.77	1.08	1.11	1.20	1	CF807746	AT1G26240	Proline-rich EXT-like protein
Gma.15996.1.S1_at	1.34E-07	0.0001	15.33	-1.22	-1.38	-1.68	8	BQ454193	AT3G62680	PRP3
Gma.15996.1.S1_x_at	1.54E-05	0.0008	8.41	1.19	-1.20	-1.01	1	BQ454193	AT3G62680	PRP3
Gma.10794.1.S1_at	5.30E-05	0.0014	4.71	-1.16	1.11	-1.05	1	BU577866	AT5G66680	DD-protein
GmaAffx.28397.1.S1_at	4.52E-05	0.0014	3.67	8.50	-1.35	6.30	5	BM568229	AT5G57560	Xet (TCH4)
Gma.16471.1.S1_at	9.75E-05	0.002	28.22	2.72	-1.68	1.62	5	BQ298739	AT4G25810	Xet (XTR6)
Gma.8565.1.S1_at	3.03E-04	0.0036	2.46	8.85	-2.41	3.67	6	AW707175	AT4G25810	Xet (XTR6)
Gma.14067.1.A1_at	4.08E-05	0.0013	5.21	1.02	-1.48	-1.45	8	CD414740	AT2G01850	Xet (XTH27)
GmaAffx.12832.1.S1_at	3.10E-05	0.0011	3.60	2.01	-2.32	-1.15	6	BG363116	AT1G10550	Xet (XTH33)
GmaAffx.89045.1.A1_s_at	4.82E-04	0.0046	2.97	1.28	-1.51	-1.17	8	CK605938	AT1G11545	Xet
Gma.10775.2.S1_s_at	2.31E-04	0.0031	16.12	2.23	-1.40	1.58	1	AI759701	AT2G40610	α -Expansin, putative (EXP8)
Gma.13110.1.S1_at	5.37E-04	0.0049	8.67	-1.19	-1.30	-1.54	1	CD394837	AT1G26770	α -Expansin, putative (EXP10)
Gma.6682.1.A1_at	1.10E-06	0.0003	2.41	4.14	1.49	6.17	7	CD417217	AT2G03090	α -Expansin, putative (EXP15)
Gma.7006.1.S1_at	1.57E-05	0.0008	3.25	3.35	-1.68	1.99	6	AF516880	AT2G39700	α -Expansin, putative (EXP4)
GmaAffx.671.1.S1_at	3.66E-04	0.004	12.81	-1.04	-1.35	-1.40	1	CA785167	AT2G28950	α -Expansin, putative (EXP6)
GmaAffx.67477.2.S1_at	7.99E-08	0.0001	403.43	-1.13	1.06	-1.06	1	AW509184	AT3G29030	α -Expansin, putative (EXP5)
GmaAffx.90009.1.S1_s_at	4.27E-04	0.0043	2.69	1.48	-1.16	1.27	5	CF805734	AT2G40610	α -Expansin, putative (EXP8)
GmaAffx.90097.1.S1_at	5.33E-07	0.0002	16.12	1.31	-1.15	1.14	1	CF805822	AT2G39700	α -Expansin, putative (EXP4)
GmaAffx.60859.1.A1_at	3.70E-06	0.0004	4.95	-1.06	1.13	1.06	1	BU550962	AT2G20370	Exostosin family protein (MUR3)
Gma.6550.1.A1_at	2.24E-04	0.0031	2.61	1.13	-1.06	1.06	1	CD402638	AT5G54160	COMT2
Gma.8251.1.S1_a_at	3.96E-04	0.0041	3.42	1.16	1.21	1.40	1	AW394443	AT4G34230	CAD5
GmaAffx.8273.1.A1_at	2.17E-06	0.0003	8.25	-1.67	-1.55	-2.59	3	BF070564	AT2G25050	FH2 domain-containing protein
Downregulated										
Gma.2044.2.S1_at	1.19E-04	0.0022	-2.20	6.17	-1.32	4.66	5	BE821230	AT4G15160	LTP family protein
Gma.3575.1.S1_at	5.08E-04	0.0048	-2.36	-1.13	-1.86	-2.10	8	BQ611599	AT3G19320	Leucine-rich repeat family protein
Gma.2507.1.S1_at	2.37E-04	0.0031	-4.10	-4.31	1.75	-2.46	2	CD409747	AT1G55850	Cellulose synthase family protein
Gma.5418.2.S1_at	4.52E-04	0.0045	-2.94	1.17	-1.63	-1.39	8	BI469627	AT4G39350	Cellulose synthase family protein
Gma.15523.1.S1_s_at	3.53E-04	0.0039	-1.39	1.52	-7.03	-4.62	8	BE475550	AT1G02810	Pectinesterase family protein
Gma.15564.3.S1_s_at	3.31E-04	0.0038	-3.74	-1.62	-3.86	-6.23	8	AW185750	AT1G26240	Proline-rich EXT-like protein
GmaAffx.34785.5.S1_s_at	4.31E-04	0.0043	-2.16	-1.07	-1.92	-2.05	8	BQ453262	AT1G23720	Proline-rich EXT-like protein
GmaAffx.34785.7.S1_s_at	1.46E-05	0.0008	-3.46	-2.94	-2.36	-6.96	3	BI497973	AT1G21310	Proline-rich EXT-like protein
GmaAffx.92830.1.S1_s_at	3.21E-04	0.0037	-3.78	-1.73	-4.01	-6.96	8	CF807748	AT1G26250	Proline-rich extensin, putative
Gma.15275.1.S1_at	1.29E-04	0.0023	-2.44	1.22	-1.60	-1.31	8	BI974583	AT3G22440	HRGP

^a PC = expression level in cells in and around the pericycle region of noninfected root tissue equivalent in development to the root tissues from which syncytia were captured 2 days postinoculation (dpi). Dark gray boxes indicate genes upregulated by at least 1.5-fold, light gray boxes indicate genes downregulated by at least 1.5-fold, and white boxes indicate genes with changes less than 1.5-fold between two timepoints.

^b Clus. = cluster number, Acces. = GenBank accession number, and AGI = Arabidopsis Homolog AGI number.

