Transcriptome Analyses of Pre-parasitic and Parasitic *Meloidogyne Chitwoodi* Race 1 to Identify Putative Effector Genes

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Abstract

*Meloidogyne chitwoodi* is a root-knot nematode that is a major pest of potato in the northwestern United States. Due to the lack of resistance against root-knot nematodes in potato, research has been undertaken to understand the *M. chitwoodi*-potato interaction at the molecular level. To identify the nematode genes that are playing roles in parasitism, we have performed transcriptome analyses on pre-parasitic and parasitic *M. chitwoodi* juveniles in susceptible potato. We compared gene expression profiles and identified genes that were significantly up- or down-regulated during nematode parasitism. Because parasitism proteins are typically secreted by the nematode to facilitate infection of host roots, we focused on the genes that encoded proteins that were predicted to be secreted. We found that approximately 34% (43/127) of the genes in the predicted secretome encoded proteins with no significant homology in the public genome databases, and 12% (15/127) encoded either a known effector, putative effectors or putative esophageal gland cell proteins. The transcriptome analyses of *M. chitwoodi* at the pre-parasitic and parasitic life stages shed light on the genes involved in nematode parasitism.

Keywords  
Differentially expressed genes, Host-parasitic relationship, Meloidogyne, Molecular biology, Transcriptome.

Root-knot nematodes are microscopic, endoparasitic roundworms that are a major limiting factor in the production of potatoes, which are the fourth most important food crop in the world (Birch et al., 2012; Lima et al., 2018). *Meloidogyne chitwoodi* (Golden et al., 1980) (Tylenchida: Meloidogynidae) is a root-knot nematode with limited worldwide distribution, and in the United States, *M. chitwoodi* is widely spread in the three states (Washington, Oregon, and Idaho) that produce over half of US potatoes (CABI/EPPO, 2012; Zasada et al., 2018).

*M. chitwoodi* second stage juveniles (J2s) hatch from the eggs and invade roots behind the root tip. They migrate intercellularly towards the differentiating vascular cylinder. In the case of tubers, the J2 will enter through wounds or lenticels. The nematode chooses 4 to 8 plant cells as feeding sites, called giant cells, which serve as the only source of nematode nutrition (Bird, 1961; Finley, 1981). Once feeding, the J2s become sedentary and molt three times into the adult life stage, and the females will lay eggs. Meanwhile, hyperplasia of surrounding root cells results in galling, which is a classic symptom of root-knot nematode infection. *M. chitwoodi* infected tubers develop galls on the skin. In addition to the galling, small dark spots appear in the tuber flesh surrounding the adult females. Tubers with 6 or more infection sites are regarded as culls (Pinkerton et al., 1986), and if as few as 5% to 15% of the tubers display these visual defects, the entire crop will be devalued or rejected (Ingham et al., 2000;
Ingham et al., 2007). Because there are no commercially available potato cultivars resistant to root-knot nematodes, conventional potato production relies heavily on chemical controls to manage nematodes (Ingham et al., 2000; King and Taberna, 2013). Although nematicides are effective against root-knot nematodes, there are concerns about the potential negative effects of these chemicals on human health and the environment (Porter et al., 2009). In order to reduce our reliance on nematicides and develop new forms of root-knot nematode resistance in potato, we must better our understanding of the plant-nematode interaction at the molecular level.

Because of their critical roles in nematode parasitism, there has been a lot of interest in identifying root-knot-nematode genes that are upregulated in expression during their parasitic life stages (Barcala et al., 2010; Caillaud et al., 2008; Dubreuil et al., 2007; Lee et al., 2019; Shukla et al., 2018). The nematode genes with roles in parasitism are referred to as ‘effector genes.’ Typically, effector genes encode secreted proteins with roles in host defense suppression and/or in feeding site formation (Vieira and Gleason, 2019).

One of the first steps in identifying effectors in root-knot nematodes is to identify genes that are expressed during the parasitic life stages. Transcriptome analysis of sedentary endoparasitic nematodes requires either the removal of the nematodes from the plant tissue prior to RNA sequencing (Kooliyottil et al., 2019) or dual RNA-seq (Petitot et al., 2016). The gene expression profiling was followed by a secreted protein prediction pipeline to identify putative *M. graminicola* effectors (Petitot et al., 2016). The dual RNA-seq approach has also been used to identify effectors from the cyst nematode *Heterodera schachtii* (Elashry et al., 2020), further emphasizing that dual RNA-sequencing is a useful tool in plant parasitic nematode effector gene discovery.

A recent publication looked at the expression of potato genes during *M. chitwoodi* race 1 infection (Bali et al., 2019). Here, our goal was to identify *M. chitwoodi* genes differentially expressed during potato parasitism by using dual RNA-seq. The list of identified genes was further narrowed by using bioinformatic tools to identify genes with putative secretion signals and no predicted transmembrane domains. These criteria are indicative of nematode genes that encode secreted proteins; this study lays the groundwork for the future identification and characterization of *M. chitwoodi* secreted effectors.

