Multiple Nodulation Genes Are Up-Regulated During Establishment of Reniform Nematode Feeding Sites in Soybean

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ABSTRACT

The semi-endoparasitic reniform nematode (*Rotylenchulus reniformis*) infects over 300 plant species. Females penetrate host roots and induce formation of complex, multinucleate feeding sites called syncytia. While anatomical changes associated with reniform nematode infection are well documented, little is known about their molecular basis. We grew soybean (*Glycine max*) in a split-root growth system, inoculated half of each root system with *R. reniformis*, and quantified gene expression in infected and control root tissue at four dates after inoculation. Over 6,000 genes were differentially expressed between inoculated and control roots on at least one date (false discovery rate [FDR] = 0.01, $|\log_2\text{FC}| \geq 1$), and 507 gene sets were significantly enriched or depleted in inoculated roots (FDR = 0.05). Numerous genes up-regulated during syncytium formation had previously

been associated with rhizobia nodulation. These included the nodule-initiating transcription factors CYCLOPS, NSP1, NSP2, and NIN, as well as multiple nodulins associated with the plant-derived peribacteroid membrane. Nodulation-related NIP aquaporins and SWEET sugar transporters were induced, as were plant CLAVATA3/ESR-related (CLE) signaling proteins and cell cycle regulators such as CCS52A and E2F. Nodulins and nodule-associated genes may have ancestral functions in normal root development and mycorrhization that have been co-opted by both parasitic nematodes and rhizobial bacteria to promote feeding site and nodule formation.

Additional keywords: RNA-seq, nodulin, plant-parasitic nematode.

Reniform nematode, *Rotylenchulus reniformis* (Linford and Oliveira), is a sedentary plant-parasitic nematode with a broad host range. Unlike more host-specific nematode parasites, reniform nematode infects over 300 species from numerous plant families in tropical, subtropical, and warm-temperate regions (Heald and Robinson 1990; Robinson et al. 1997). Among its hosts are multiple economically important crops, including soybean, cotton, and pineapple. Reniform nematode has a significant impact on global crop production and food security; in the United States alone, its infection causes millions of dollars in annual yield losses (Robinson 2007).

Similar to other sedentary plant-parasitic nematodes, reniform nematode induces the formation of permanent feeding structures in host root tissue. Immature females penetrate the epidermis and cortex of a host root, stopping when they reach an endodermal cell. There, each inserts its stylet and releases a proteinaceous secretion that promotes development of a permanent feeding site. Partial cell wall lysis begins at the initial endodermal cell and progresses to multiple cells in the pericycle, ultimately creating a multinucleate syncytium. The amalgamated cells undergo multiple structural and metabolic changes over the course of several days, giving rise to a metabolically active feeding site that nourishes the nematode during her development and reproduction (Rebois 1980; Rebois et al. 1975).

The anatomical changes associated with reniform nematode syncytia have been extensively documented (Agudelo et al. 2005; Jones and Dropkin 1975; Rebois 1980; Rebois et al. 1975), but less is known about their molecular basis, especially in comparison with better-studied parasites like the cyst nematodes (*Heterodera* and *Globodera* spp.) and root-knot nematodes (RKN) (*Meloidogyne* spp.). Cyst nematodes (CN) manipulate host genes to orchestrate partial cell wall lysis in procambium or young vascular tissue,

inducing the formation of a multinucleate, hypermetabolic syncytium. RKN alter the expression of host genes involved in cell cycle control, cell wall modification, and hormone regulation to induce the formation of “giant cells” in root galls (Cabrera et al. 2015; de Almeida Engler et al. 2015; Wieczorek 2015). Both groups of nematodes suppress defense-related signaling pathways throughout the lifetime of the feeding structure (Jaouannet et al. 2013; Quentin et al. 2013). Whether reniform nematode manipulates host genes in a manner analogous to that of CN or RKN is unknown, although recent work suggests that broadly similar groups of genes may be involved (Li et al. 2015).

Previous work has highlighted overlap in physiology and gene expression between rhizobia root nodules and CN/RKN feeding sites. RKN giant cells in *Medicago truncatula* exhibit altered expression of the nodule-associated transcriptional regulators PHAN and KNOX, the early nodulin ENOD40, the cell cycle regulator CCS52a, and the cytokinin primary-response gene ARR5 (Lohar et al. 2004). Hyper-nodulated (*har1*) *Lotus japonicus* mutants exhibit both increased nodulation and increased RKN infection (Lohar and Bird 2003). Similarly, the production of rhizobia-signaling isoflavonoids is up-regulated during CN infection, and a beet CN resistance gene is highly expressed during both CN and rhizobia infection (Kennedy et al. 1999; Mitra et al. 2004).

Mounting evidence supports the hypothesis that diverse below-ground symbionts and parasites have evolved to manipulate a similar genetic toolkit in their hosts. Genes normally involved in lateral root development and substrate transport are co-opted to support the formation of root nodules, mycorrhizae, and feeding sites (Lohar and Bird 2003; Lohar et al. 2004; Mathesius 2003; Wasson et al. 2009). In the context of nodulation, these genes have been termed nodulins. They include many sugar, nutrient, amino acid, and hormone transport proteins, as well as transcription factors, phosphatidylinositol transfer family proteins, and cell cycle regulators (Denance et al. 2014; Desbrosses and Stougaard 2011; Ferguson et al. 2010; Ghosh et al. 2015; Maroti and Kondorosi 2014; Verma et al. 1986). While most extensively studied in the context of nodulation, nodulins and nodule-associated genes are also present in the genomes of nonleguminous plants and are

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*The e-Xtra logo stands for “electronic extra” and indicates that nine supplementary files are published online.

Fig. 1. Micrographs of acid fuchsin-stained soybean roots and reniform nematodes taken at 100× magnification at 3, 6, 9, and 12 days after inoculation (DAI).

Fig. 2. Visualization of gene expression data using principal components analysis of rlog-transformed counts for all genes in control and inoculated soybean roots at four time points after inoculation.

expressed in nonnodulation contexts (Denance et al. 2014; Reddy et al. 1999; Vieira-de-Mello et al. 2011).

Here we report the reference-guided assembly of a soybean (*Glycine max*) transcriptome from control and reniform nematode infected roots over a 12-day time course. We describe a split-root growth system that allows us to harvest infected and noninfected control roots from the same plant. We identify individual genes and gene sets whose expression is altered in reniform nematode-infected roots, and we report the up-regulation of multiple well-known nodulins and nodule-associated genes during reniform nematode parasitism of soybean.

MATERIALS AND METHODS

Split-root growth system and nematode inoculation. To minimize the effects of plant-to-plant variation on identification of genes related to reniform nematode infection, we developed a split-root growth system that permitted the collection of infected and control root tissue from the same plant. Surface-sterilized seeds of the reniform nematode-susceptible *Glycine max* ‘Hutcheson’ were germinated on sterilized vermiculite and maintained in a growth room at $28 \pm 2^\circ\text{C}$ and 50% relative humidity (RH) with a 14-h/10-h (light/dark) photoperiod. The soybean seed had been grown and collected in our greenhouses and was not coated with the rhizobial inoculum commonly present on commercial seed. After cotyledon emergence, a horizontal cut was made at the base of each primary root to encourage

lateral root proliferation. After 1 week, the lateral roots were grouped into two equal bundles, threaded through the arms of a Y-shaped plastic tube (diameter 2.22 cm), and directed into adjacent 300 cm³ pots filled with pasteurized fine sand. Plants were grown for 1 week, and 12 uniform plants were then selected for use in the experiment.

Reniform nematode inoculum was prepared from soil collected in a St. Matthews, SC agricultural field that had been under cotton and corn rotation for many years. Nematodes were extracted by sugar centrifugal flotation, rinsed, and collected in a 500 μm mesh sieve (Jenkins 1964). Following inspection and quantification, nematodes were suspended in tap water at a concentration of 1,500 individuals/ml. One pot in each split-root system was selected at random and inoculated with 2 ml of the nematode suspension (3,000 individuals); the other pot received an equivalent volume of tap water as a negative control.

Three replicate plants were harvested at 3, 6, 9, and 12 days after inoculation (DAI) for microscopy and RNA extraction as described below. Thorough examination of scanned root images on each harvest date confirmed that no nodules were present on any root samples. Additional inoculated and noninoculated plants that were grown for 18 days in the same pasteurized sand system also showed no evidence of nodulation.