^c RGP = reversibly glycosylated polypeptide, FUT1 = xyloglucan α -(1,2)-fucosyltransferase, PRP = proline-rich family protein, EXT = extensin-like family, DD = dolichyl-diphosphooligosaccharide, Xet = xyloglucan endotransglucosylase transferase, COMT = caffeic acid/5-hydroxyferulic acid *O*-methyltransferase, CAD = Cinnamyl-alcohol dehydrogenase, FH2 = formin homology 2, LTP = protease inhibitor/seed storage/lipid transfer protein, and HRGP = hydroxyproline-rich glycoprotein

these secondary metabolites in plant–nematode interactions is not clear. However, their upregulation may reflect a general stress response of the plant to nematode infection. Alternatively, these compounds may play an important role in the establishment of a compatible interaction. Flavonoids, for example, which are produced from a branch of the phenylpropanoid pathway, have been implicated in the local accumulation of auxin at the feeding sites by blocking auxin efflux carriers (Hutangura et al. 1999), a process that may be essential for syncytium formation. In addition, at late stages of syncytium development, there is extensive thickening of syncytial cell walls (Jones and Payne 1977). The composition of the newly deposited wall material has not been characterized; however, in histological studies, Jones and Northcote (1972) observed that the center of the outer cortical walls as well as the center of some wall stubs and fragments left inside syncytia stained with toluidine blue, indicating the presence of lignin or some polyphenol. Thus, increased lignin production may contribute to the formation of secondary wall deposits to help protect enlarging syncytia from increasing turgor pressure. Similarly, the transcript abundance of genes encoding peroxidases also was upregulated in syncytia. Peroxidases function as scavengers of reactive oxygen species typically produced as an early resistance response in plants against a wide range of pathogens and may be involved in blocking oxidant-mediated programmed cell death (Blokhina et al. 2003; Marrs 1996). Peroxidase family members also are involved in cell wall rigidification through the cross-linking of cell wall polymers by oxidative coupling (Darley et al. 2001; Schopfer 1996). This peroxidase-mediated cell wall rigidification might occur through polymerization of extensins or through the cross-linking of polysaccharides through phenolic dimerisation (Darley et al. 2001) to strengthen syncytial cell walls.

The phytohormones auxin and ethylene are known to play an important role in the establishment and maintenance of

syncytia (Goverse et al. 2000; Wubben et al. 2001). However, there is little evidence to support a role for other classes of phytohormones in syncytium formation. The observed upregulation of soybean homologs of *Arabidopsis* cytokinin- and GA-regulated genes such as the *Arabidopsis* response regulator (ARR4), a negative regulator of cytokinin response, and GASAs, GA-induced cysteine-rich putative cell wall proteins (Kotilainen et al. 1999), as well as the downregulation of a soybean homolog of *Arabidopsis* SLEEPY1, an F-box protein required for GA signaling and homologs of *Arabidopsis* histidine kinases, which function as cytokinin receptors, suggest potential alterations to the status of cytokinin and GA biosynthesis and signaling pathways in developing syncytia that warrants further investigation. In addition, our data suggests alterations to JA biosynthesis and signaling within developing syncytia. JA is a small signaling molecule with roles in plant development and responses to biotic and abiotic stresses. JA is involved in a wide range of plant developmental processes, such as pollen development, anther dehiscence, fruit ripening, tuber formation, and tendril coiling. In addition, JA is a primary component of the plant wound response and is well known for its role in plant defense against insects (Howe et al. 1996). In recent years, JA has been shown to play an important role during resistance in other plant–biotic interactions, including interactions with pathogenic organisms. Most notably, JA-dependent defense responses recently have been shown to play a role in induced resistance against obligate biotrophic pathogens, challenging the notion that JA-dependent defense responses play a predominant role in basal resistance against insects and necrotrophic pathogens (Pozo et al. 2005). Earlier studies utilizing a range of JA mutants defective in either biosynthesis or signaling clearly point out that JA-dependent defense responses are effective against a wide range of pathogens and pests spanning diverse lifestyles, including the fungi *Alter-*

Table 3. Differential expression of phenylpropanoid and lignin biosynthetic pathway genes in syncytia

Affymetrix probeset ID ^c	Values		Fold change between dpi ^a				Numbers ^b			Annotation ^d
	<i>P</i>	<i>q</i>	PC to 2	2 to 5	5 to 10	2 to 10	Clus.	Acces.	AGI	
Upregulated										
Gma.2266.1.S1_s_at*	1.67E-04	0.0026	5.42	1.02	1.15	1.17	1	BG839284	AT2G37040	PAL1
Gma.2887.2.S1_x_at	1.80E-04	0.0027	18.17	−1.57	1.42	−1.11	1	BI701520	AT2G37040	PAL1
GmaAffx.89508.1.A1_s_at	4.77E-05	0.0014	8.50	−1.04	1.23	1.19	1	CK606172	AT2G37040	PAL1
GmaAffx.93094.1.S1_s_at	3.21E-05	0.0012	7.92	−1.08	−1.49	−1.62	8	CF805936	AT2G37040	PAL1
Gma.3881.1.S1_at*	1.19E-04	0.0022	3.74	−1.04	1.08	1.04	1	X92437	AT2G30490	C4H
Gma.3881.2.S1_x_at	5.34E-04	0.0049	2.36	1.02	1.51	1.54	9	AW349366	AT2G30490	C4H
Gma.4928.1.S1_a_at	2.59E-05	0.001	4.71	1.34	−1.26	1.06	1	AF002259	AT3G21240	4CL2
Gma.8472.1.S1_at*	8.80E-05	0.0019	8.24	−1.45	1.42	−1.02	1	BI942098	AT1G65060	4CL3
Gma.2206.1.S1_at*	1.10E-04	0.0021	3.32	−1.04	−1.01	−1.05	1	BG650498	AT2G02400	CCR
Gma.8251.1.S1_a_at*	3.96E-04	0.0041	3.42	1.16	1.21	1.40	1	AW394443	AT4G34230	CAD5
GmaAffx.92070.1.S1_at	1.52E-04	0.0025	3.90	3.03	−1.13	2.69	5	CF807049	AT4G34050	CCoA-OMT
Gma.3604.1.S1_at*	2.87E-06	0.0004	5.81	1.70	−1.04	1.63	5	AW349604	AT4G34050	CCoA-OMT
Gma.6550.1.A1_at	2.24E-04	0.0031	2.61	1.13	−1.06	1.06	1	CD402638	AT5G54160	COMT
Gma.10216.1.S1_a_at*	2.55E-04	0.0032	5.26	1.60	−2.59	−1.62	8	BI969369	AT5G54160	COMT1
Gma.10216.3.A1_a_at	6.31E-05	0.0016	4.35	1.79	−1.42	1.26	5	BI969715	AT5G54160	COMT2
Gma.15180.1.S1_at*	2.25E-03	0.0115	4.53	1.62	1.19	1.92	ND	CD408129	AT4G36220	F5H
Gma.5578.1.S1_x_at*	1.99E-03	0.0107	1.75	1.28	1.31	1.68	ND	BE659192	AT2G40890	C3H
Downregulated										
Gma.14136.1.A1_at*	1.15E-04	0.0022	−6.23	1.92	2.46	4.71	7	CD416189	AT5G38120	4CL
Gma.1527.1.S1_a_at	4.41E-04	0.0044	−13.46	−1.49	−1.36	−2.03	1	BI968185	AT2G02400	CCR
Gma.1527.1.S1_x_at*	1.29E-04	0.0023	−25.79	−1.08	−1.14	−1.23	1	BI968185	AT2G02400	CCR

^a PC = expression level in cells in and around the pericycle region of noninfected root tissue equivalent in development to the root tissues from which syncytia was captured 2 days postinoculation (dpi). Dark gray boxes indicate genes upregulated by at least 1.5-fold, light gray boxes indicate genes downregulated by at least 1.5-fold, and white boxes indicate genes with changes less than 1.5-fold between two timepoints.