### Materials and Methods

#### Nematode cultures and inoculation of potato plants

The *Meloidogyne chitwoodi* isolate race 1 egg inoculum was initially provided by Dr. Charles Brown (USDA-ARS), and *M. chitwoodi* race 1 was maintained on the susceptible tomato *Solanum lycopersicum* cv. Rutgers under greenhouse conditions. To obtain nematode eggs from inoculated tomato plants, tomato roots were cut into small pieces and rinsed in a 0.6% sodium hypochlorite solution for 3 min. The solution was then passed through a set of sieves (pore size of 125, 45, 25 μm). Nematode eggs were collected on the 25 μm sieve and then further purified using sucrose floatation (Jacob and van Bezooijen, 1984). The eggs were suspended in 0.1% Plant Preservative Mixture (Plant Cell Technology) and incubated in a modified Baermann beaker (Figure S1) in darkness at room temperature. Hatched infective J2s were collected after 3 days of incubation.

For inoculation of potato plants with *M. chitwoodi*, three-week-old tissue culture Russet Burbank plantlets were transferred to 500-mL cone-tainers filled with sand and grown in growth chambers (14-hr light/10-hr darkness, 23°C) for 14 days. The Russet Burbank plants were then inoculated with 500 freshly hatched *M. chitwoodi* race 1 J2s. Root samples were harvested at 4-, 8- and 16-day-post-inoculation (dpi) and were stained with acid fuchsin solution (0.35% acid fuchsin, 25% acetic acid) to visualize nematodes within roots (Bybd et al., 1983) on a Nikon Eclipse Ti inverted microscope at ×40 magnification.

#### Sample preparation for RNA-seq experiment

For RNA-seq experiment, newly hatched *M. chitwoodi* race 1 J2s and galled potato root tissues at 8 dpi were collected, respectively. Three biological samples of *M. chitwoodi* race 1 J2s were also collected. Total nematode RNA was extracted using PureLink™ RNA Mini Kit (ThermoFisher). Following the inoculation of potato plants with 500 *M. chitwoodi* J2s (procedure described above), Russet Burbank roots were harvested at 8 dpi, and the infected roots were checked under a stereomicroscope (Zeiss zoom stereomicrope) to collect galled root tissues where nematodes resided. Galled root tissues from two individual potato plants were combined as one biological replicate, and three biological replicates were collected for 8 dpi samples. Total RNA was extracted from root samples using RNeasy Plant Mini Kit (QIAGEN, Germany).
quality total RNA (1-2 µg, 260/280 = 1.8-2.2, with RIN value of RNA of at least 8.5) was packed on dry ice and sent to Novogene Co. Ltd for library preparation and sequencing (30M reads per sample with PE150) using the Illumina platform.

RNA-seq data analysis

The raw sequencing data were trimmed using Trimmomatic-v0.38 to remove adapters and low-quality sequences (Bolger et al., 2014). The quality of trimmed sequences was checked using FastQC-v0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The trimmed sequences were evaluated according to the summary output of FastQC, and threshold of per base sequence quality was set as 20 by default. The trimmed data was analyzed using TopHat/2.1.1 to map to the M. chitwoodi race 1 draft genome (Bali et al., 2021). Mapped read counts were converted to tab-delimited text file (Trapnell et al., 2012). The differentially expressed genes (DEGs, adjusted p-value < 0.05) between M. chitwoodi J2s and at 8 dpi (n = 3 biological samples for each time point) were analyzed using the Cuffdiff.

Prediction of secreted proteins

We predicted secreted proteins from the M. chitwoodi race 1 genes up-regulated at 8 dpi based on the criteria: presences of N-terminus signal peptide and absence of transmembrane domains. First, the coding DNA sequences (CDS) of M. chitwoodi race 1 genes were predicted using the TransDecoder v5.1.0 with minimal length of deduced proteins set as 30 amino acids (Haas et al., 2013). The deduced protein sequences of up-regulated genes were used to predict presence of N-terminus signal peptide for secretion using the software SignalP version 5.0 (Almagro Armenteros et al., 2019; Nielsen, 2017). Proteins predicted to have Signal Peptide (Sec/SP1) likelihood above 0.6 were retained for further analyses. These protein sequences were then subjected to TMHMM v. 2.0 analysis, which predicts membrane proteins (Krogh et al., 2001). Proteins were classified as secreted proteins if they either had no predicted transmembrane domains or only had predicted transmembrane domains in the secretion signal sequence.

Real time PCR validation

For gene expression analysis, potato galled root tissues inoculated with 500 M. chitwoodi race 1 J2s were collected at 4-, 8- and 16-day-post-inoculation. Simultaneously, freshly hatched M. chitwoodi race 1 pre-parasitic J2s were also collected. Total RNA was extracted from galled root tissues and J2s separately and was treated with DNA-free™ DNA Removal Kit (ThermoFisher) to remove residual DNA. cDNA was synthesized using ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, USA) with oligo-dT primer. qRT-PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The qRT-PCR conditions were: 95°C for 3 min, 40 cycles of 95°C for 15 sec, 53°C for 15 sec and 72°C for 20 sec, and followed by a melting curve analysis from 65°C to 95°C with 0.5°C increased incrementally at 5 sec increments. Expression levels of genes of interest in nematode were normalized to the expression of M. chitwoodi housekeeping gene Internal transcribed spacer 2 (ITS2) rRNA. The relative expression levels of each target gene at 4-, 8- or 16-dpi were calculated by comparing with those in M. chitwoodi race 1 J2s using the 2−ΔΔCt method (Livak and Schmittgen, 2001). The primer pairs used for RT-qPCR are summarized in Table 1. Each qRT-PCR experiment consisted of two technical replicates per time-course of each gene, and the average of three biological replicates. Statistical tests were performed using GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