Microscopy. Inoculated root samples were obtained from three replicate plants on each harvest date. Roots were gently rinsed free of soil, cleared with chloride bleach, and stained with acid fuchsin following the protocol of Byrd et al. (1983). Briefly, cleaned root

Fig. 3. Volcano plots of false discovery rate (FDR) adjusted P values graphed against $\log_2(\text{fold change})$ estimates for all expressed genes at four time points after inoculation. Black symbols: $p\text{-adj} > 0.01$; yellow symbols: $p\text{-adj} \leq 0.01$ and $|\log_2\text{FC}| < 1$; red symbols: $p\text{-adj} \leq 0.01$ and $|\log_2\text{FC}| \geq 1$. Numbers in the top left of each panel reflect numbers of significantly up- and down-regulated genes in infected roots on each date.

samples were incubated in 0.9% NaOCl for 4 min, rinsed, and placed in tap water for 15 min to remove residual bleach. After a second rinse, roots were heated to boiling in beakers with 30 ml of tap water and 1 ml of acid fuchsin stain (3.5 g of acid fuchsin, 250 ml of acetic acid, and 750 ml of water). After boiling for 30 s, the beakers were cooled to room temperature, and the roots were rinsed in tap water, placed in acidified glycerol, and heated until destained. Once cooled, roots and stained nematodes were photographed on an inverted microscope at 100× magnification (Motic AE2000 with Cannon EOS Rebel T3i Camera). Neither nodules nor nodule primordia were observed in any root sample.

Transcriptome sequencing. Approximately 500 mg fresh weight (FW) of infected and control root tissue was harvested from each of three replicate plants at 3, 6, 9, and 12 days after reniform nematode inoculation, for a total of 24 samples (two infection statuses × four dates × three biological replicates). Tissue was stored in RNeasy Lysis Reagent (Qiagen, Austin, TX) and sent

to the University of Arizona Genetics Core (Tucson, AZ). RNA was extracted using the Qiagen RNeasy Mini Kit, and RNA quality was assessed with the Agilent 2100 Bioanalyzer prior to 100-bp paired-end library preparation with the Illumina TruSeq 2 RNA kit. Samples were sequenced across two lanes of the Illumina HiSeq 2000 platform (Illumina, San Diego, CA).

A total of 309.5 million paired-end 100-bp reads were generated across the experiment, with an average of 12.8 million reads per sample. Read quality was assessed with FastQC v0.10.1, sequencing adaptors were trimmed with Trimmomatic v0.32 (Bolger et al. 2014), and low-quality bases were removed using the default settings of ConDeTri v2.2 (Smeds and Kunstner 2011). The average per-read PHRED quality score after trimming was 36.7, and the minimum per-read score was 36.02. All raw reads were uploaded to the NCBI Sequence Read Archive under accession number SRP091708.

Transcriptome assembly and annotation. Trimmed reads from each sample were mapped to the Wm82.a1.v1 build of the

TABLE 1. Top 20 genes whose expression was significantly up-regulated in reniform nematode-infected soybean roots at four time points after inoculation (false discovery rate = 0.01, genes ranked based on log₂FC estimate)^a

Gene ID	Description	Mean normalized counts				L ₂ FC estimate
		Inoculated	SD	Control	SD	
3 days after inoculation (DAI)						
XLOC_003387	Ring-h2 finger protein atl34-like	1,623	2,264	1	1	8.75
XLOC_002605	Prolyl 4-hydroxylase subunit alpha-1	1,048	895	0	1	8.63
XLOC_000614	casp-like protein	1,456	1,448	1	1	8.53
XLOC_012146	Vacuolar iron transporter homolog 4-like/nodulin 21	2,352	2,392	3	0	8.44
XLOC_033005	Probable 2-isopropylmalate synthase	929	1,344	0	0	8.35
XLOC_039620	Aspartic protease CDR-1-like /nodulin 41	637	489	0	0	8.31
XLOC_049195	2-Isopropylmalate synthase	558	512	0	0	8.07
XLOC_019074	Selenium-binding protein 1	1,998	2,542	2	1	8.05
XLOC_005135	Nodulin 16	808	394	1	0	8.04
XLOC_011554	Carboxy-terminal region remorin	547	629	0	0	7.92
XLOC_037046	Ring-h2 finger protein atl34-like	909	996	1	1	7.76
XLOC_020450	Carboxy-terminal region remorin	595	585	1	1	7.62
XLOC_009155	Nodule inception protein (NIN)	1,170	861	3	1	7.56
XLOC_028021	Polyol transporter 3/6-like	344	216	0	0	7.54
XLOC_049849	Subtilisin-like serine protease	4,337	2,568	18	7	7.52
XLOC_012147	EKN protein	3,489	4,401	3	2	7.42
XLOC_013559	Defensin-like protein 1	289	173	0	0	7.26
XLOC_030420	tmv resistance protein n/TIR-NBS-LRR	391	449	1	1	7.01
XLOC_028458	PGN polygalacturonase specific to nodule	328	305	0	1	7.00
XLOC_036345	Root determined nodulation 1 homolog	229	176	0	0	6.95
6 DAI						
XLOC_042297	Uncharacterized glycosyl transferase	1,421	2,455	1	2	4.11
XLOC_042566	Serine acetyltransferase 2-like	916	1,580	1	2	4.10
XLOC_022985	Cell number regulator 2/FW2.2-like	408	687	0	0	3.61
XLOC_022216	Peroxidase 15	342	151	7	5	3.58
XLOC_051027	Protein iq-domain 31-like	170	292	0	0	2.69
XLOC_014144	Transcription factor bhlh30-like	88	147	1	1	2.44
XLOC_004535	Ammonium transporter 3 member 1-like	56	40	3	1	2.43
XLOC_015272	Expansin A15	81	134	1	1	2.40
XLOC_031107	Putative cyclin-dependent kinase inhibitor 1c-like	239	397	6	8	2.39
XLOC_051990	Peroxidase 12-like	424	482	39	17	2.25
XLOC_045581	Mitochondrial calcium uniporter	104	179	0	1	2.22
XLOC_035511	Allantoinase 1	420	720	2	3	2.22
XLOC_051360	Zinc transporter protein ZIP1	296	394	13	9	2.18
XLOC_040432	g-type lectin s-receptor-like serine threonine-protein kinase SD2-5-like	74	123	0	0	2.18
XLOC_040169	Glutamine synthetase	745	805	45	18	2.15
XLOC_011394	IRK-interacting protein-like	69	116	0	0	2.03
XLOC_018724	e3 ubiquitin-protein ligase lin-1/CERBERUS	42	64	0	1	1.99
XLOC_017748	Putative ABC transporter C family member 15	1,321	1,022	205	39	1.95
XLOC_049982	abc transporter b family member 15	232	374	11	11	1.86
XLOC_050071	Hyoscyamine 6-dioxygenase-like	57	15	8	1	1.85
9 DAI						
XLOC_000425	udp-glycosyltransferase 83a1-like	88	107	0	0	4.06

(Continued on next page)

^a Bolded genes have previously been identified as nodulins or as playing a role in legume-rhizobia nodulation.

soybean reference genome using Bowtie2 v2.1.0 and TopHat v2.0.11 with default parameters (Trapnell et al. 2012). The -G option was used to supply TopHat with the Glyma1.0 reference gene models as a GTF file (Schmutz et al. 2010). Transcripts were assembled separately for each sample using Cufflinks v2.2.1, then merged with the Glyma1.0 reference annotation to generate a soybean root transcriptome that included both previously described and newly assembled transcripts (Trapnell et al. 2012). The new reference transcriptome was annotated in Blast2GO Pro v3.0, which performs a BLASTx search against the NCBI nr protein database and assigns sequence descriptions, gene ontology (GO) terms, Interpro IDs, enzyme codes (EC), and KEGG pathways to transcripts with significant blast hits ($E < 10^{-6}$) (Conesa et al. 2005).

Gene expression was quantified by mapping reads from individual samples back to the new transcriptome using HTSeq v0.6.1 (Anders et al. 2015). Reads from all splice forms of a given gene were pooled, and count data were imported to DESeq2 for differential expression analysis using default parameters (Love et al. 2014). Data were analyzed separately by date to identify genes differentially expressed between inoculated and noninoculated roots, while controlling for the effect of individual plants (false

discovery rate [FDR] = 0.01). Genes were considered to be significantly differentially expressed when they fulfilled two criteria: (i) an FDR-adjusted P value of ≤ 0.01 and (ii) a $|\log_2(\text{fold change})|$ estimate of ≥ 1 . The $\log_2\text{FC}$ estimates were calculated in DESeq2 and implemented empirical Bayes shrinkage to permit accurate comparison of effect sizes across all genes (Love et al. 2014).

In parallel with the identification of individual DE genes, gene set enrichment analysis (GSEA) was performed to identify predefined gene sets whose expression was significantly enriched or depleted in inoculated roots (Subramanian et al. 2005). A custom GSEA database of 1,499 predefined gene sets, each containing at least 15 genes, was created from GO terms, enzyme codes, and KEGG pathway annotations of the root transcriptome. Gene sets whose expression was enriched or depleted in inoculated roots on each date were identified using an FDR of 0.05.