^b Clus. = cluster number, Acces. = GenBank accession number, and AGI = Arabidopsis Homolog AGI number. ND = not determined. Cluster analysis was performed only on genes with *q* value < 0.005

^c Asterisk (*) indicates differential expression confirmed by quantitative real-time reverse-transcription polymerase chain reaction.

^d PAL1 = phenylalanine ammonia-lyase 1, C4H = cinnamic acid 4-hydroxylase, 4CL = 4-coumarate–CoA ligase 2, CCR = cinnamoyl-CoA reductase family protein, CAD = cinnamyl-alcohol dehydrogenase, CCoA-OMT = caffeoyl-CoA 3-*O*-methyltransferase, COMT = caffeic acid/5-hydroxyferulic acid *O*-methyltransferase, F5H = ferulate-5-hydroxylase, and C3H = coumarate 3-hydroxylase.

naria brassicicola (Thomma et al. 1998), *Botrytis cinerea* (Thomma et al. 1999), and *Erysiphe spp.* (Ellis et al. 2002); the bacterium *Pseudomonas syringae* (Ellis et al. 2002); and insect pests such as fungal gnats (McConn et al. 1997) and

aphids (Ellis et al. 2002). Recent studies have demonstrated that exogenous JA treatment of spinach, oat, and tomato increased their resistance against plant-parasitic nematodes (Cooper et al. 2005; Soriano et al. 2004a and b). Interestingly,

Table 4. Differential expression of hormone-related genes in syncytia

Affymetrix probeset ID ^c	Values		Fold change between dpi ^a				Numbers ^b			Annotation ^d
	<i>P</i>	<i>q</i>	PC to 2	2 to 5	5 to 10	2 to 10	Clus.	Accession	AGI	
Upregulated										
Auxin										
Gma.13060.1.S1_s_at	9.17E-05	0.0019	11.25	-1.46	-1.55	-2.27	1	AW348940	AT5G43700	IAA4/AUX2-11
GmaAffx.40399.1.S1_at	1.80E-05	0.0009	2.44	-2.75	-1.08	-2.97	2	BU548178	AT1G70940	PIN3
GmaAffx.2744.1.S1_at	1.68E-04	0.0026	4.62	1.36	-1.19	1.15	1	AW349891	AT2G46690	ARG7
Gma.15985.1.S1_at	5.96E-05	0.0015	5.00	-3.94	1.26	-3.13	2	CD402460	AT3G07390	AIR12
Gibberellin										
Gma.15958.1.S1_at	1.62E-04	0.0026	7.77	-1.68	1.54	-1.09	2	BF008858	AT3G02885	GASA5
Gma.4565.1.S1_s_at	1.68E-06	0.0003	17.12	2.34	-1.13	2.08	5	BI968013	AT4G09600	GASA3
Gma.4565.2.S1_at	4.64E-05	0.0014	10.59	1.21	-2.18	-1.80	8	AW348176	AT4G09600	GASA3
Gma.3433.1.S1_at	1.43E-04	0.0024	3.00	2.72	-1.45	1.88	5	BU091148	AT2G39540	GRFP
GmaAffx.90343.1.S1_at	6.75E-05	0.0016	8.08	-2.36	1.25	-1.90	2	CF806068	AT2G39540	GRFP
GmaAffx.90343.1.S1_s_at	2.80E-05	0.0011	12.18	-2.69	-1.15	-3.10	2	CF806068	AT2G39540	GRFP
Jasmonic acid										
GmaAffx.6142.1.S1_at*	9.41E-06	0.0006	10.80	1.03	-1.11	-1.07	1	BU765938	AT2G06050	OPR3
Cytokinin										
GmaAffx.44301.1.S1_at	1.86E-05	0.0009	7.69	-1.99	1.57	-1.27	4	AW395344	AT3G57040	ARR9
Downregulated										
Auxin										
GmaAffx.91588.1.S1_at	4.39E-04	0.0044	-2.86	-1.13	-1.36	-1.54	1	CF807313	AT2G22670	IAA8
GmaAffx.91229.1.S1_s_at	4.37E-05	0.0013	-2.14	-3.06	-1.11	-3.39	2	CF806954	AT3G04730	IAA16
GmaAffx.91229.1.S1_x_at	8.96E-05	0.0019	-2.03	-2.83	-1.45	-4.10	2	CF806954	AT3G04730	IAA16
Gma.11326.1.S1_s_at	2.35E-04	0.0031	-3.78	2.20	-1.17	1.88	5	CD406958	AT5G19140	AUX/AIRP
Gma.6662.1.S1_at	4.64E-05	0.0013	-5.21	-1.46	-1.14	-1.67	1	BI968600	AT4G23980	ARF9
Gma.7493.1.A1_at	2.67E-05	0.0010	-6.42	1.22	-1.40	-1.15	1	BQ611096	AT4G23980	ARF9
Gma.2563.1.S1_a_at	3.13E-04	0.0036	-2.05	1.02	-1.60	-1.57	8	BQ742867	AT5G62000	ARF2
Gma.2563.2.S1_at	2.03E-05	0.0009	-2.80	1.13	-1.30	-1.15	8	BI316885	AT5G62000	ARF2
Gibberellin										
Gma.3519.1.S1_at	1.82E-04	0.0027	-4.31	2.92	6.30	18.36	7	CD403581	AT5G59845	GRFP
Gma.12497.1.S1_s_at	4.92E-04	0.0047	-3.71	1.54	-1.54	1.00	6	CD395205	AT4G24210	SLY1
Jasmonic acid										
Gma.10969.1.S1_at	5.09E-04	0.0048	-11.82	-10.70	-1.13	-12.06	2	U36191	AT1G55020	LOX1
GmaAffx.89826.1.S1_at	1.50E-05	0.0008	-3.29	-2.83	-2.10	-5.93	3	CK606399	AT1G55020	LOX1
GmaAffx.90937.1.A1_at*	2.36E-04	0.0031	-3.46	-1.99	-3.60	-7.17	8	CF807838	AT1G55020	LOX1
GmaAffx.86334.1.S1_at*	1.84E-04	0.0027	-2.48	-1.90	1.51	-1.26	4	CD418191	AT5G42650	AOS
Gma.12166.1.S1_at*	1.93E-02	0.0428	-0.85	-3.19	1.62	-1.97	ND	CD409881	AT1G13280	AOC4
Gma.4728.1.A1_at*	1.00E-04	0.002	-11.70	-2.41	-1.79	-4.31	2	BI968944	AT1G76680	OPR1
GmaAffx.89772.8.S1_at	1.16E-03	0.0078	-9.39	-1.02	-2.53	-2.59	ND	CK605778	AT5G24780	VSP1
GmaAffx.89772.8.S1_x_at	2.24E-03	0.0114	-4.10	-1.13	-1.40	-1.58	ND	CK605778	AT5G24780	VSP1
Cytokinin										
Gma.6700.1.S1_at	3.10E-04	0.0036	-2.39	1.63	-1.49	1.09	6	AW597543	AT1G27320	AHK3
Gma.1514.1.S1_s_at	3.72E-04	0.004	-2.72	1.43	-2.66	-1.86	8	BI967628	AT2G01830	AHK4
Gma.1584.1.S1_at	8.54E-07	0.0002	-4.39	-1.26	-2.25	-2.83	8	AW309395	AT2G01830	AHK4
Gma.4725.1.S1_at	3.54E-04	0.0039	-1.72	-1.21	-1.30	-1.57	8	AW309254	AT2G01830	AHK4
Abscisic acid										
Gma.18000.1.S1_at	4.79E-06	0.0005	-8.76	1.58	1.39	2.20	7	BG406413	AT1G64060	RbohF
Gma.3154.1.S1_at	6.18E-05	0.0015	-4.18	-1.23	-1.27	-1.57	1	BQ272719	AT2G22430	HB-6
Gma.3154.2.S1_at	2.16E-05	0.001	-16.78	11.13	-2.05	5.42	6	CA784542	AT2G22430	HB-6
Ethylene										
Gma.3591.1.S1_a_at	3.52E-05	0.0012	-10.38	-1.51	1.11	-1.36	1	BE440266	AT1G05010	ACO
Gma.3591.2.S1_at	4.35E-05	0.0013	-8.33	-1.36	-1.90	-2.59	8	BE473658	AT1G05010	ACO
Gma.16623.2.A1_at	4.96E-04	0.0047	-3.71	1.30	-1.42	-1.09	1	BG157524	AT2G27050	EIL1
Gma.68.1.S1_at	2.95E-04	0.0036	-5.42	1.62	-2.64	-1.63	8	AF357211	AT2G44840	ERF