Differential gene expression of Meloidogyne chitwoodi during early infection stage in potato

Root-knot nematode effector genes critical for parasitism are typically up-regulated during early infection stages, when the nematodes enter the root, evade plant defenses, and form their giant cells. To identify M. chitwoodi genes expressed during early infection stages, we first established infection timepoints in our experimental system. We monitored nematode infections at 4-, 8- and 16-days post-inoculation (dpi) by staining the roots with acid fuchsin, allowing us to observe nematodes within the roots. At 4 dpi, J2 could be seen within the roots, indicating that root penetration had occurred. At 8 dpi, most nematodes within the roots were swollen, but still in the J2 stage. The swollen bodies indicated that the nematodes had begun to feed but had not yet molted to the next life stage. At 16 dpi, galls on the roots were clearly visible and the nematodes were in the globose J4 or young
female stage (Fig. 1). Based on our observations and experimental setup, at 4 dpi, the juveniles were still migrating through the roots, and by 8 dpi, the nematodes had settled and started to feed. Because we wanted to capture the J2 stage when initial feeding and giant cell formation occurs, we chose 8 dpi as the timepoint for the RNA-seq experiment.

Our next goal was to compare gene expression profiles between pre-parasitic and parasitic J2 to determine which nematode genes are differentially regulated during parasitism. We collected freshly hatched *M. chitwoodi* Race 1 J2 as the pre-parasitic J2 samples. To obtain the parasitic nematodes, Russet Burbank potato plants were inoculated with *M. chitwoodi* Race 1 J2 and galled root tissues were collected at 8 dpi. We collected whole galls to avoid isolating nematodes from the roots, which meant we had a mixture of plant and nematode tissue in the sample. Six cDNA libraries were constructed – three libraries of pre-parasitic J2 and three libraries of galls isolated at 8 dpi. The libraries were sequenced using an Illumina platform for paired end 150-bp reads, generating between 43 and 55 million raw read pairs for each library. The RNA-seq raw reads were pre-processed to remove low-quality bases and adapter sequences, resulting in between 40 and

### Table 1. Primers for qRT-PCR.

| Name              | 5′-3′                  |
|-------------------|------------------------|
| McITS2 forward    | GGGTCAAACCTTTGGCAGTCTGG|
| McITS2 reverse    | GCGGTTGATCTCTGACTGAGTTCG|
| Mc00773 forward  | AAGTTGCCGATAGCATTGCG    |
| Mc00773 reverse   | TCCGGAAGAGCATGACGAAT    |
| Mc03439 forward  | TGCAAGTTGCTGAGAGTTGTC   |
| Mc03439 reverse   | TGCATGTTGGAATAACCAAAGTG |
| Mc03992 forward  | TGATCGTTGCACTTCTGGACA   |
| Mc03992 reverse   | GACGAGTGCCTTGAACTTG     |
| Mc04677 forward  | CGTCACATCGAGAAAAATGCT   |
| Mc04677 reverse   | TCCACAGGGTCAACACTTCC    |
| Mc04921 forward  | TCTTGCAAGTGAGTTGTT      |
| Mc04921 reverse   | CGTTTCCGTGCAAGTTTCCAG   |
| Mc08895 forward  | TCTTTGCAAGTGAGTTG       |
| Mc08895 reverse   | TTTAATCCATTTACAAAAAGCTC |
| Mc10400 forward  | CAAGGAGGTGAAAAACCCG     |
| Mc10400 reverse   | GGGTCTTTGATTCACACCCG    |
| Mc01565 forward  | CAGCTGAATCTTCTGCACCA    |
| Mc01565 reverse   | AATAGCAACGGCTGGAGCTT     |
| Mc06869 forward  | CCACATCATCCTATCACTTCAAGT|
| Mc06869 reverse   | CCCATGGGCAAGTGAGACCC    |
| Mc08299 forward  | GAATTACGCCGCTTTCTTTGGT  |
| Mc08299 reverse   | TGCATACCTAGGCCACTCAT    |
| Mc07118 forward  | TGGTTGTTATGAACTTCTGCG   |
| Mc07118 reverse   | CCAGGTTTGTAGTAAAGGCAG   |
| Mc10102 forward  | TCCCATCATACTCTGGAGTC     |
| Mc10102 reverse   | ATGAAAAAGCACCAGCCCCT    |
52 million clean read pairs (Table 2). Interestingly, the GC content of individual libraries were different among samples: an average of 34% GC content for the *M. chitwoodi* J2 samples and 41% of GC content for the 8 dpi samples. The low GC content of the *M. chitwoodi* J2 transcripts corresponds to the low GC content of the *M. chitwoodi* and other root-knot nematode genomes (Koutsovoulos et al., 2020; Paganini et al., 2012). The higher GC content of the 8 dpi samples may be due to the mixture of plant and nematode reads at 8 dpi.