To assess the possibility of rhizobial contamination, raw reads from individual soybean root samples were mapped to the sequenced genomes of rhizobial bacteria and other prokaryotic and eukaryotic model organisms. The reference genomes of eight nodulating bacterial species (*Bradyrhizobium diazoefficiens*, *B. elkanii*, *B. japonicum*, *Mesorhizobium loti*, *Rhizobium etli*, *R. leguminosarum*, *Sinorhizobium*

TABLE 1. (Continued from previous page)

Gene ID	Description	Mean normalized counts				
		Inoculated	SD	Control	SD	L ₂ FC estimate
XLOC_019174	SNF1-related serine threonine-protein kinase SRK2A-like	191	243	5	8	3.87
XLOC_045079	Subtilisin-like protease SDD1	622	881	25	41	3.85
XLOC_025801	Cytochrome P450 geraniol 8-hydroxylase-like	111	147	2	1	3.80
XLOC_048378	Caffeic acid o-methyltransferase	145	240	2	3	3.80
XLOC_025121	Thaumatococcus-like protein 1b	139	232	1	2	3.78
XLOC_049783	Homoglutathione synthetase	174	200	4	7	3.70
XLOC_041566	NEP1-interacting protein 2	99	129	1	2	3.68
XLOC_029854	Annexin-like protein rj4	150	157	7	11	3.51
XLOC_038757	p34 thiol protease	49	49	0	0	3.47
XLOC_007899	Basic endochitinase-like	563	531	28	34	3.43
XLOC_019850	Dirigent protein 9-like	220	159	10	6	3.41
XLOC_001973	Sec14p-like phosphatidylinositol transfer protein	103	139	2	2	3.41
XLOC_040201	stg1-like protein	92	141	2	3	3.39
XLOC_024512	duf642 family protein	103	107	3	6	3.34
XLOC_011098	Remorin family protein	64	80	1	2	3.25
XLOC_020628	Probable serine threonine-protein kinase	79	43	2	4	3.22
XLOC_035322	Mate efflux family protein limate1-like	1,037	1,012	94	102	3.15
XLOC_038934	14 Kda proline-rich protein	69	75	2	4	3.15
XLOC_013019	Squalene monooxygenase	388	420	40	56	3.13
12 DAI						
XLOC_026556	Protein flug/nodulin 61	1,326	2,291	3	4	4.72
XLOC_006138	ent-copalyl diphosphate synthase	116	100	0	0	4.01
XLOC_043161	Copper diamine oxidase	5,314	5,056	159	86	3.99
XLOC_016066	Gibberellin 20 oxidase 1	207	121	7	3	3.76
XLOC_008289	Pleiotropic drug resistance protein 1-like	75	16	0	0	3.61
XLOC_034123	Universal stress protein a-like protein	285	492	1	1	3.54
XLOC_042753	Peroxidase 12-like	189	321	2	4	3.44
XLOC_036762	Potassium channel akt1-like	214	368	1	1	3.42
XLOC_033005	2-Isopropylmalate synthase-like	173	295	0	0	3.40
XLOC_010580	Extensin-like/14 kda proline-rich DC2.15 protein	433	229	34	21	3.20
XLOC_045110	Extensin-like/14 kda proline-rich DC2.15 protein	230	226	7	2	3.19
XLOC_043784	Extensin-like/14 kda proline-rich DC2.15 protein	125	88	7	5	3.16
XLOC_049373	ent-copalyl diphosphate synthase	71	59	1	1	3.09
XLOC_004314	LRR receptor-like protein kinase At3g28040-like	64	62	0	0	3.08
XLOC_022215	Peroxidase 15	641	501	31	7	3.07
XLOC_003387	Ring-h2 finger protein atl34-like	119	204	1	1	2.93
XLOC_026325	1-Aminocyclopropane-1-carboxylate oxidase-like	57	56	0	1	2.89
XLOC_038982	Peroxidase 15	204	93	17	2	2.88
XLOC_051032	gdp-mannose-epimerase 1	100	62	6	2	2.85
XLOC_013663	Carbonic anhydrase	255	404	7	7	2.83

fredii, and *S. meliloti*) and six additional model organisms (*Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Xenopus laevis*, and *Danio rerio*) were obtained from NCBI. Trimmed reads from each soybean root sample were mapped to each reference genome using Bowtie2 v2.1.0 with default parameters (Langmead and Salzberg 2012). The percentage of reads from each sample that mapped to each genome was calculated with HTSeq v0.6.1. Two-way ANOVAs were used to test for differences in percent mapped reads between inoculated and control samples for each genome and date.

The percent reads that could be successfully aligned to these nonplant genomes was extremely low, never exceeding 2.06E-05 percent for any sample. Across all dates and genomes, there were

no significant differences in the percent mapped reads between inoculated and control samples. The percent reads that could be aligned to nodulating bacterial genomes (range: 0 to 2.06E-05) were similar in magnitude to the percent that could be aligned to *E. coli*, animal, and fungal genomes (range: 0 to 2.01E-05). It is likely that these reads derive from genes and gene segments that are highly conserved across species.

RESULTS

Microscopy. Histology of infected roots documented the progress of reniform nematode infection through time and provided context for the gene expression results (Fig. 1). At 3 DAI, all

TABLE 2. Top 20 genes whose expression was significantly down-regulated in reniform nematode-infected soybean roots at four time points after inoculation (false discovery rate = 0.01, genes ranked based on log₂FC estimate)

Gene ID	Description	Mean normalized counts				L ₂ FC estimate
		Inoculated	SD	Control	SD	
3 days after inoculation (DAI)						
XLOC_031122	High affinity nitrate transporter 2.4	123	78	4,096	2,780	-4.91
XLOC_014708	Molybdate transporter 1-like	5	3	158	112	-4.37
XLOC_009515	Molybdate transporter 1-like	32	5	626	235	-4.09
XLOC_015632	Light-dependent short hypocotyls 10-like transcription factor	1	1	55	43	-3.98
XLOC_005541	Vinorine synthase	8	4	149	37	-3.80
XLOC_016249	K+ efflux antiporter	2	2	62	16	-3.78
XLOC_044150	12-Oxophytodienoate reductase 11	3	4	91	38	-3.75
XLOC_012899	Subtilisin-like protease cucumisin-like	13	5	224	110	-3.74
XLOC_022590	LRR receptor-like serine/threonine-protein kinase	3	3	70	11	-3.70
XLOC_012604	CONSTANS-like zinc finger transcription factor	2	4	55	48	-3.63
XLOC_002565	Homeobox protein knotted-1-like 6 transcription factor	2	2	53	12	-3.58
XLOC_006330	Ethylene-responsive transcription factor erf024-like	10	12	149	44	-3.56
XLOC_049553	Ethylene-responsive transcription factor erf024-like	13	9	170	25	-3.43
XLOC_029737	Bel1-like homeodomain protein 1 transcription factor	60	8	589	24	-3.21
XLOC_002405	wrky transcription factor 47-like	9	5	103	11	-3.21
XLOC_030749	Bel1-like homeodomain protein 1 transcription factor	82	67	766	72	-3.18
XLOC_010357	7-Deoxyloganetin glucosyltransferase-like	11	4	112	44	-3.08
XLOC_004579	Glutaredoxin C13-like	0	0	17	14	-3.05
XLOC_021885	ABC transporter c family member 3	3	2	44	15	-3.02
XLOC_052219	Metal-nicotianamine transporter YSL1	3	1	44	12	-2.99
6 DAI						
XLOC_006330	Ethylene-responsive transcription factor erf024-like	13	3	319	236	-3.38
XLOC_010579	Extensin-like/14 kda proline-rich DC2.15 protein	0	0	69	65	-2.76
XLOC_007587	Cytochrome p450 94a1-like	24	20	247	127	-2.50
XLOC_016551	Laccase 17 isoform 1	35	26	304	99	-2.34
XLOC_049553	Ethylene-responsive transcription factor erf024-like	10	9	201	190	-2.34
XLOC_012899	Subtilisin-like protease cucumisin-like	43	14	336	260	-2.25
XLOC_030148	Laccase 5-like	43	25	311	79	-2.25
XLOC_009515	Molybdate transporter 1-like	94	60	734	541	-2.25
XLOC_038094	Blue copper protein/phytoeyanin	88	62	526	199	-2.18
XLOC_025501	Germin-like protein 17	68	52	436	56	-2.15
XLOC_051714	Probable carboxylesterase 6	62	27	357	46	-2.14
XLOC_021377	Palmitoyl-monogalactosyldiacylglycerol delta-7 (FAD5)	156	147	797	387	-2.14
XLOC_045792	Laccase 5-like	41	20	263	57	-2.14
XLOC_027563	Probable carboxylesterase 17	8	5	72	39	-2.04
XLOC_033266	Glucomannan 4-beta-mannosyltransferase 9-like	14	15	97	53	-2.03
XLOC_048157	Mannan endo-beta-1,4-mannosidase 6	17	6	130	44	-2.03
XLOC_046719	Protein LURP-one-related 15-like	46	33	237	75	-2.02
XLOC_010120	UDP-glucuronate:xylan alpha-glucuronosyltransferase 1-like	27	11	167	35	-2.01

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inoculated samples contained multiple vermiform females embedded in the root cortex with their head regions adjacent to the stele. At 6 DAI, individual root samples contained a mixture of infection stages: both vermiform females and swollen females were present, indicating a mixture of new and mature feeding sites. At 9 DAI, all females were swollen into the characteristic mature reniform shape, and at 12 DAI, egg masses were present at the majority of infection sites. In no samples was there evidence of rhizobial nodules or nodule primordia.