^a PC = expression level in cells in and around the pericycle region of non-infected root tissue equivalent in development to the root tissues from which syncytia were captured 2 days postinoculation (dpi). Dark gray boxes indicate genes upregulated by at least 1.5-fold, light gray boxes indicate genes downregulated by at least 1.5-fold. White boxes indicate genes with changes less than 1.5-fold between two timepoints.

^b Clus. = cluster number, Accession = GenBank accession number, and AGI = Arabidopsis Homolog AGI number. ND = not determined. Cluster analysis was performed only on genes with *q* value < 0.005.

^c Asterisks (*) indicate differential expression confirmed by quantitative real-time reverse-transcription polymerase chain reaction.

^d IAA = Indoleacetic acid-induced protein, AUX = auxin-induced protein, PIN3 = putative auxin transport protein, ARG = auxin-responsive family protein, AIR12 = auxin-responsive protein/auxin-induced protein, GASA = gibberellin-regulated protein, GRFP = gibberellin-regulated family protein, OPR3 = 12-oxophytodienoate reductase, ARR = two-component responsive regulator/response reactor, AUX/AIRP = putative auxin/aluminum-responsive protein, ARF = auxin-responsive factor, SLY1 = F-box family protein, LOX1 = lipoxygenase, AOS = allene oxide synthase, AOC = allene oxide cyclase, OPR1 = 12-oxophytodienoate reductase, VSP1 = vegetative storage protein, AHK = histidine kinase, RbohF = respiratory burst oxidase protein F, HB-6 = homeobox-leucine zipper protein 6, ACO = 1-aminocyclopropane-1-carboxylate oxidase, EIL1 = ethylene-insensitive3-like 1, ERF = ethylene response factor, subfamily B-3 of the ERF/AP2 transcription factor family.

Table 5. Comparison of differentially expressed genes from this study (false discovery rate = 0.5%) with the results from a microarray study (false discovery rate = 5%) using soybean cyst nematode-infected excised root tissues containing syncytia (Ithal et. al. 2007)