The differentially expressed genes (DEGs) of *M. chitwoodi* between J2 and 8 dpi were analyzed using the TopHat and Cufflinks/Cuffdiff pipeline (Trapnell et al., 2012). First, the RNA-seq read pairs were mapped to the *M. chitwoodi* race 1 genome using TopHat2. Mapping the reads from the J2 libraries generated 21 to 25 million mapped read pairs. Meanwhile, mapping reads from the 8dpi gall library resulted in 1.2 to 1.5 million mapped read pairs. By mapping to the *M. chitwoodi* race 1 genome, we removed the plant sequences from our dual RNA-seq experiment (Table 2). The reduction in the read pairs suggest that the majority of the reads from the dual sequencing were from potato. Next, we used Cufflinks to merge the information from all six libraries to assemble a *M. chitwoodi* transcriptome. DEGs between pre-parasitic and parasitic (8 dpi) J2 were determined by Cuffdiff. In the parasitic nematodes, we found 693 *M. chitwoodi* genes up-regulated (fold-change ≥ 4 and adjusted $P < 0.05$) and 1727 *M. chitwoodi* genes down-regulated (at least a 4-fold decrease and adjusted $P < 0.05$) (Supplementary Table 1, https://figshare.com/articles/dataset/Zhang_and_Gleason/16746286). To limit the number of genes in our subsequent analyses, we focused on genes encoding proteins of 30 or more amino acids. This left us with 679 genes up-regulated and 1570 genes down-regulated. A BLASTx-fast search of the NCBI non-redundant (nr) database found that of the 679 upregulated genes, 341 (50%) did not have any Blast hits (E value ≤ 1.0E-3) (Supplementary Table 2, Table 2. *Meloidogyne chitwoodi* RNA-seq libraries and mapping to genome.

| Library                | Bio-replicate | Read pairs | Read pairs mapped to genome | Mapping rate (%) |
|------------------------|---------------|------------|------------------------------|------------------|
| Pre-parasitic J2       | A             | 51 950 815 | 25 570 659                   | 49.60%           |
| Pre-parasitic J2       | B             | 43 883 143 | 21 477 892                   | 49.00%           |
| Pre-parasitic J2       | C             | 44 867 529 | 22 072 545                   | 49.20%           |
| Mc1-potato 8 dpi       | A             | 46 115 017 | 15 72 404                    | 3.40%            |
| Mc1-potato 8 dpi       | B             | 40 726 172 | 1 294 772                    | 3.20%            |
| Mc1-potato 8 dpi       | C             | 40 068 646 | 1 357 759                    | 3.40%            |
https://figshare.com/articles/dataset/Zhang_and_Gleason/16746286). Second level GO term analysis of the up-regulated genes indicate that sequences related to cellular, metabolic, and developmental processes were enriched during nematode parasitism (Fig. 2A). Of the 1570 down regulated genes, 408 (26%) did not have any Blast hits (E value ≤ 1.0E-3) (Supplementary Table 3, https://figshare.com/articles/dataset/Zhang_and_Gleason/16746286). Second level GO term analysis of the down-regulated genes indicate that

Figure 2: Gene Ontology (GO) term level 2 categories of genes for up (A) and down (B) regulated genes from *M. chitwoodi* parasitic stage compared to pre-parasitic J2. Bars show the number of sequences with annotations under each term. BP = biological processes, MP = molecular processes, CC = cellular function.
sequences related to cellular, signaling, and response to stimulus were enriched in pre-parasitic nematodes (Fig. 2B). When comparing genes expressed in the pre-parasitic J2 versus genes expressed in the parasitic nematode, we comprised a list of the top 20 most differentially expressed genes and their annotations from the BLASTx homology search (Table 3).

**Prediction of secreted proteins among *M. chitwoodi* genes up-regulated at early infection stage**

We hypothesized that nematode genes induced at early infection stages may be important for successful infection of host plants, therefore, we focused on the 679 genes that were significantly up-regulated at 8 dpi (Fig. 3, Supplementary Table 2, https://figshare.com/articles/dataset/Zhang_and_Gleason/16746286). We performed a bioinformatic analysis on the 679 *M. chitwoodi* genes up-regulated at 8 dpi to predict if the genes encode secreted proteins. The prediction of secretion was based on two criteria: the presence of a signal peptide at the N-terminus of the peptide sequence and the lack of any transmembrane domains. The SignalP 5 program predicted that 133 of the proteins contained a signal peptide at the N-terminus. Among these 133 sequences, 6 were excluded from further analysis because the TMHMM2 predictions indicated they contained one or more transmembrane domains within the protein sequence, minus the signal peptide sequence. Therefore, *in silico* analyses identified 127 *M. chitwoodi* genes (18.7% of the 679 up-regulated genes) encoding predicted secreted proteins (Supplemental Table 4, https://figshare.com/articles/dataset/Zhang_and_Gleason/16746286).