Assembly and differential gene expression. Illumina sequencing of 24 RNA samples from reniform nematode-inoculated and control roots generated over 300 million 100-bp reads. After filtering and trimming, clean reads from all samples were mapped back to the soybean reference genome, and transcripts were assembled

using soybean reference gene models as guidance. The final soybean root transcriptome contained both previously annotated and novel transcripts. In total, we assembled 46,995 unique genes and 120,565 alternate splice forms of these genes, with an N50 of 1,430 bp and a mean length of 984 bp. Ninety-seven percent of the assembled genes received a significant hit to the NCBI nr database ($E < 10^{-6}$), and 79% were annotated with at least one GO term. Enzyme codes and KEGG pathways were assigned to 21 and 26% of the assembled genes, respectively. The FASTA sequences of all assembled transcripts are provided in Supplementary File S1, and Blast2GO transcript annotations, including Glyma names of previously annotated genes, are provided in Supplementary File S2.

Overall patterns of differential gene expression. Exploratory data visualization with principal components analysis (PCA)

TABLE 2. (Continued from previous page)

Gene ID	Description	Mean normalized counts				
		Inoculated	SD	Control	SD	L ₂ FC estimate
XLOC_035373	Sugar transporter ERD6-like 16	15	8	611	985	-2.00
XLOC_025490	ATP binding cassette B family member 4	28	13	147	75	-1.96
9 DAI						
XLOC_006330	Ethylene-responsive transcription factor erf024-like	4	2	123	19	-4.05
XLOC_002565	Homeobox protein knotted-1-like 6 transcription factor	0	0	38	1	-3.38
XLOC_012899	Subtilisin-like protease cucumisin-like	7	5	161	133	-3.23
XLOC_007587	Cytochrome p450 94a1-like	31	17	282	82	-2.93
XLOC_049553	Ethylene-responsive transcription factor erf024-like	5	3	67	17	-2.89
XLOC_027563	Probable carboxylesterase 17	3	1	53	33	-2.85
XLOC_039814	Ethylene-responsive transcription factor erf024-like	2	1	44	1	-2.85
XLOC_031122	High affinity nitrate transporter 2.4	129	54	1,464	853	-2.72
XLOC_044314	WUSCHEL-related homeobox 4-like	1	1	31	22	-2.58
XLOC_002206	E3 ubiquitin-protein ligase RHA1B-like	29	15	189	55	-2.48
XLOC_019263	Auxin-induced protein 5NG4	47	27	278	90	-2.40
XLOC_047728	Nitrate transporter 1.2-like	14	3	89	15	-2.28
XLOC_035906	High affinity nitrate transporter 2.4-like	1,108	530	7,295	4,942	-2.27
XLOC_043785	Extensin-like/14 kDa proline-rich DC2.15 protein	9	10	99	62	-2.25
XLOC_038778	Fasciclin-like arabinogalactan protein 11-like	185	220	915	773	-2.20
XLOC_000520	Sugar phosphate translocator at3g11320-like	3	5	39	53	-2.14
XLOC_029801	High affinity nitrate transporter 2.4	458	364	2,069	167	-2.14
XLOC_035435	Fasciclin-like arabinogalactan protein 11-like	297	303	1,435	1,140	-2.12
XLOC_030739	Momilactone A synthase	63	33	300	86	-2.11
XLOC_030910	Pleckstrin domain-containing family N member 1	7	8	46	26	-2.06
12 DAI						
XLOC_036897	Alcohol dehydrogenase 1	1	2	49	41	-2.51
XLOC_012481	Ring-h2 finger protein ATL78-like	5	2	55	39	-2.23
XLOC_028025	Polyol transporter 3-like	285	173	1,338	560	-2.11
XLOC_041911	CEN-like protein 1	100	60	465	104	-2.00
XLOC_024315	Dehydrin-like protein	7	7	65	81	-1.87
XLOC_020262	Trypsin inhibitor A	11	12	178	287	-1.84
XLOC_021091	Ring-h2 finger protein ATL78-like	36	22	185	75	-1.83
XLOC_020147	gDSL esterase lipase	78	41	474	341	-1.83
XLOC_007303	Glucan endo-1,3-beta-glucosidase-like	996	993	4,017	2,077	-1.81
XLOC_000098	Hydroxyproline-rich glycoprotein	61	43	249	96	-1.78
XLOC_018422	Ankyrin repeat protein	12	3	65	16	-1.73
XLOC_043206	Late embryogenesis abundant protein LEA5	127	88	771	790	-1.71
XLOC_031220	Cysteine-rich repeat secretory protein 38-like	23	12	102	54	-1.65
XLOC_049742	Galactinol synthase	434	215	1,624	995	-1.64
XLOC_006648	cbl-interacting serine threonine-protein kinase 9-like	32	14	122	42	-1.63
XLOC_013822	LRR receptor-like serine threonine-protein kinase at2g24130-like	57	58	191	76	-1.59
XLOC_013725	Cyclic dof factor 3-like transcription factor	69	29	258	132	-1.58
XLOC_039767	Ovate family protein transcription factor	70	25	236	64	-1.56
XLOC_003515	LRR receptor-like serine threonine-protein kinase at1g06840-like	161	8	540	163	-1.54
XLOC_014835	Cryptochrome 1	71	17	260	105	-1.51

confirmed that samples could be separated by infection status along one of the first two principal components at 3, 9, and 12 DAI (Fig. 2). There was no clear separation of samples by infection status at 6 DAI (see below). Sequence descriptions, normalized counts, $\log_2$ FC estimates, and FDR adjusted *P* values for all genes on all dates are provided in Supplementary Files S3, S4, S5, and S6.

A total of 6,628 genes were differentially expressed (DE) between inoculated and control roots on at least one sampling date (Fig. 3; FDR = 0.01; $|\log_2\text{FC}| \geq 1$). The greatest number of DE genes (6,021) was observed early in the infection process on DAI 3. There were far fewer DE genes on later dates (Fig. 3). The magnitude of differential expression was also highest on DAI 3, when estimated $\log_2\text{FC}$ values for individual DE genes ranged from -5.24 to 8.75 . The fold changes of DE genes on subsequent dates were orders of magnitude smaller: $\log_2\text{FC}$ ranges were -3.38 to 4.11 (6 DAI), -4.05 to 4.06 (9 DAI), and -3.00 to 4.72 (12 DAI).

More genes were up-regulated than down-regulated in infected roots: 4,000 up-regulated versus 2,021 down-regulated (3 DAI), 773 versus 296 (9 DAI), and 458 versus 64 (12 DAI). The exception occurred on DAI 6, when only a small number of DE genes were identified, and more were down-regulated (72 versus 138). In a separate experiment, we observed a similar phenomenon during reniform nematode infection of cotton roots: high variability among samples and fewer DE genes at 6 DAI compared with 3, 9, and 12 DAI. This result may reflect the presence of multiple infection stages at 6 DAI (Fig. 1), a result we have documented histologically in both soybean and cotton.

Top differentially expressed genes. Tables 1 and 2 present the top 20 DE genes with the largest positive and negative $\log_2\text{FC}$ estimates on each sampling date. Many of the top 20 up-regulated genes on each date coded for nodulins, nodulation-specific proteins, and/or proteins with a documented role in nodulation (Table 1). This trend was most apparent at 3 DAI, when 16 of the top 20 up-regulated genes were known to be associated with nodulation. A total of 5, 8, and 11 of the top 20 up-regulated genes at 6, 9, and 12 DAI also had documented roles in nodulation. The magnitude of up-regulation for some nodulation-related genes was extremely high: at 3 DAI, $\log_2\text{FC}$ estimates for eight nodulation-related genes were greater than 8.0 (i.e., a 256-fold increase).

The top up-regulated gene list contained nodulin-16 (a membrane protein), nodulin-21 (an iron transporter), nodulin-41 (an aspartic protease), and nodulin-61 (a FltG glutamate-ammonia ligase). Other well-known nodulation genes included CERBERUS/LIN-1, the NODULE INCEPTION transcription factor NIN, the nodule-specific zinc transporter ZIP1, and a soybean homolog of ROOT DETERMINED NODULATION 1.

The top up-regulated gene list also contained multiple genes related to defense (defensin- and thaumatin-like proteins, endochitinase, and tmv resistance protein), stress and reactive oxygen detoxification (universal stress protein A, numerous peroxidases, and GDP-mannose-epimerase), modification and degradation of proteins (several proteases and ring-h2 finger proteins), and the metabolism of gibberellic acid (ent-copalyl diphosphate synthase and gibberellin 20 oxidase 1) and ethylene (1-aminocyclopropane-1-carboxylate oxidase).