Gene ID	Fold change between dpi ^a				Numbers		Annotation
	LCM	Tissue			GenBank	Arabidopsis	
	PC to 2	2 dpi	5 dpi	10 dpi	Accession	Homolog AGI	
Upregulated genes in LCM experiment							
Cell-wall related							
GmaAffx.92021.1.S1_x_at	85.04	1.31	1.94	1.24	CF807746	AT1G26240	Proline-rich extensin-like family protein
GmaAffx.67477.2.S1_at	403.33	2.76	2.31	1.4	AW509184	AT3G29030	Alpha-expansin, putative (EXP5)
GmaAffx.50677.1.S1_s_at	60.58	1.84	4.93	1.4	BI971744	AT4G08400	Proline-rich extensin-like family protein
Gma.6682.1.A1_at	2.40	1.2	2.32	3.7	CD417217	AT2G03090	Alpha-expansin, putative (EXP15)
Gma.15636.2.S1_x_at	54.00	1.28	1.92	1.37	BU577532	AT1G23720	Proline-rich extensin-like family protein
Hormone biosynthesis, signaling, and response							
GmaAffx.6142.1.S1_at	10.76	4.1	4.44	2.4	BU765938	AT2G06050	12-oxophytodienoate reductase (OPR3)
Metabolism: synthesis, degradation/assimilation/utilization							
Carbohydrate/sugar/starch							
GmaAffx.494.1.S1_at	298.20	1.07	3.57	2.4	AI938330	AT5G24090	Acidic endochitinase (CHIB1)
Gma.10531.1.S1_at	4.86	1.01	1.42	1.2	BI967212	AT4G10260	pfkB-type carbohydrate kinase family protein
Amino acids							
Gma.4893.1.S1_at	7.59	1.01	1.22	1.24	AF049706	AT1G31230	Bifunctional aspartate kinase/homoserine dehydrogenase / AK-HSDH
Other							
GmaAffx.19349.1.A1_at	8.44	1.12	1.71	1.39	BQ080157	AT1G10400	UDP-glucuronosyl/UDP-glucosyl transferase family protein
Gma.9288.1.S1_at	33.32	1.63	3.02	1.67	BG155460	AT2G36780	UDP-glucuronosyl/UDP-glucosyl transferase family protein
Gma.8472.1.S1_at	8.18	1.09	4.9	1.22	BI942098	AT1G65060	4-coumarate--CoA ligase 3
Gma.7423.2.S1_a_at	4.38	1.47	1.61	1.72	BG046270	AT3G48990	AMP-dependent synthetase and ligase family protein, similar to peroxisomal-coenzyme A synthetase (FAT2)
Gma.7423.1.S1_a_at	4.91	1.29	1.41	1.76	BU547609	AT3G48990	AMP-dependent synthetase and ligase family protein, similar to peroxisomal-coenzyme A synthetase (FAT2)
Gma.5283.1.S1_at	15.42	-1.06	6.79	11.67	BM091956	AT2G18660	Expansin family protein (EXPR3)
Gma.5014.1.S1_at	3.32	1.09	1.37	1.39	AI443304	AT1G01050	Inorganic pyrophosphatase, putative (soluble)
Gma.2096.1.S1_at	29.11	2.22	1.77	1.06	CD403339	AT2G29340	Short-chain dehydrogenase/reductase (SDR) family protein
Gma.12574.1.S1_at	4.83	1.03	1.5	1.23	CD391606	AT2G43360	Biotin synthase (BioB) (BIO2)
Proteasome pathway components							
Gma.10241.1.S1_at	1.19	1.03	3.21	1.02	BF069083	AT3G07990	Serine carboxypeptidase S10 family protein
Transport							
Sugar transporters							
GmaAffx.92466.1.A1_at	18.69	1.79	2.49	1.23	CF808191	AT5G26340	Hexose transporter, putative
GmaAffx.11897.1.S1_at	5.05	1.52	1.64	1.56	BE819868	AT5G13750	Transporter-related
Amino acid transporters							
Gma.10199.1.A1_at	8.42	1.55	1.58	1.38	CD409733	AT4G15236	ABC transporter family protein
Electron transport							
Gma.4434.1.S1_at	11.77	1.56	2.33	1.29	BE659015	AT5G26330	Plastocyanin-like domain-containing protein
Other transporters							
GmaAffx.55267.1.S1_at	7.55	1.19	1.76	1.23	BE020105	AT1G33090	MATE efflux family protein
Stress, defense response							
GmaAffx.93392.1.S1_s_at	109.68	3.27	13.43	1.86	CF807760	AT5G42500	Disease resistance-responsive family protein
GmaAffx.90703.1.A1_at	122.60	2.31	21.51	1.15	CF809087	AT5G05340	Peroxidase, putative
GmaAffx.90028.1.S1_s_at	14.97	1.34	2.78	2.18	CF805753	AT1G01720	No apical meristem (NAM) family protein
GmaAffx.15130.1.A1_at	8.23	1.6	1.85	1.14	BU545350	AT4G11470	Protein kinase family protein
Gma.5519.1.S1_at	12.40	1.3	1.92	1.44	BI967466	AT1G01720	No apical meristem (NAM) family protein
Gma.4919.1.S1_at	62.92	2.93	4.29	1.48	AW350788	AT5G05340	Peroxidase, putative
Gma.2593.1.S1_s_at	13.83	1.43	2.11	1.73	CF922368	AT2G29480	Glutathione S-transferase, putative
Gma.2344.1.S1_at	13.21	1.45	2.29	1.75	BG511051	AT5G49480	Sodium-inducible calcium-binding protein (ACPI)
Gma.1917.1.S1_s_at	30.63	2.66	3.22	2.5	AF243365	AT1G78380	Glutathione S-transferase, putative
Gma.1917.1.S1_at	33.29	2.89	5.11	3.13	AF243365	AT1G17180	Glutathione S-transferase, putative
Gma.17744.1.S1_at	64.10	2.22	2.4	1.29	AW349581	AT1G10360	Glutathione S-transferase, putative
Gma.11004.1.S1_at	21.50	1.2	1.73	1.71	AW102356	AT2G35980	Harpin-induced family protein (YLS9)
Transcription factors and components of signal transduction pathways							
GmaAffx.2712.1.A1_x_at	48.52	1.09	1.46	1.18	AW30923	AT5G02170	Amino acid transporter family protein

Continued on following page

^a LCM = laser capture microdissection. Tissue = excised root tissue experiment; fold-change compared with mock at each timepoint. PC = expression level in cells in and around the pericycle region of noninfected root tissue equivalent in development to the root tissues from which syncytia were captured 2 days postinoculation (dpi). Dark gray boxes indicate genes upregulated by at least 1.5-fold, light gray boxes indicate genes downregulated by at least 1.5-fold, and white boxes indicate genes with changes less than 1.5-fold between two timepoints.