Using the sequence information for the 127 identified putative secreted proteins, we performed a Blastx search of the NCBI nr database. We found that 62.9% (80/127) did not have any BLAST hits (E value ≤ 1.0E-3)

**Table 3. Top 10 most differentially expressed genes in parasitic *M. chitwoodi* juveniles.**

| Log FC | Description                                                                 |
|-------|-----------------------------------------------------------------------------|
|       | **Up-regulated**                                                            |
| 1     | 12.2878 Putative cuticular collagen                                          |
| 2     | 10.8863 ---NA---                                                             |
| 3     | 10.6047 Putative esophageal gland cell secretory protein 14                  |
| 4     | 10.5679 ---NA---                                                             |
| 5     | 10.5294 Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial                |
| 6     | 9.2692 ---NA---                                                              |
| 7     | 9.21531 ---NA---                                                             |
| 8     | 9.21423 Nematode cuticle collagen domain protein                             |
| 9     | 9.1679 Saposin-like type B, 1 domain and Saposin B domain                   |
|       | and Saposin-like domain-containing protein                                   |
| 10    | 9.0144 ---NA---                                                              |
|       | **Down-regulated**                                                          |
| 1     | -11.012 C-type lectin domain-containing protein                             |
| 2     | -10.711 Mucin-like protein-1                                                 |
| 3     | -10.681 SCP domain-containing protein                                        |
| 4     | -10.443 ---NA---                                                             |
| 5     | -10.308 Beta-1,4-endoglucanase                                               |
| 6     | -10.234 C-type lectin domain-containing protein                             |
| 7     | -10.205 Mucin-like protein-1                                                 |
| 8     | -9.9796 ---NA---                                                             |
| 9     | -9.9755 ---NA---                                                             |
| 10    | -9.8201 Hypothetical protein Mgra_00005110, partial                         |
Meloidogyne chitwoodi transcriptome: Zhang and Gleason

Mc J2, 3 libraries

~ 23 M paired reads mapped per library

TopHat2

Mc-potato 8 dpi, 3 libraries

~ 1.4 M paired reads mapped per library

Cufflinks Cuffdiff

679 Mc genes up-regulated at 8 dpi, (FC ≥ 4)

SignalP 5.0 TMHMM 2.0

127 Mc genes encoding secreted proteins

FPKM ≥ 2 Highly up-regulated

11 genes

RT-qPCR at different life stages

6 genes upregulated in the early parasitic stages only

Future characterization of possible effector functions

Figure 3: Flowchart summary of RNA-seq analysis and prediction of Meloidogyne chitwoodi genes involved in parasitism. Bioinformatic and other tools are boxed. FC, fold change. FPKM, fragments per kilobase of transcript per million mapped reads.

Twelve of the genes (12/127) were annotated as “putative esophageal gland cell secretory protein” (Huang et al., 2003). Three known effectors were found in the annotations of the 127 M. chitwoodi genes, including VAP1, which was originally discovered in cyst nematodes as an effector that suppresses basal plant defense responses (Lozano-Torres et al., 2014). A gene encoding a chorismate mutase domain-containing protein was also identified in our in-silico M. chitwoodi secretome analysis. Root-knot nematode chorismate mutases may be altering salicylic acid levels within the host cells to suppress plant immune responses (Huang et al., 2005; Lambert et al., 1999; Wang et al., 2018). The third known effector found in our list of M. chitwoodi genes is homologous to the candidate secreted effector Minc11888, which was previously identified in M. incognita and is highly expressed in the parasitic juvenile stages (Nguyen et al., 2018).

Similarly, to assess protein homology, a BLASTp analysis of the 127 proteins using the nr database was performed. The analysis revealed that 66% (84/127) had significant hits (60% query coverage and E value ≤ 1.0E-3), to plant parasitic nematodes (Supplemental Table 5, https://figshare.com/articles/dataset/Zhang_and_Gleason/16746286). Interestingly, 53.5% (45/84) of those genes were hypothetical or unnamed proteins from the rice root-knot nematode (M. graminicola), which is a close relative to M. chitwoodi in the Meloidogynidae (Bert et al., 2008; Phan et al., 2020). We also found that 35/84 genes in our BLASTp analysis had top hits to genes encoding unnamed proteins in M. enterolobii, perhaps owing to the fact that M. enterolobii has one of the most complete genomes available for root-knot nematodes at this time (Koutsovoulos et al., 2020).

Real-time PCR validation on highly up-regulated genes

To validate our list of up-regulated genes, we selected 11 genes from the list of 127 that were highly up-regulated at 8 dpi (FC > 10 and FPKM ≥ 2, Table 4). We measured the expression of these 11 genes by quantitative reverse transcription polymerase chain reaction (RT-qPCR) in different nematode life stages: pre-parasitic J2 and parasitic nematodes in potato roots at 4, 8, and 16 dpi. Expression of 6 genes (Mc03439, Mc03992, Mc04677, Mc04921, Mc08895 and Mc10400) peaked at the early infection stages (4 or 8 dpi) and decreased at a later life stage, 16 dpi (Fig. 4). For the other genes, the expression levels were either similar across all infection stages (Mc01565, Mc06869, Mc08299) or highly expressed at the later infection stage, 16 dpi (Mc07118 and Mc10102) (Fig. 4). All 11 genes were confirmed to be expressed at higher levels at 8 dpi by RT-qPCR compared to the J2 stage, confirming our transcriptome data (Fig. 4). These results highlight the fidelity of dual RNA-seq analyses to identify differentially expressing genes.
Table 4. Information about the 11 *M. chitwoodi* genes analyzed by qRT-PCR at different life stages.