Two strongly induced genes coded for transcription factors: NIN and a bHLH30-like transcription factor. In contrast, 12 transcription factors appeared on the top down-regulated gene list, three of them ethylene response factors (ERFs, Table 2). ERFs control plant responses to pathogens through the regulation of jasmonate (JA)/ethylene (ET) responsive defense genes (Huang et al. 2016); their down-regulation is consistent with nematode-induced suppression of the plant immune system. Also, strongly down-regulated were genes associated with jasmonic acid metabolism (12-oxophytodienoate reductase and cytochrome p450 94a1) and the transport and response to auxin (ATP binding cassette B family member 4, auxin-induced protein 5NG4, and cryptochrome 1).

Numerous cell wall genes were down-regulated, including those associated with lignin metabolism (laccases), hemicellulose

metabolism (glucanase 4- β -mannosyltransferase, mannan endo- β -1,4-mannosidase, and UDP-glucuronate:xylan α -glucuronosyltransferase), cell adhesion (fasciclin-like arabinogalactan proteins), and cell wall strengthening (extensins and hydroxyproline-rich glycoproteins).

Gene set enrichment analysis. In parallel with the identification of individual DE genes, GSEA was used to identify predefined gene sets whose expression differed between inoculated and control roots on each date (FDR = 0.05; Fig. 4). While DESeq2 identifies individual genes with large, statistically significant fold changes, GSEA identifies gene sets whose individual members show concordant, but potentially smaller, differences in expression between treatments (Subramanian et al. 2005). Five hundred and seven gene sets were significantly enriched or depleted in infected roots on at least one sampling date: 303 enriched in infected roots, 169 depleted, and 35 either enriched or depleted, depending on the date. The top 20 gene sets significantly enriched in infected roots on each date are presented in Table 3, and normalized enrichment scores for all gene sets are provided in Supplementary File S7.

Some enriched gene sets confirmed the results of the differential gene expression analysis. The gene set GO:0009877 (nodulation) was significantly enriched in infected roots on all sampling dates and was among the top 20 enriched gene sets at 6 and 9 DAI (Table 3). The related gene set GO:0009399 (nitrogen fixation) was also significantly enriched on all sampling dates. BLASTx results confirmed that all genes with these GO annotations were plant genes, most of them previously annotated in the soybean reference genome. None were of bacterial origin.

The gene set MAP00940 (phenylpropanoid biosynthesis) was significantly enriched in infected roots on all dates, and the related gene sets GO:0016210 (naringenin-chalcone synthase activity), MAP00943 (isoflavonoid biosynthesis), MAP00941 (flavonoid biosynthesis), and GO:0009813 (flavonoid biosynthetic process) were all significantly enriched at 3 DAI. While flavonoids in general have broad roles in plant development and defense, isoflavonoids such as naringenin serve as chemical messengers between legumes and their bacterial symbionts and have been shown to increase during nematode infection (Hutangura et al. 1999; Kennedy et al. 1999; Liu and Murray 2016).

By far the largest number of top enriched gene sets were related to DNA: its packaging, modification, and replication, as well as the accompanying process of mitosis (Table 3). Many of these sets were significantly enriched on at least three dates, including GO:0003678 (DNA helicase activity), GO:0006270 (DNA replication initiation), GO:0000786 (nucleosome), GO:0051567 (Histone H3-K9 methylation), GO:0042555 (MCM complex), and GO:0010389 (regulation of G2/M transition of mitotic cell cycle).

Enrichment at 3 DAI of gene sets related to large ribosomal subunits (GO:0015934), small ribosomal subunits (GO:0015935), and rRNA binding (GO:0019843) suggested that early stages of reniform nematode symbiosis were characterized by wholesale induction of translational machinery and de novo protein synthesis (Table 3). Enrichment of genes sets related to aerobic respiration (GO:0009060, GO:0005747, and GO:0004129) on multiple dates was consistent with enhanced energy demand and cellular respiration. Enrichment of gene sets related to pectin catabolism suggested that pectin degradation may occur in cell walls and middle lamellae during reniform nematode infection (EC:3.1.1.11, GO:0030599, and GO:0045490).

While pectin catabolism genes were enriched, far more cell wall-related gene sets were depleted. At 3 DAI, four gene sets related to 1,3-beta-glucan (callose) synthase activity were depleted in infected roots (Table 4). At 6 DAI, expression of multiple cell wall gene sets was depleted, including those associated with general wall properties (GO:0042546, GO:0071555), pectin synthesis (GO:004589, EC:2.4.1.43), hemicellulose synthesis (GO:0010417, GO:0010413), and lignin metabolism (EC:1.10.3.2, GO:0046274). Many of these same sets were enriched at 12 DAI, consistent with a switch from wall thinning and lysing in early infection to later wall thickening in mature feeding sites (Rebois et al. 1975).

Also depleted in infected roots were gene sets related to blue light signaling. These included phototropism (GO:0009638), blue light signaling pathway (GO:0009785), and blue light photoreceptor activity (GO:0009882). Inspection of the leading-edge genes from the depleted sets (i.e., the specific genes responsible for the depletion signal) showed that in all cases the signal was related to repression of four cryptochrome 1 genes (XLOC_009646, XLOC_014716, XLOC_034413, and XLOC_037027), two cryptochrome 2 genes (XLOC_039443 and XLOC_002396), and one phytochrome A gene (XLOC_025512). Down-regulation of these genes was most marked at 3 DAI but, in some cases, continued into later stages of syncytium formation.

Expression of nodulation-related genes. Of the 59 genes in the Nodulation (GO:0009877) gene set, 50 contributed to its leading edge on at least one date. Expression data for these and 81 additional nodulation-related genes identified from the assembled transcriptome are presented as clustered heat maps in Figure 5 (up-regulated genes) and Figure 6 (down-regulated and unchanged genes). The most pronounced differences in nodulation-related gene expression occurred early in the infection process at 3 DAI. Ninety-nine putative nodulation-related genes were significantly up-regulated at 3 DAI, compared with 6, 20, and 18 at 6, 9, and 12 DAI, respectively. Nineteen nodulation-related genes were down-regulated at 3 DAI compared with three, five, and zero at 6, 9, and 12 DAI.

Multiple genes that participate in the canonical signal transduction pathway of nodule initiation were strongly up-regulated in infected roots. These included the transcriptional activator CYCLOPS (up-regulated 5.5-fold at 3 DAI) and four soybean homologs of the NODULE INCEPTION (NIN) transcription factor to which CYCLOPS binds (up-regulated 6- to 188.7-fold at 3 DAI). Also induced were soybean homologs of the GRAS-family transcription factors NODULATION SIGNALING PATHWAY 1 (NSP1, up 3.5-fold) and NODULATION SIGNALING PATHWAY 2 (NSP2, up 10.3-fold), which bind the promoters of early nodulin genes. Components of signal transduction events associated with the autoregulation of nodulation (AON) program were also induced. These included a homolog of ROOT DETERMINED NODULATION 1 (RDN1, up 123.6-fold at 3 DAI) and five plant CLAVATA3/ESR family (CLE) proteins (up 2.7- to 8.3-fold at 3 DAI).

Additional nodulins and nodulation-related genes, many of which act downstream of the transcription factors above, were also induced by reniform nematode parasitism. These spanned a wide range of functions, from membrane transporters (NIP aquaglyceroporins, SWEET bidirectional sugar uniporters, vacuolar iron transporters, and a nodule-specific zinc transporter) to lipid raft components (remorins and flotillins) to proteins involved in remodeling of the extracellular matrix (pectate lyases and extensin-like/proline-rich proteins). Some

Fig. 4. Gene set enrichment plot for the GO:0009877 (nodulation) gene set at 9 days after inoculation (DAI). All expressed genes are ordered into a ranked list based on their relative expression in inoculated versus control samples. Genes at the beginning of the list (left) are up-regulated in inoculated roots; those at the end of the list (right) are down-regulated. The middle portion of the graphic shows where the 59 genes of the GO:0009877 gene set appear in the ranked list of all genes. The upper portion of the graphic illustrates the calculation of a running enrichment score for the GO:0009877 gene set. Its enrichment score peaks far to the left, indicating that genes from this set tend to be up-regulated in infected roots. The lower portion of the graph shows the value of each gene's ranking metric, a measure of its correlation with inoculation status.

were nodulins of unknown function that have been previously annotated only as peribacteroid membrane components (e.g., ENOD 55-2, nodulin-24, nodulin-20, and nodulin-16). Others were associated with cell cycle control and endoreduplication (cyclin-dependent kinase inhibitor 7, E2Fa, and FIZZY-RELATED 3). Of particular interest was the significant up-regulation of nodulation genes that have no obvious role in nematode parasitism, such as the three leghemoglobin genes induced 8.3- to 11.5-fold at 3 DAI. It is unclear whether these genes are required for reniform nematode parasitism, or whether their induction is incidental to nematode manipulation of high level regulatory programs also involved with nodulation.