Table 5. Continued from preceding page

Gene ID	Fold change between dpi ^a				Numbers		Annotation
	LCM	Tissue			GenBank	Arabidopsis	
	PC to 2	2 dpi	5 dpi	10 dpi	Accession	Homolog AGI	
Transcription factors and components of signal transduction pathways (<i>Continued</i>)							
Gma.7631.1.A1_at	43.20	1.41	3.84	1.46	AW309495	AT1G79750	Malate oxidoreductase, putative
Others/unclassified							
Gma.2505.2.S1_x_at	82.40	2.02	2.87	3.49	BU548684	AT3G11050	Ferritin, putative
Gma.17263.1.A1_at	7.62	1.27	1.91	1.34	AW310602	AT1G70520	Protein kinase family protein
Gma.1654.1.S1_s_at	22.41	1.67	1.61	1.42	BI971603	AT1G52360	Coatomer protein complex, subunit beta 2 (beta prime), putative
Gma.1096.1.S1_at	117.30	2.11	2.36	2.16	CD407195	AT5G59310	Lipid transfer protein 4 (LTP4)
Gma.9956.1.S1_at	5.06	1.67	13.48	-2.04	AI938045	AT1G35710	Leucine-rich repeat transmembrane protein kinase
GmaAffx.91749.1.S1_s_at	12.74	1.49	20.03	-1.95	CF807474	AT1G35710	Leucine-rich repeat transmembrane protein kinase, putative
Unknown function							
Gma.10747.2.S1_at	4.86	1.05	1.14	1.03	BG881394	AT5G17190	Expressed protein
Gma.12398.1.A1_s_at	109.01	1.05	5.32	8.06	BG509862	AT5G39050	Transferase family protein
Gma.11278.1.S1_at	3.11	-1.02	1.14	1.32	BI971791	AT2G35900	Expressed protein
Gma.11116.1.S1_at	7.20	2.33	5	-1.07	BG156999	AT1G08380	Expressed protein
Gma.2252.1.S1_at	1.41	1.15	1.33	1.19	CD406598	AT4G16146	Expressed protein
Gma.3604.1.S1_at	5.84	1.15	1.17	1.15	AW349604	AT4G34050	Caffeoyl-CoA 3-O-methyltransferase, putative
GmaAffx.63137.1.S1_at	16.45	-1.25	-1.27	-1.29	BG043541	AT5G29560	Ca+2-binding EF hand family protein
GmaAffx.90984.1.S1_s_at	97.12	2.44	15.9	8.9	CF806709	AT5G39110	Germin-like protein, putative
GmaAffx.90785.1.S1_s_at	1,113.35	1.86	5.15	2.61	CF807476	AT1G03220	Extracellular dermal glycoprotein, putative
GmaAffx.92082.1.A1_s_at	10.73	1.73	2.93	1.44	CF807807	AT1G17860	Trypsin and protease inhibitor family protein
GmaAffx.92722.1.S1_at	3.41	1.06	1.93	1.28	CF807561	AT3G48690	Expressed protein
No hits							
GmaAffx.91442.1.S1_at	176.87	3.3	41.93	1.46	CF805736	No Hit	...
GmaAffx.90222.1.S1_at	10.36	1.28	2.22	1.34	CF805947	No Hit	...
GmaAffx.89772.10.A1_s_at	55.33	5.2	37.8	1.27	CK606097	No Hit	...
GmaAffx.88979.1.A1_s_at	4.61	1.11	1.31	1.41	CK605704	No Hit	...
GmaAffx.26010.1.S1_at	18.74	1.48	2.46	1.42	AW756277	No Hit	...
GmaAffx.18769.1.S1_at	25.54	2.43	5.19	1.42	BI972364	No Hit	...
Gma.987.1.S1_at	230.60	1.09	26.09	-1.26	AW100853	No Hit	...
Gma.6999.3.S1_s_at	92.15	2.8	28.57	1.52	CF921432	No Hit	...
Gma.6999.2.S1_s_at	143.21	3.77	37.45	1.4	X60043	No Hit	...
Gma.6999.1.S1_x_at	68.46	2.93	24.45	1.28	AF529303	No Hit	...
Gma.6999.1.S1_s_at	123.71	3.71	35.08	1.45	AF529303	No Hit	...
Gma.6515.1.S1_s_at	2.14	1	1.24	1.16	BQ627548	No Hit	...
Gma.14304.1.S1_at	3.00	1.47	2.9	2.01	CA852438	No Hit	...
Gma.11119.2.S1_s_at	31.36	1.53	4.19	1.23	CF920743	No Hit	...
Downregulated genes in LCM experiment							
Metabolism: synthesis, degradation/assimilation/utilization							
<i>Protein</i>							
GmaAffx.80899.1.S1_at	-69.64	-1.3	-1.45	-1.34	AW348263	AT2G28305	Expressed protein
Gma.8370.1.S1_at	-2.34	1.1	1.61	1.1	BQ454087	AT3G15260	Protein phosphatase 2C, putative
<i>Other</i>							
Gma.3429.1.S1_at	-9.93	-1.24	-1.26	-1.25	BI974673	AT5G09300	2-oxoisovalerate dehydrogenase
Proteasome pathway components							
GmaAffx.62364.1.S1_at	-2.29	-1.05	2.42	-1.06	AW733764	AT5G36210	Expressed protein
Transport							
<i>Sugar transporters</i>							
GmaAffx.487.1.S1_at	-3.52	-1.25	-1.56	-1.49	BG511495	AT1G30220	Sugar transporter family protein
Stress, defense response							
Gma.8053.1.S1_at	-7.07	-1.17	-1.31	-1.05	AW351130	AT1G04400	Cryptochrome 2 apoprotein (CRY2)
Transcription factors and components of signal transduction pathways							
Gma.4821.1.A1_s_at	-2.38	-1.26	-1.32	-1.15	BI969245	AT5G43130	Transcription initiation factor IID (TFIID) component TAF4 family protein
Gma.4279.1.S1_at	-6.82	-1.11	-1.19	-1.05	CA935007	AT4G38690	1-phosphatidylinositol phosphodiesterase-related
Others/unclassified							
GmaAffx.77737.1.S1_at	-9.84	-1.37	-1.67	-1.74	BU084758	AT1G65480	Flowering locus T protein (FT)
Unknown function							
GmaAffx.87793.1.S1_at	-18.27	-1.18	-1.37	-1.23	BI941782	AT5G63500	Expressed protein
Gma.3893.3.S1_at	-3.95	1.08	2.5	1.14	BG651854	AT1G74950	Expressed protein
Gma.17662.2.S1_s_at	-9.32	-1.07	-1.27	-1.08	BQ630190	AT3G14280	Expressed protein
Gma.11026.2.S1_s_at	-2.05	1.2	11.84	-1.76	BQ080041	AT1G17860	Trypsin and protease inhibitor family protein
No hits							
Gma.17258.1.S1_s_at	-12.00	-1.39	-1.58	-1.13	BG509247	No Hit	
Gma.5838.1.S1_s_at	-5.36	-1.03	-1.35	-1.54	BG405383	No Hit	
GmaAffx.13490.1.S1_at	-6.65	-1.12	-1.41	-1.09	AW831242	No Hit	

we observed downregulation of multiple genes encoding enzymes involved in JA biosynthesis and response within developing syncytia, suggesting that active suppression of JA biosynthesis and signaling may be important for their development. The only discrepancy was the observed upregulation of an *OPR3* homolog while homologs of *OPR1/OPR2* were downregulated in syncytia. In *Arabidopsis*, it was shown that *OPR3* effectively converts the natural 12-oxophytodienoic acid (OPDA) to JA, whereas *OPR1* and *OPR2* convert OPDA with greatly reduced efficiency compared with *OPR3* (Schaller et al. 2000). Due to the fact that the JA biosynthetic pathway has not been characterized in soybean and full-length JA biosynthetic genes have not yet been cloned, it is not clear which *OPR* homolog is involved in JA biosynthesis in soybean. Further studies will be required to understand the potential role of JA in plant–nematode interactions and the possible mechanism of suppression of JA biosynthesis and signaling within syncytia. In addition to a downregulation of JA biosynthesis, we also observed downregulation of *GmEREBP* (Mazarei et al. 2002) and other genes associated with abiotic and biotic stress responses such as senescence-associated proteins, pathogen-responsive receptor-like protein kinases, and wound and osmotic stress-responsive genes within syncytia consistent with recent microarray studies in other pathosystems that suggest a general suppression of plant defense mechanisms by pathogens during compatible interactions (Jammes et al. 2005; Moy et al. 2004).

In this study, LCM facilitated the developmental transcript profiling of syncytia to provide new insights into the complex molecular mechanisms that determine the properties of a cell type unique to plant–nematode interactions. Our analysis has identified a large panel of soybean genes expressed in syncytia, revealing pathways that appear to play important roles in their induction, formation, and function as nutrient sinks to support nematode development and reproduction. In addition, a significant proportion of differentially expressed genes identified in this study is of unknown function or have no significant homology to any known genes or proteins in the sequence databases. This is a significant advancement in our understanding of gene expression changes specific to feeding cells because all previous microarray studies of cyst nematode–plant interactions were conducted using whole root or excised root tissues (Alkarouf et al. 2006; Ithal et al. 2007; Khan et al. 2004; Puthoff et al. 2003). These earlier studies identified differentially expressed genes during cyst nematode infection in *Arabidopsis* and soybean; however, it is difficult to differentiate expression changes specific to feeding cells from systemic responses when feeding cells represent only a small fraction of whole root tissue. In addition, small changes in gene expression within feeding cells may be undetectable even in excised root tissue studies. A comparison between soybean gene expression changes identified using excised root tissues infected with nematodes over a time course of infection (Ithal et al. 2007) and those identified specifically within feeding cells in this study using LCM identified 85 genes in common between the two studies. For the majority of the genes in common, the magnitude of change was several-fold higher in this study compared with the excised root tissue experiment, demonstrating the sensitivity of the LCM approach. Moreover, an additional 1,680 gene expression changes occurring within developing syncytia were identified in this study. Therefore, coupling of LCM with microarray analysis proved to be an effective tool for identifying gene expression changes in developing syncytia.