| Gene ID (aa) | BLASTx nr | BLASTx % query cover | BLASTx e-value | Predicted domains | FPKM J2 | FPKM 8 dpi |
|-------------|-----------|----------------------|----------------|-------------------|---------|-----------|
| Mc07118 (171) | *Meloidogyne graminicola* MSP18 mRNA, complete cds, Genbank: MK628546.1 | 84 | 7.00E-40 | N/A | 2 | 73.9 |
| Mc10102 (103) | Unnamed protein product [*Meloidogyne enterolobii*] Genbank: CAD2180454.1 | 79 | 4.00E-46 | Cytochrome b5, heme binding domain | 16.7 | 418.6 |
| Mc08299 (207) | Cathepsin B [*Meloidogyne graminicola*] Genbank KAF7630769.1 | 89 | 7.00E-45 | cysteine proteinase | 26.4 | 476 |
| Mc04921 (544) | Hypothetical protein Mgra_00005223 [*Meloidogyne graminicola*] Genbank KAF7635403.1 | 34 | 6.00E-74 | Thioredoxin-like fold | 2.9 | 50.4 |
| Mc06869 (63) | N/A | | | | 39.2 | 1704.9 |
| Mc03992 (306) | Hypothetical protein Mgra_00005223 [*Meloidogyne graminicola*] Genbank KAF76334147.1 | 49 | 3.20E-02 | N/A | 4.3 | 69.8 |
| Mc01565 (430) | Unnamed protein product [*Meloidogyne enterolobii*] Genbank: CAD2124200.1 | 18 | 2.00E-22 | N/A | 7.3 | 288.1 |
| Mc08895 (332) | Unnamed protein product [*Meloidogyne enterolobii*] Genbank: CAD2174631.1 | 32 | 5.00E-25 | N/A | 3.5 | 123.7 |
| Mc04677 (315) | Hypothetical protein Mgra_00005223 [*Meloidogyne graminicola*] Genbank KAF7635986.1 | 59 | 7.00E-54 | N/A | 2.3 | 51.8 |
| Mc03439 (79) | Hypothetical protein Mgra_00005223 [*Meloidogyne graminicola*] Genbank KAF7630992.1 | 97 | 1.00E-23 | N/A | 25.8 | 1319.5 |
| Mc10400 (130) | N/A | | | | 7.2 | 154.8 |

Discussion

Nematode success depends on the production and secretion of effectors. Therefore, effector identification is critical for understanding the compatible interaction with the host. The first step in effector identification is the discovery of differentially expressed genes during the nematode parasitic life stages. In a previous report, effector genes of *M. chitwoodi* were mined from an EST library constructed from *M. chitwoodi* eggs, J2s and females, and over three hundred predicted secreted effectors were identified (Roze et al., 2008). This study did not analyze gene expression specifically at the early parasitic life stages of the nematode, and as a result, the large list of genes in the secretome may include genes not related to parasitism. Because root-knot nematodes are not transformable, it would be a very labor-intensive task...
Figure 4: The expression level of selected *M. chitwoodi* genes in J2 and in parasitic nematodes in potato roots at 4, 8, and 16 dpi. McITS2 was used as the reference gene. The results show the fold change relative the juvenile stage, n = 3 ± SEM, *P* < 0.05 using two-tailed Student’s *t* test.
to try to functionally characterize over three hundred nematode genes. In this study we have compared the transcriptomic datasets between pre-parasitic and parasitic *M. chitwoodi* juveniles and generated the first comprehensive analysis of *M. chitwoodi* genes that are expressed during parasitism and that encode predicted secreted proteins. We have narrowed our list to 127 genes, of which the majority encode pioneer proteins. We focused on upregulated genes because we are interested in nematode effectors, which are secreted during infection to promote parasitism through the suppression of the host defense responses and/or the creation of the feeding site (Vieira and Gleason, 2019). Although the list of upregulated genes encoding secreted proteins is still relatively large, future experiments that would narrow this list further include performing additional gene expression analyses over a time course or finding transcripts that localize to the nematode secretory organs through in situ hybridization experiments.

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**References**

Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G. and Nielsen, H. 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nature Biotechnology 37:420–3.

Bali, S., Hu, S., Vining, K., Brown, C. R., Majtahedi, H., Zhang, L., Gleason, C. and Sathuvalli, V. 2021. Nematode Genome Announcement: Draft genome of *Meloidogyne chitwoodi*, an economically important pest of potato in the Pacific Northwest. Molecular Plant-Microbe Interactions March 29, doi: 10.1094/MPMI-12-20-0337-A.

Bali, S., Vining, K., Gleason, C., Majtahedi, H., Brown, C. R. and Sathuvalli, V. 2019. Transcriptome profiling of resistance response to *Meloidogyne chitwoodi* introgressed from wild species *Solanum bulbocastanum* into cultivated potato. BMC Genomics 20:907.

Barcala, M., García, A., Cabrera, J., Casson, S., Lindsey, K., Favery, B., García-Casado, G., Solano, R., Fenoll, C. and Escobar, C. 2010. Early transcriptomic events in microdissected Arabidopsis nematode-induced giant cells. The Plant Journal 61:698–712.

Bert, W., Leliatert, F., Vierstraete, A. R., Vanfeyerlen, J. R. and Borgonie, G. 2008. Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda: Rhabditida). Molecular Phylogenetics and Evolution 48:728–44.