A smaller number of putative nodulation-related genes were down-regulated during reniform nematode infection. Several were from families also present in the up-regulated gene set, such as remorins, SWEET sugar transporters, a CLAVATA/ESR (CLE) protein, and an RDN-1/o-arabinosyl transferase. Others were from families not present in the up-regulated gene list, and many of these had putative roles in defense. Five of the down-regulated nodulation-related genes coded for phospholipase D, which generates phosphatidic acid (PA) in response to bacterial Nod factors, and functions in perception and response to bacterial, fungal, and nematode pathogens (Zhao 2015). Two genes coding for nodulin-related protein 1, previously linked to

heat stress and defense signaling, were also significantly down-regulated during infection (Fu et al. 2010).

DISCUSSION

Reniform nematode infection begins when an immature female penetrates the root epidermis and cortex to reach an endodermal “initial cell.” There she inserts her stylet and releases a mixture of effectors, including both CLE and CEP peptide mimics (Eves-Van den Akker et al. 2016; Wubben et al. 2015). The initial cell and adjacent pericycle cells exhibit areas of cell wall lysing, which ultimately converge into full breaks, allowing multiple cytoplasms to coalesce into a single syncytium. Previous work suggests that feeding site formation is initiated by 2 to 3 DAI and that syncytia are well established by 6 to 9 DAI (Agudelo et al. 2005; Jones and Dropkin 1975; Rebois 1980; Rebois et al. 1975). Our histological results were consistent with this time course.

Uninfected root pericycle cells are dominated by a large central vacuole, with a relatively small amount of cytoplasm confined to the cell periphery. During feeding site formation, the central vacuoles of affected cells are disassembled and the numbers of ribosomes, mitochondria, plastids, and small vacuoles increase. Together with an increase in the size of endoplasmic reticula and nuclei, these changes

TABLE 3. Top 20 gene sets whose expression was significantly enriched in reniform nematode-infected soybean roots at four time points after inoculation (false discovery rate = 0.05; gene sets ranked by gene set enrichment analysis normalized enrichment score)

Gene set name	Gene set description	Number of genes in set	Type	Normalized enrichment score
3 days after inoculation (DAI)				
GO:0006334	Nucleosome assembly	97	BP	2.68
GO:0009853	Photorespiration	152	BP	2.50
GO:0008283	Cell proliferation	136	BP	2.47
GO:0006270	DNA replication initiation	67	BP	2.39
GO:0080129	Proteasome core complex assembly	114	BP	2.32
GO:0043086	Negative regulation of catalytic activity	222	BP	2.26
GO:0045490	Pectin catabolic process	145	BP	2.24
GO:0009060	Aerobic respiration	36	BP	2.24
GO:0000786	Nucleosome	114	CC	2.91
GO:0005747	Mitochondrial respiratory chain complex I	53	CC	2.42
GO:0015935	Small ribosomal subunit	61	CC	2.26
EC:3.1.1.11	Pectinesterase	205	EC	2.39
MAP00940	Phenylpropanoid biosynthesis	326	KEGG	2.31
GO:0003678	DNA helicase activity	47	MF	2.46
GO:0046982	Protein heterodimerization activity	204	MF	2.44
GO:0019843	rRNA binding	129	MF	2.44
GO:0030599	Pectinesterase activity	205	MF	2.40
GO:0004857	Enzyme inhibitor activity	177	MF	2.34
GO:0045330	Aspartyl esterase activity	123	MF	2.24
GO:0004601	Peroxidase activity	256	MF	2.24
6 DAI				
GO:0009399	Nitrogen fixation	29	BP	2.59
GO:0009877	Nodulation	59	BP	2.34
GO:0080129	Proteasome core complex assembly	114	BP	2.26
GO:0006542	Glutamine biosynthetic process	16	BP	2.11
GO:0006334	Nucleosome assembly	97	BP	2.11
GO:0051276	Chromosome organization	75	BP	2.10
GO:0009060	Aerobic respiration	36	BP	2.08
GO:0006189	De novo IMP biosynthetic process	23	BP	2.01
GO:0006511	Ubiquitin-dependent protein catabolic process	264	BP	1.93
GO:0009853	Photorespiration	152	BP	1.92
GO:0000786	Nucleosome	114	CC	2.32
GO:0005747	Mitochondrial respiratory chain complex I	53	CC	2.19
GO:0031012	Extracellular matrix	30	CC	1.86
EC:6.3.1.2	Glutamate-ammonia ligase	16	EC	2.10
EC:1.9.3.1	Cytochrome-c oxidase	25	EC	1.99
GO:0003678	DNA helicase activity	47	MF	2.14
GO:0004356	Glutamate-ammonia ligase activity	16	MF	2.05
GO:0019825	Oxygen binding	15	MF	2.03
GO:0004129	Cytochrome-c oxidase activity	25	MF	2.00
GO:0019843	rRNA binding	129	MF	1.89

(Continued on next page)

fill syncytial cells with a dense, organelle-rich cytoplasm (Agudelo et al. 2005; Rebois et al. 1975). The number of incorporated pericycle cells increases outwardly from the initial cell as the syncytium develops, ultimately encompassing 100 to 200 cells. Remaining portions of the syncytial cell walls thicken and may display in-growths and complex “boundary formations” (Agudelo et al. 2005; Rebois et al. 1975). Although the broad ultrastructural changes that accompany infection have been documented, relatively little is known about infection-related changes in plant gene expression.

General trends in gene expression during reniform nematode infection. The most substantial changes in gene expression occurred early in infection. A subset of genes was differentially expressed throughout the full 12-day time course, but the largest number of DE genes and the largest differences in gene expression were observed at 3 DAI.

Many DE genes and gene sets were consistent with ultrastructural changes previously reported during infection. Genes involved in cell wall synthesis and organization were suppressed early in infection when cell walls have been shown to lyse, then up-regulated in later stages when they have been reported to thicken and develop in-growths (Agudelo et al. 2005; Rebois et al. 1975). Likewise, genes and gene sets associated with aerobic respiration and ribosomal biogenesis were markedly up-regulated in infected roots, consistent with the documented proliferation of mitochondria and ribosomes in syncytial cells. It should be noted that we sampled whole infected roots, not syncytia alone. Gene expression changes therefore represent the aggregate response of the

root system as a whole, including but not limited to, the responses of syncytial cells.

Many genes and gene sets induced during infection had previously documented roles in rhizobial nodule formation. Nodulation-related genes are not unique to legumes, nor are they expressed only during nodulation (Denance et al. 2014). They have been most extensively studied in the context of legume nodulation, but they have homologs in nonlegume species and function in multiple developmental and symbiotic contexts. Many instances of overlap among genes involved in mycorrhization, nodulation, and nematode parasitism have been reported previously (reviewed in Mathesius 2003). For example, nodulin N-26, which encodes an aquaglyceroporin, is also induced during mycorrhizal infection, RKN gall formation, and reniform nematode parasitism (Albrecht et al. 1999; Favery et al. 2002). Likewise, the leucine-rich repeat (LRR) receptor kinase SYMRK/NORK is part of a receptor complex that functions in plant perception of rhizobia, mycorrhizal fungi, and RKN (Endre et al. 2002; Stracke et al. 2002; Weerasinghe et al. 2005). Nonetheless, the number and magnitude of differentially expressed nodulation-related genes observed during reniform nematode parasitism of soybean was unexpected.

Reniform nematodes may exploit aspects of the same signal exchange pathways used by rhizobia to communicate with their legume hosts. This possibility has previously been raised for the endoparasitic RKN *Meloidogyne incognita*, which possesses a horizontally acquired gene homologous to that of rhizobial nodL (Bellafiore et al. 2008; McCarter et al. 2003). In response to root-derived flavonoids, rhizobial nodL

TABLE 3. (Continued from previous page)