Analyzing this dataset with a stringent q value cut-off ($q = 0.005$) and validating differentially expressed genes by qRT-PCR and in situ hybridization gives us very high confidence in the validity of our approach. In the near future, availability of

the complete soybean genome sequence, more accurate annotations of soybean genes, and reverse genetic tools will provide an even better understanding of the function of differentially expressed genes identified in this study in syncytia development. With the set of genes enriched in syncytia, it should be possible to screen for and identify factors critical for the establishment and maintenance of syncytia, an understanding of which is essential for developing novel, target-specific plant resistance against nematodes.

MATERIALS AND METHODS

Plant and nematode material.

Soybean (*Glycine max* (L.) Merr) cv. Williams 82, an SCN-susceptible cultivar, was used in this study. SCN (*H. glycines* Ichinohe) inbred line PA3 (HG-type 0; race 3) was obtained from a publicly available collection maintained at the University of Illinois at Urbana-Champaign and mass selected in greenhouse cultures on Williams 82 according to standard procedures (Niblack et al. 1993). HG-type testing was conducted according to established protocols (Niblack et al. 2002) to confirm the population as HG-type 0 (race 3).

Nematode inoculations.

Soybean seed were soaked in running tap water for 30 min, surface-sterilized in 10% bleach for 10 min, and rinsed again in running tap water for 30 min. Seed were germinated for 2 days in rag dolls at 28°C in the dark. Cysts of *H. glycines* were extracted from infested soil by flotation in water and collected on a 250- μ m sieve. Harvested cysts were gently crushed using a drill press and the eggs collected on a 25- μ m sieve (Faghihi and Ferris 2000). Eggs were purified further on a sucrose-density gradient. The eggs were surface sterilized in 0.02% sodium azide for 20 min, washed extensively in sterile water, and set up to hatch in the presence of gentamicin at 1.5 mg/ml and nystatin at 0.05 mg/ml at 28°C on a sterile modified Baermann pan for 2 days. Parasitic J2 nematodes were collected, washed with sterile water, and resuspended at a concentration of 300 J2 per 100 μ l in 0.01% sterile agarose. Two-day-old seedlings with a 2- to 3-cm-long uniform root were inoculated with freshly hatched juveniles according to Mahalingam and associates (1998) with the following modifications. To mark the precise location of root infection and facilitate the excision of nematode infection sites at later timepoints, a black dot was made 1 cm from the root tip of each soybean seedling using a black Sharpie pen. A 100- μ l drop of inoculum was applied at this site. Infection was synchronized by washing the roots exactly 24 h after inoculation. Seedlings were rolled into rag dolls and placed vertically in sterile Hoagland's solution (1/10 dilution) and allowed to grow in a plant growth chamber at 26°C with a photoperiod of 16 h of light and 8 h of darkness with constant aeration. The infection process was monitored in a portion of the collected roots by clearing and staining with acid fuchsin (Daykin and Hussey 1985). Samples were collected at 2, 5, and 10 dpi by excising root pieces of approximately 1 cm in length at the zone of maximum infection and transferring to fixative. Control samples were mock inoculated using 100 μ l of 0.1% agarose and samples were collected at 2 dpi. In total, three independent biological replicates were obtained for each condition.

Tissue processing.

The root tissues excised from SCN-infected roots at 2, 5, and 10 dpi and mock-inoculated roots at 2 dpi were fixed immediately in freshly prepared 3:1 ethanol/glacial acetic acid for 19 h at 4°C and transferred to 70% ethanol at 4°C. Tissues were mounted in cassettes and processed by the MU Histology laboratory using an automated tissue processor. Fixed tissues were

dehydrated at room temperature in a graded series of ethanol (1 h each [vol/vol], 65, 80, 80, 95, 95, 100, 100, and 100%) followed by two changes of Clear Rite III (Richard Allan, Richland, MI, U.S.A.) for 1 h each. After the second change, root tissues were transferred to paraffin (Surgipath, Richmond, IL, U.S.A.) at 58°C. The paraffin was replaced four times at 50-min intervals. Root tissue samples were embedded in paraffin and allowed to set at 4°C. Sections 10 µm in thickness were cut from the blocks with an HM325 rotary microtome (Richard Allen) and floated in diethylpyrocarbonate (DEPC)-treated water at 50°C. Sections were mounted on positively charged Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA, U.S.A.) and dried at 42°C for 24 h on a slide warmer. Slides with dried sections were processed for LCM.

LCM.

Just prior to LCM, slides were deparaffinized twice in xylene for 5 min each, allowed to dry at room temperature, and transferred to an airtight container. LCM was carried out using a PixCell Ite LCM system (Arcturus, Mountain View, CA, U.S.A.). The cells within the pericycle region of mock-inoculated roots (2 dpi) and syncytial cells (2, 5, and 10 dpi) were laser capture microdissected from the dried root tissue sections according to the manufacturer's instructions. LCM conditions used were as follows: laser spot size of 7.5 µm, laser power of 70 to 80 mW, and laser pulse duration of 700 µs. CapSure HS LCM caps (Arcturus) were used for capturing cells. A minimum of 10 syncytia from at least 10 plants was used per biological replicate.

RNA extraction and amplification.

Total RNA was isolated immediately from LCM-syncytia captured on CapSure HS LCM caps using the Picopure RNA isolation kit (Arcturus) and stored in extraction buffer at -80°C. Total RNA was isolated following the manufacturer's instructions and treated with RNase-free DNase I (Qiagen, Valencia, CA, U.S.A.). A T7 promoter-based RNA amplification approach was used to generate biotin-labeled cRNA. Double-stranded cDNA synthesis and RNA amplification in the first cycle of the two-cycle RNA amplification procedure was performed using the Riboamp HS RNA amplification kit (Arcturus) following the manufacturer's instructions. Double-stranded cDNA was synthesized from the amplified cRNA using the Riboamp HS RNA amplification kit (Arcturus). Biotinylated cRNA was generated from the double-stranded cDNA by a second round of RNA amplification using the GeneChip IVT labeling kit (Affymetrix, Santa Clara, CA, U.S.A.) following the manufacturer's protocol. Labeled cRNA was column purified using the Riboamp HS RNA amplification kit (Arcturus). Amplified RNA was quantified spectrophotometrically using a Nanodrop 1000 system (Nanodrop Technologies, Wilmington, DE, U.S.A.). Because a syncytium is made up of a complex of fused cells and the number of cells incorporated into the syncytium increases over time, it is difficult to determine the exact number of individual cells (or individual syncytia) required to obtain a sufficient amount of amplified cRNA for microarray hybridization. Therefore, we evaluated the correlation between the amount of captured syncytial cells and the quantity of amplified RNA recovered by comparing the cRNA yield obtained from 16 independent LCM experiments with the surface area of the LCM cap covered by captured syncytial cells. To estimate the area covered by the syncytial cells on each cap, following RNA extraction, LCM caps were photographed at the same magnification and the cap images were analyzed using Scion image software (Scion Corporation, Frederick, MD, U.S.A.).