Birch, P. R. J., Bryan, G., Fenton, B., Gilroy, E. M., Hein, I., Jones, J. T., Prashar, A., Taylor, M. A., Torrance, L. and Toth, I. K. 2012. Crops that feed the world 8: Potato: are the trends of increased global production sustainable? Food Security 4:477–508.

Bird, A. F. 1961. The ultrastructure and histochernistry of a nematode-induced giant cell. The Journal of Biophysical and Biochemical Cytology 11:701–15.

Bolger, A. M., Lohse, M. and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–20.

Bybd, D. W., Kirkpatrick, T. and Barker, K. R. 1983. An improved technique for clearing and staining plant tissues for detection of nematodes. Journal of Nematology 15:142–3.

CABI/EPPO. 2012. *Meloidogyne chitwoodi*. [Distribution map]. Distribution Maps of Plant Diseases, No. April, Wallingford: CABI, Map 803 (Edition 2).

Caillaud, M.- C., Dubreuil, G., Quentin, M., Perfus-Barbeoch, L., Lecomte, P., de Almeida Engler, J., Abad, P., Rosso, M.- N. and Favery, B. 2008. Root-knot nematodes manipulate plant cell functions during a compatible interaction. Journal of Plant Physiology 165:104–13.

Dubreuil, G., Magliano, M., Deleury, E., Abad, P. and Rosso, M. N. 2007. Transcriptome analysis of root-knot nematode functions induced in the early stages of parasitism. New Phytologist 176:426–36.

Elashry, A. M., Habash, S. S., Vijayapalani, P., Brocke-Ahmadinejad, N., Blümel, R., Seetharam, A., Schoof, H. and Grundler, F. M. W. 2020. Transcriptome and parasitome analysis of beet cyst nematode *Heterodera schachtii*. Scientific Reports 10:3315.

Finley, A. M. 1981. Histopathology of *Meloidogyne chitwoodi* (Golden et al.) on Russett Burbank Potato. Journal of Nematology 13:486–91.

Golden, A. M., Obannon, J. H., Santo, G. S. and Finley, A. M. 1980. Description and some observations of *Meloidogyne chitwoodi* n. sp. (Meloidogynidae), a root-knot nematode on potato in the Pacific Northwest. Journal of Nematology 12:319–27.

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowdern, J., Couger, M. B., Eccles, D., Li, B., Lieber, M., MacManes, M. D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C. N., Henschel, R., LeDuc, R. D., Friedman, N. and Regev, A. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols 8:1494–512.

Huang, G., Dong, R., Allen, R., Davis, E. L., Baum, T. J. and Hussey, R. S. 2005. Two chorismate mutase genes from the root-knot nematode *Meloidogyne incognita*. Molecular Plant Pathology 6:23–30.
Huang, G., Gao, B., Maier, T., Allen, R., Davis, E. L., Baum, T. J. and Hussey, R. S. 2003. A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode *Meloidogyne incognita*. Molecular Plant–Microbe Interactions 16:376–81.

Ingham, R. E., Hamm, P. B., Williams, R. E. and Swanson, W. H. 2000. Control of *Meloidogyne chitwoodi* in potato with fumigant and nonfumigant nematicides. Journal of Nematology 32:556–65.

Ingham, R. E., Hamm, P. B., Baune, M., David, N. L. and Wade, N. M. 2007. Control of *Meloidogyne chitwoodi* in potato with shank-injected metam sodium and other nematicides. Journal of Nematology 39:161–8.

Jacob, J. J. and van Bezooijen, J. 1984. Manual for Practical Work in Nematology. LH.

King, B. A. and Taberna, J. P. Jr 2013. Site-specific management of *Meloidogyne chitwoodi* in Idaho potatoes using 1,3-Dichloropropene; approach, experiences, and economics. Journal of Nematology 45:202–13.

Kooliyottil, R., Dandurand, L.-M., Kuhl, J. C., Caplan, A., Xiao, F., Mimee, B. and Lafond-Lapalme, J. 2019. Transcriptome analysis of *Globodera pallida* from the susceptible host *Solanium tuberosum* or the resistant plant *Solanus sisymbriifolium*. Scientific Reports 9:13256.

Koutsovoulos, G. D., Poulet, M., Elashry, A., Kozlowski, D. K. L., Sallet, E., Da Rocha, M., Perfus-Barbeoch, L., Martin–Jimenez, C., Frey, J. E., Ahrens, C. H., Kiewnick, S. and Danchin, E. G. J. 2020. Genome assembly and annotation of *Meloidogyne enterolobii*, an emerging parthenogenetic root-knot nematode. Scientific Data 7:324.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. Journal of Molecular Biology 305:567–80.

Lambert, K. N., Allen, K. D. and Sussex, I. M. 1999. Cloning and characterization of an esophageal-gland-specific chorismate mutase from the phytoparasitic nematode *Meloidogyne javanica*. Molecular Plant–Microbe Interactions 12:328–36.

Lee, I. H., Shim, D., Jeong, J. C., Sung, Y. W., Nam, K. J., Yang, J.-W., Ha, J., Lee, J. J. and Kim, Y.-H. 2019. Transcriptome analysis of root-knot nematode (*Meloidogyne incognita*)-resistant and susceptible sweetpotato cultivars. Planta 249:431–44.