Gene set name	Gene set description	Number of genes in set	Type	Normalized enrichment score
9 DAI				
GO:0008283	Cell proliferation	136	BP	2.11
GO:0006270	DNA replication initiation	67	BP	2.10
GO:0010951	Negative regulation of endopeptidase activity	87	BP	2.07
GO:0007018	Microtubule-based movement	173	BP	2.06
GO:0000911	Cytokinesis by cell plate formation	117	BP	2.06
GO:0009399	Nitrogen fixation	29	BP	2.06
GO:0045490	Pectin catabolic process	145	BP	2.00
GO:0009877	Nodulation	59	BP	1.94
GO:0051567	Histone H3-K9 methylation	129	BP	1.93
GO:0000786	Nucleosome	114	CC	2.10
GO:0005871	Kinesin complex	171	CC	2.07
GO:0042555	MCM complex	15	CC	2.04
GO:0005819	Spindle	45	CC	1.99
EC:1.11.1.7	Peroxidase	256	EC	2.02
MAP00940	Phenylpropanoid biosynthesis	326	KEGG	2.11
MAP00360	Phenylalanine metabolism	364	KEGG	1.94
GO:0003678	DNA helicase activity	47	MF	2.44
GO:0003777	Microtubule motor activity	171	MF	2.07
GO:0008017	Microtubule binding	225	MF	2.04
GO:0004601	Peroxidase activity	256	MF	2.02
12 DAI				
GO:0007018	Microtubule-based movement	173	BP	2.77
GO:0000911	Cytokinesis by cell plate formation	117	BP	2.75
GO:0008283	Cell proliferation	136	BP	2.73
GO:0006275	Regulation of DNA replication	93	BP	2.68
GO:0051567	Histone H3-K9 methylation	129	BP	2.67
GO:0006270	DNA replication initiation	67	BP	2.63
GO:0006334	Nucleosome assembly	97	BP	2.46
GO:0006306	DNA methylation	148	BP	2.42
GO:0007067	Mitotic nuclear division	113	BP	2.40
GO:0010389	Regulation of G2/M transition	42	BP	2.40
GO:0045490	Pectin catabolic process	145	BP	2.32
GO:0006260	DNA replication	126	BP	2.29
GO:0016572	Histone phosphorylation	47	BP	2.28
GO:0005871	Kinesin complex	171	CC	2.78
GO:0000786	Nucleosome	114	CC	2.67
GO:0005874	Microtubule	281	CC	2.60
GO:0042555	MCM complex	15	CC	2.32
GO:0003777	Microtubule motor activity	171	MF	2.76
GO:0003678	DNA helicase activity	47	MF	2.75
GO:0008017	Microtubule binding	225	MF	2.67

promotes production of Nod factors that trigger nodule formation on host roots. It is not yet clear whether the *M. incognita* nodL gene promotes synthesis of nematode “Nod factors” that similarly trigger aspects of feeding site formation. Nonetheless, the wholesale induction of nodulation-related genes reported here suggests that reniform nematode may also modulate high-level regulators shared with the nodulation program.

Nodulation-related genes: Early signaling events and autoregulation of nodulation. Legumes signal to rhizobia through the secretion of specific flavonoids that promote synthesis and release

of bacterial Nod factors (Ferguson et al. 2010). In *L. japonica*, Nod factors diffuse to the root epidermis, where they bind to a receptor complex consisting of NFR1, NFR5, and SYMRK/NORK recruited by flotillin FLOT4 to lipid rafts (Madsen et al. 2003). Protein interactions and phosphorylations generate a signaling cascade that ultimately initiates a cytosolic calcium spike perceived by calcium/calmodulin-dependent protein kinase (CCaMK). CCaMK binds and phosphorylates the transcription factor CYCLOPS, which in turn induces expression of NIN, a transcriptional regulator of multiple nodulation genes including pectate lyase (Singh et al. 2014; Xie et al.

TABLE 4. Top 20 gene sets whose expression was significantly depleted in reniform nematode-infected soybean roots at four time points after inoculation (false discovery rate = 0.05; gene sets ranked by gene set enrichment analysis normalized enrichment score)^a

Gene set name	Description	Total genes in set	Type	Normalized enrichment score
3 days after inoculation (DAI)				
GO:0043531	ADP binding	380	MF	-2.86
GO:0004012	Phospholipid-translocating ATPase activity	34	MF	-2.55
GO:0045332	Phospholipid translocation	34	BP	-2.52
GO:0019829	Cation-transporting ATPase activity	60	MF	-2.51
EC:3.6.3.1	Phospholipid-translocating ATPase	34	EC	-2.50
GO:0000956	Nuclear-transcribed mRNA catabolic process	66	BP	-2.43
EC:2.4.1.34	1,3-Beta-glucan synthase	30	EC	-2.43
GO:0006075	1,3-Beta-D-glucan biosynthetic process	30	BP	-2.37
GO:0003843	1,3-Beta-D-glucan synthase activity	30	MF	-2.37
GO:0000148	1,3-Beta-D-glucan synthase complex	30	CC	-2.33
GO:0008026	ATP-dependent helicase activity	159	MF	-2.31
GO:0098655	Cation transmembrane transport	108	BP	-2.30
GO:0036459	Ubiquitinyl hydrolase activity	57	MF	-2.25
GO:0004386	Helicase activity	181	MF	-2.25
GO:0009638	Phototropism	18	BP	-2.24
GO:0010119	Regulation of stomatal movement	46	BP	-2.22
GO:0006487	Protein N-linked glycosylation	107	BP	-2.21
GO:0023014	Signal transduction by protein phosphorylation	60	BP	-2.17
GO:0009785	Blue light signaling pathway	25	BP	-2.17
EC:2.7.3	Phosphotransferases with a nitrogenous group as acceptor	55	EC	-2.16
6 DAI				
GO:0009834	Plant-type secondary cell wall biogenesis	46	BP	-2.52
EC:1.10.3.2	Laccase	58	EC	-2.38
GO:0052716	Hydroquinone:oxygen oxidoreductase activity	58	MF	-2.37
GO:0046274	Lignin catabolic process	58	BP	-2.36
GO:0010417	Glucuronoxylan biosynthetic process	22	BP	-2.29
GO:0010089	Xylem development	81	BP	-2.29
GO:0045489	Pectin biosynthetic process	26	BP	-2.19
GO:0042546	Cell wall biogenesis	49	BP	-2.15
GO:0010413	Glucuronoxylan metabolic process	138	BP	-2.13
GO:0009832	Plant-type cell wall biogenesis	94	BP	-2.12
GO:0045492	Xylan biosynthetic process	139	BP	-2.12
GO:0000139	Golgi membrane	207	CC	-2.11
GO:0018298	Protein-chromophore linkage	81	BP	-2.06
GO:0071555	Cell wall organization	362	BP	-2.06
EC:2.4.1.43	Polygalacturonate 4-alpha-galacturonosyltransferase	28	EC	-2.06
GO:0048519	Negative regulation of biological process	44	BP	-2.05
MAP00532	Glycosaminoglycan biosynthesis chondroitin sulfate	27	KEGG	-2.05
GO:0008361	Regulation of cell size	58	BP	-2.04
GO:0031225	Anchored component of membrane	97	CC	-2.03
GO:0010051	Xylem and phloem pattern formation	59	BP	-2.02
9 DAI				
GO:0043531	ADP binding	380	MF	-2.29
GO:0009785	Blue light signaling pathway	25	BP	-2.20
GO:0071702	Organic substance transport	20	BP	-1.98
GO:0044444	Cytoplasmic part	88	CC	-1.94
GO:0009882	Blue light photoreceptor activity	15	MF	-1.94
12 DAI				
GO:0043531	ADP binding	380	MF	-2.17
GO:0000956	Nuclear-transcribed mRNA catabolic process	66	BP	-2.03

^a On 9 and 12 DAI, fewer than 20 gene sets were significantly depleted.

Fig. 5. Clustered heatmap of log₂FC estimates for up-regulated nodulins and nodulation-related genes in soybean roots. Red and blue indicate up- and down-regulation in infected root tissues, respectively. Asterisks indicate significant differences in expression (adjusted *P* value of ≤0.01 and |log₂FC| ≥ 1). Sequence IDs for each gene in the assembled transcriptome are given in parentheses.

2012). GRAS family transcription factors NSP1 and NSP2 are also induced by the calcium spike and promote expression of early nodulation genes (Catoira et al. 2000; Oldroyd and Long 2003). Finally, CERBERUS, a putative E3 ubiquitin-protein ligase, is expressed early in nodule formation and appears to be required for proper infection thread development (Yano et al. 2009).

Soybean homologs of many genes described above were differentially expressed in response to reniform nematode infection, including FLOT4, CYCLOPS, NIN, pectate lyase, NSP1, NSP2, and CERBERUS (Fig. 5). Early events in reniform nematode infection appear to share components of the signal transduction pathways involved in perception of rhizobial Nod factors. Indeed, the evolution of these signal transduction pathways may predate both nematode parasitism and rhizobial nodulation, as a recent review suggests that nodulation arose through co-option of genes involved in the ancient mycorrhizal symbiosis (Suzaki et al. 2015).

Once formed, nodules can be costly to maintain. The extent of rhizobial nodulation is therefore limited through the autoregulation of nodulation (AON) program, a long-distance feedback loop in which NIN promotes synthesis of CLE signaling peptides that negatively regulate nodule number (Soyano et al. 2014, Suzaki et al. 2015). Also

involved in AON is the o-arabinosyl transferase, RDN1, which adds arabinose to CLE peptides (Kassaw et al. 2015).

Infection by reniform nematode was associated with induction of both NIN and RDN1, as well as up-regulation of five CLE peptides and down-regulation of one CLE peptide (Fig. 5). All CLE peptides were of plant origin and had been previously annotated in the soybean reference genome. Two CLE receptors, homologs of CALVATA1 and ACR4, were also down-regulated (Fig. 6).