Regression analysis yielded a correlation coefficient of 0.90, indicating a strong positive linear relationship between the RNA yield and corresponding syncytial cell area harvested by LCM. These results indicated that approximately 7 µg of amplified cRNA could be obtained per square millimeter of captured cell area. The amplified cRNA products were distributed within the size range of approximately 100 bases to over 1,500 bases. RT-PCR analysis and successful amplification of several low- or high-abundant soybean cDNAs provided confirmation of the high quality and integrity of the cRNA (data not shown). Labeled cRNA (20 µg) was used for hybridization. The samples were sent to the Iowa State University GeneChip microarray core facility for fragmentation, hybridization, staining, and scanning.

Microarray chip description.

The GeneChip soybean genome array (Affymetrix) was used in study. This oligonucleotide array contains 37,593 probe sets representing 35,611 soybean transcripts, 7,530 probe sets representing 7,431 SCN transcripts, and over 15,800 probe sets representing 15,421 *Phytophthora sojae* transcripts.

Data analysis.

The data were transformed by taking the natural log of the GeneChip Operating Software (v. 1.0) expression measurements. Normalization was performed by subtracting, from each value on an array, the median of all the values from that array. Analysis was performed on a gene-by-gene basis by fitting a simple analysis of variance (ANOVA) model with one mean for each treatment (noninfected and 2, 5, and 10 dpi). A *t* test contrasting the means for the noninfected and 2-dpi treatments yielded a *P* value for each gene. These *P* values were converted to *q* values using the method of Storey and Tibshirani (2003). The *q* values can be used to approximate the false discovery rate (FDR) associated with a list of genes. The FDR represents the expected proportion of genes declared to be differentially expressed that are, in fact, not differentially expressed. We selected genes with *q* values < 0.005; thus, the FDR is estimated to be 0.5% for the method we used to obtain a list of genes with statistically significant differences between the cells from noninfected root tissue and the syncytia at 2 dpi. This gene list was further filtered using a fold-change cutoff of 1.5 to identify 1,765 significantly differentially expressed genes. The microarray data is deposited in the ArrayExpress database at the European Bioinformatics Institute under accession number E-MEXP-876.

Genes upregulated in the syncytia at 2 dpi relative to the cells from noninfected root tissue were clustered based on their expression patterns across 2, 5, and 10 dpi. Specifically, tests for differences between adjacent timepoints (2 versus 5 dpi and 5 versus 10 dpi) were used to classify each gene into one of the nine categories labeled in Figure 2. Genes in the category labeled 2 ≈ 5 ≈ 10 exhibited no significant expression changes between 2 and 5 dpi or between 5 and 10 dpi. Genes in the category labeled 2 > 5 ≈ 10 exhibited significantly greater expression at 2 dpi than at 5 dpi but no significant change in expression between 5 and 10 dpi. The other categories can be interpreted similarly. Throughout our clustering procedure, *P* values < 0.05 from the contrast of treatment means in our ANOVA analysis were used to determine significant differences. The identical clustering approach also was used for genes downregulated in the syncytia at 2 dpi relative to the cells from noninfected root tissue.

Note that our clustering strategy is different from clustering approaches commonly employed in microarray data analysis (e.g., hierarchical clustering). Standard approaches are based

on correlations between expression profiles which would not be well estimated in our case because we have expression measures at only three timepoints. Thus, we have chosen to use *P* values for tests of differences between adjacent timepoints to provide a clustering that accounts for uncertainty in our estimated mean expression profiles.

The consensus nucleotide sequences corresponding to the differentially expressed genes identified from microarray analysis were obtained from the Affymetrix NetAffix analysis center. Soybean consensus sequences were used as a query to search for *Arabidopsis* homologs in the TAIR database using WU-BLAST2 search. The soybean genes were annotated based on the *Arabidopsis* top hit with an *e* value < 10⁻³.

qRT-PCR.

Real-time qRT-PCR was carried out using the Applied Biosystems 7500 Real-Time PCR system. Specific primers for each gene selected were designed using the Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.). Total RNA prepared from an independent inoculation and LCM experiment was amplified by a two-cycle RNA amplification procedure using the Riboamp HS RNA amplification kit (Arcturus) following the manufacturer's instructions. For quantification using real-time PCR, template was prepared by reverse transcription of amplified cRNA using random primers. PCR was conducted in triplicate in 20 µl using SYBR Green PCR master mix (Applied Biosystems). The qRT-PCRs were conducted in triplicate. PCR conditions used were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A soybean gene encoding a cytochrome P450 family protein (accession no. AF135484) was used as an internal control. This is one of the seven soybean internal control genes represented on the Genechip Soybean Genome Array, and we determined by qRT-PCR that expression of this gene is stable across the treatment groups in our experiment. However, additional probesets for cytochrome P450 family members are printed on the chip and several of these displayed differential expressions across treatment groups. The quantification of gene expression was performed using the relative $\Delta\Delta C_T$ method by comparing the data with the internal control gene.

In situ hybridization.

Two genes encoding putative CWMPs, expansin (accession no. AF516880) and xyloglucan endotransglycosylase (accession no. BM568229), and a third gene encoding a putative pathogen-responsive protein transporter (accession no. AI941474) were selected for in situ localization of transcripts in SCN-infected soybean roots. Inoculated root tissues collected at 5 dpi with SCN were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.0) by vacuum infiltration and further incubated in fresh 4% paraformaldehyde for 12 h at room temperature followed by 24 h at 4°C. Fixed tissues were mounted in cassettes and processed by the MU Histology laboratory using an automated tissue processor as described above for LCM. Sections of 5 µm in thickness were cut from the blocks with an HM325 rotary microtome (Richard Allen) and floated in DEPC-treated water at 50°C. Sections were mounted on positively charged Superfrost Plus glass slides (Fisher Scientific), dried at 42°C for 24 h on a slide warmer, and stored in slide boxes at 4°C with desiccant. Slides were deparaffinized twice in xylene for 5 min each and rehydrated at room temperature in a graded series of ethanol (4 min each, 100, 100, 95, 85, 70, 60, and 30%) followed by 4 min in DEPC-treated water. Riboprobe synthesis and in situ hybridizations were performed as described by Goellner and associates (2001).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Affymetrix GeneChip array website:
www.affymetrix.com/products/arrays/specific/soybean.affx
 Affymetrix NetAffix analysis center:
www.affymetrix.com/analysis/netaffix/index.affx
 ArrayExpress database: www.ebi.ac.uk/arrayexpress/