Lima, F. S. O., Mattos, V. S., Silva, E. S., Carvalho, M. A. S., Teixeira, R. A., Silva, J. C. and Correa, V. R. 2018. “Nematodes affecting potato and sustainable practices for their Management”, In Yildiz, M. (Ed.), Potato - From Incas to All Over the World, IntechOpen, Available at: https://www.intechopen.com/chapters/59075.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. Methods 25:402–8.

Lozano-Torres, J. L., Wilbers, R. H., Warmerdam, S., Finkers-Tomczak, A., Diaz-Granados, A., van Schaik, C. C., Heider, J., Bakker, J., Goverse, A., Schots, A. and Smant, G. 2014. Apoplastic venom allergen-like proteins of cyst nematodes modulate the activation of basal plant innate immunity by cell surface receptors. PLoS Pathogens 10:e1004569.

Nguyen, C.-N., Perfus-Barbeoch, L., Quentin, M., Zhao, J., Magliano, M., Marteu, N., Da Rocha, M., Nottet, N., Abad, P. and Favery, B. 2018. A root-knot nematode small glycine and cysteine-rich secreted effector, MlSGCR1, is involved in plant parasitism. New Phytologist 217:687–99.

Nielsen, H. 2017. Predicting Secretory Proteins with SignalP Methods in Molecular Biology 1611:59–73.

Paganini, J., Campan-Fournier, A., Da Rocha, M., Gouret, P., Pontarotti, P., Wajnberg, E., Abad, P. and Danchin, E. G. J. 2012. Contribution of lateral gene transfers to the genome composition and parasitic capacity of root-knot nematodes. PLoS ONE 7:e50875.

Petitot, A. S., Dereeper, A., Agbessi, M., Da Silva, C., Guy, J., Ardisson, M. and Fernandez, D. 2016. Dual RNA-seq reveals *Meloidogyne graminicola* transcriptome and candidate effectors during the interaction with rice plants. Molecular Plant Pathology 17:860–74.

Phan, N. T., Orjuela, J., Danchin, E. G. J., Klopp, C., Perfus-Barbeoch, L., Koutsovoulos, G. D., Lopez-Roques, C., Bouchez, O., Zahm, M., Besnard, G. and Bellafiore, S. 2020. Genome structure and content of the rice root-knot nematode (*Meloidogyne graminicola*). Ecology and Evolution 10:11006–21.

Pinkerton, J. N., Santo, G. S., Ponti, R. P. and Wilson, J. S. 1986. Control of *Meloidogyne chitwoodi* in commercially grown Russet Burbank potatoes. Plant Disease 70:860–3.

Porter, I., Banks, J., Mattner, S. and Fraser, P. 2009. “Global phaseout of methyl bromide under the Montreal protocol: implications for bioprotection, biosecurity and the ozone layer”, In Gisi, U., Chet, I. and Gullino, M. L. (Eds), Recent Developments in Management of Plant Diseases, Springer Netherlands, Dordrecht, pp. 293–309.

Roze, E., Hanse, B., Mitreva, M., Vanholme, B., Bakker, J. and Smart, G. 2008. Mining the secretome of the root-knot nematode *Meloidogyne chitwoodi* for candidate parasitism genes. Molecular Plant Pathology 9:1–10.

Shukla, N., Yadav, R., Kaur, P., Rasmussen, S., Goel, S., Agarwal, M., Jagannath, A., Gupta, R. and Kumar, A. 2018. Transcriptome analysis of root-knot nematode (*Meloidogyne incognita*)-infected tomato (*Solanum lycopersicum*) roots reveals complex gene expression profiles and metabolic networks of both host and nematode during susceptible and resistance responses. Molecular Plant Pathology 19:615–33.

Trappell, C., Roberts, A., Goff, L., Pertieta, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L. and Pachtet, L. 2012. Differential gene and transcript
expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols 7:562–78.

Vieira, P. and Gleason, C. 2019. Plant-parasitic nematode effectors—insights into their diversity and new tools for their identification. Current Opinion in Plant Biology 50:37–43.

Wang, X., Xue, B., Dai, J., Qin, X., Liu, L., Chi, Y., Jones, J. T. and Li, H. 2018. A novel Meloidogyne incognita chorismate mutase effector suppresses plant immunity by manipulating the salicylic acid pathway and functions mainly during the early stages of nematode parasitism. Plant Pathology 67:1436–48.

Westermann, A. J., Gorski, S. A. and Vogel, J. 2012. Dual RNA-seq of pathogen and host. Nature Reviews Microbiology 10:618–30.

Zasada, I. A., Dandurand, L. M., Gleason, C., Hagerty, C. H. and Ingham, R. E. 2018. “Plant Parasitic Nematodes of the Pacific Northwest: Idaho”, In S. S. and C. J. (Eds), Oregon and Washington, Plant Parasitic Nematodes in Sustainable Agriculture of North America, Springer, Cham.

Figure S1: Modified Baermann funnel apparatus diagram. The modified Baermann funnel apparatus uses a 100 mL beaker instead of the traditional funnel. Mesh is placed into the mouth of the beaker and up to 4 layers of Kimwipes are placed on the mesh. Nematode eggs (or soil) are placed onto the paper and immersed in water. After three days, the paper and mesh are removed and the nematodes sitting at the bottom of the beaker are collected. The modified apparatus allows for J2 collection from small volumes of soil or isolated eggs.