This is not the first evidence for a role of CLE peptides in nematode parasitism: previous studies have documented the production of CLE peptide mimics by CN, RKN, and RN (Bakhtia et al. 2007; Guo et al. 2015; Patel et al. 2008; Wang et al. 2005; Wubben et al. 2015). Hg-SYV46 was the first nematode-derived CLE to be identified from the secretions of the soybean CN *Heterodera glycines* (Wang et al. 2005). Its role and that of other CLE mimics was subsequently established through RNAi and receptor knockout studies (Bakhtia et al. 2007; Guo et al. 2015; Patel et al. 2008). RKNs secrete the CLE-like peptide 16D10, and its RNAi silencing causes lower RKN infection (Huang et al. 2006a; Huang et al. 2006b). Recently, the presence of at least three CLE-motif genes has been demonstrated in the dorsal esophageal gland cell of sedentary reniform nematode females (Wubben et al. 2015). The present work suggests that the

Fig. 6. Clustered heatmap of log₂FC estimates for a combination of down-regulated and nondifferentially expressed nodulation-related genes in soybean roots. Red and blue indicate up- and down-regulation in infected root tissues, respectively. Asterisks indicate significant differences in expression (adjusted *P* value of ≤0.01 and |log₂FC| ≥ 1). Genes are a combination of those significantly down-regulated on at least one sampling date and genes that did not show significant differential expression but that were identified through leading-edge analysis or homology. XLOC_050155 showed mix regulation being down on 3 days after inoculation (DAI) and up on 12 DAI.

plant's own CLE peptides are also involved in the response to RN parasitism.

Other nodulation-related genes. Multiple transporter genes whose expression changed in response to reniform nematode also had documented roles nodulation: three nodulin 26-like intrinsic proteins (NIPs, a family of aquaporins), two vacuolar iron transporters, a nodule-specific zinc transporter and numerous sugar transporters (Fig. 5). Eight of these sugar transporters belonged to the SWEET (sugars will eventually be exported transporters) family of bidirectional sugar uniporters. The first SWEET gene to be identified was a nodulin from *Medicago truncatula* (MtN3), but SWEETs are known to function in a variety of processes, including phloem loading, nectar secretion, pollen nutrition, and seed filling (Eom et al. 2015; Gamas et al. 1996). Their up-regulation by bacterial and fungal pathogens is thought to promote the release of sugars into the apoplast during early infection stages (Chen et al. 2010).

In addition to SWEET sugar transporters, homologs of the sucrose-proton symporters SUT4 and SUC2 were also up-regulated. Both genes have previously been implicated in phloem loading and unloading (Truernit and Sauer 1995; Weise et al. 2000), and their expression has been documented in nodules and nodule-associated phloem (Complainville et al. 2003; Flemetakis et al. 2003). AtSUC2 expression has also been observed in syncytia induced by the sugar beet cyst nematode, *Heterodera schachtii* (Juergensen et al. 2003).

The construction of nodules and syncytia necessitates significant changes to cellular membrane systems. In nodules, these changes involve root hair curling, infection thread formation, and production of a plant-derived peribacteroid membrane (Ferguson et al. 2010). In reniform nematode feeding sites, syncytial cells disassemble their central vacuoles, expand the endoplasmic reticulum membrane system, and increase the number of membrane-bound organelles (Agudelo et al. 2005; Rebois et al. 1975). Multiple genes involved in lipid signaling, membrane trafficking, and membrane organization are up-regulated during both biotic interactions.

Four Sec14-like phosphatidylinositol transfer proteins with the GO term nodulation (GO:0009877) were up-regulated in infected roots (Fig. 5). Sec14-like proteins coordinate lipid signaling with membrane trafficking and facilitate the nonenzymatic transfer of phosphatidylinositols and phosphatidylcholine monomers between membrane bilayers (Ghosh and Bankaitis 2011). Sec14-nodulins were first identified in *L. japonicus* nodules and have been shown to influence polar membrane trafficking and root hair development in *Arabidopsis thaliana* (Ghosh et al. 2015). Two up-regulated soybean Sec14-like genes contained a GOLD (Golgi dynamics) domain. In *Arabidopsis*, such genes are termed patellins and are believed to function in vesicle and membrane trafficking, including cell plate formation during cytokinesis (Peiro et al. 2014; Peterman et al. 2004).

A large number of remorins were also differentially expressed in infected roots: nine up-regulated and four down-regulated (Figs. 5 and 6). While their functions are not fully understood, remorins appear to be associated almost exclusively with plasma membrane lipid rafts; several members of the family are strongly induced during nodulation in *M. truncatula* and *L. japonicus* (Lefebvre et al. 2010). One nodulation-related remorin is thought to function as a scaffolding protein, mediating spatial relationships among the core Nod factor receptor-like kinases NFP, LYK3, and DMI2 in *M. truncatula*.

Cell cycle and endoreduplication. During rhizobial infection, host cells undergo several rounds of endoreduplication, i.e., replication of the genome without mitosis or cytokinesis, leading to increased nuclear size (Maroti and Kondorosi 2014). The mitotic inhibitor CCS52 is required for this process. It activates the APC ubiquitin protein ligase that degrades mitotic cyclins, blocking the mitotic cell cycle and promoting endoreduplication (Engler and Gheysen 2013; Vinardell et al. 2003).

Reniform nematode infected roots showed up-regulation of two CCS52B homologs (FIZZY-RELATED 3) at 12 DAI (Fig. 5). At 3 DAI, a CCS52A homolog was up-regulated with an FDR-adjusted *P* value of ≤ 0.01 , although it did not meet our $\log_2FC \geq 1$ cutoff;

another CCS52A homolog was up-regulated with an FDR adjusted *P* value between 0.01 and 0.05. Also up-regulated at 3 DAI was the transcription factor E2Fa, which is required for endoreduplication (Engler and Gheysen 2013; Vinardell et al. 2003) and whose expression in the *Arabidopsis* pericycle controls lateral root initiation (Benkova and Bielach 2010).

The up-regulation of endoreduplication genes, enrichment of multiple DNA replication-related gene sets, and the increase in nuclear size previously observed in syncytial cells is consistent with endoreduplication in reniform nematode syncytia. This finding comports with studies of RKN and CN, both of whose feeding structures exhibit increased nuclear size, primarily due to endoreduplication (Edgar et al. 2014). Endoreduplication appears to be vital for successful RKN and CN feeding site formation, as a knockdown of CCS52 delays feeding site development and decreases reproduction for both nematodes (Vieira et al. 2013).

Genes associated with nitrogen fixation. While similarities in gene expression between nodulation and reniform nematode parasitism may reflect common developmental mechanisms, extensive nodule-related gene expression in parasitized roots may also be a by-product of nematode interference with nodulation signaling pathways. Some up-regulated nodulation-related genes may be "brought along for the ride" when transcription factors such as CYCLOPS, NIN, NSP1, and NSP2 are induced early in reniform nematode parasitism. Evidence for this hypothesis is provided by the induction of nitrogen fixation genes that have no obvious role in nematode parasitism. A case in point is the expression of three leghemoglobin genes up-regulated from 8.3-fold to 11.5-fold at 3 DAI. Leghemoglobins bind oxygen to protect nitrogenase and to manage the movement of oxygen for bacterial respiration (Ott et al. 2005). They have no documented function outside of nitrogen-fixing root nodules, and it is unclear what role they would play in the absence of nitrogenase or rhizobia.

Several lines of evidence suggest that rhizobial contamination was not responsible for changes in gene expression reported here. Surface-sterilized soybean seeds were produced in our lab, free from rhizobial inoculum, and soil was pasteurized prior to seedling transplant. Reniform nematode inoculum was prepared from a field that had been under a continuous nonlegume corn and cotton rotation for many years. Careful visual inspection and microscopy revealed no evidence of nodules or nodule primordia on any sample throughout an 18-day time course (Supplementary File S8). The very small percentage of Illumina reads that could be aligned to rhizobial genomes did not differ in magnitude from the percentage that could be aligned to genomes from *E. coli* and a variety of eukaryotic model organisms (Supplementary File S9). In no case did the small percentage of rhizobial-mappable reads differ between inoculated and control root samples. While some microbial RNA is inevitably sequenced in plant tissue transcriptomes, we found no evidence to suggest there was significant rhizobial contamination.

Conclusion. Our understanding of reniform nematode biology and feeding site formation is limited compared with that of other sedentary endoparasitic nematodes such as RKN and CN. Nonetheless, the overall patterns of gene expression reported here share features with transcriptomes from better-studied nematode–host interactions. Many genes affected by reniform nematode infection also share similarities with mutualistic belowground symbioses such as nodulation and mycorrhization, and it has been suggested that multiple belowground symbionts and parasites manipulate aspects of the lateral root organogenesis program to form symbiotic structures (Mathesius 2003). If an ultimate goal is nematode management, then the similarities among diverse belowground processes may be less important than their specific differences. A comparative approach to the transcriptomic changes induced by lateral root organogenesis, mycorrhization, nodule formation, and nematode parasitism will allow us to pinpoint how one organism induces formation of a beneficial nodule while another induces formation of a harmful feeding site. Breeding and genetic engineering efforts can be focused on disrupting molecular events that

are unique to nematode infection while avoiding pathways that function in normal organogenesis and beneficial symbioses.

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