1-2013

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MPMI Vol. 26, No. 1, 2013, pp. 31–35. http://dx.doi.org/10.1094/MPMI-05-12-0121-FI.

e-Xtra*

TECHNICAL ADVANCE

Isolation of Whole Esophageal Gland Cells from Plant-Parasitic Nematodes for Transcriptome Analyses and Effector Identification

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Submitted 18 May 2012. Accepted 24 July 2012.

Esophageal glands of plant-parasitic nematodes are highly specialized cells whose gene expression products include secreted effector proteins, which govern nematode parasitism of host plants. Therefore, elucidating the transcriptomes of esophageal glands with the goal of identifying nematode effectors is a promising avenue to understanding nematode parasitism and its evolutionary origins as well as to devising nematode control strategies. We have developed a method to separate and isolate individual esophageal gland cells from multiple species of plant-parasitic nematodes while preserving RNA quality. We have used such isolated gland cells for transcriptome analysis via high-throughput DNA sequencing. This method relies on the differential histochemical staining of the gland cells after homogenization of phytonematode tissues. Total RNA was extracted from whole gland cells isolated from eight different plant-parasitic nematode species. To validate this approach, the isolated RNA from three plant-parasitic nematode species—*Globodera rostochiensis*, *Pratylenchus penetrans*, and *Radopholus similis*—was amplified, gel purified, and used for 454 sequencing. We obtained 456,801 total reads with an average read length of 409 bp. Sequence analyses revealed the presence of homologs of previously known nematode effectors in these libraries, thus validating our approach. These data provide compelling evidence that this technical advance can be used to relatively easily and expediently discover effector repertoires of plant-parasitic nematodes.

Plant-parasitic nematodes have evolved complex parasitic relationships with their host plants to obtain nutrients that are required for nematode development and reproduction. Parasitic strategies can be relatively simple, as in the case of some migratory nematodes that withdraw cytoplasm contents directly from mostly unaltered plant cells. On the other hand, sedentary nematodes have evolved more sophisticated parasitic relationships with host plants, in which they induce normal plant cells to develop into highly modified feeding sites. In both migratory as well as sedentary plant-parasitic nematodes, plant–parasite interactions are hugely influenced and mediated by so-called effectors, proteins synthesized in the esophageal glands and secreted through the nematode stylet into the plant (Davis et al. 2004; Hussey 1989).

The stylet and the very large esophageal secretory glands are the most visible and obvious adaptations of plant-parasitic nematodes for plant parasitism (Hussey 1989; Hussey and Mims 1990). The stylet is a hollow needle-like structure adapted to penetrate plant cell walls and inject gland secretions (effectors) into parasitized host plants and cells, and also to withdraw nutrients from the cytoplasm. The esophageal glands, one dorsal and two subventral cells in tylenchid and aphelenchid nematodes and a basal bulb in dorylaimid nematodes, are the main source of effectors involved in plant parasitism. These gland cells are thought to have evolved from early bacterial-feeding nematode precursors, enlarging considerably and containing ever more complex transcripts coding for comprehensive arrays of nematode effectors which, in turn, enabled the ability of nematodes to parasitize higher plants (Hussey et al. 2002). The morphological changes between the dorsal and subventral gland cells over time and at different life stages indicate that the activities of these glands change as the life cycle progresses. In the highly evolved sedentary tylenchid nematodes such as cyst (*Heterodera* and *Globodera* spp.) and root-knot (*Meloidogyne* spp.) nematodes, the subventral gland cells are most active in infective second-stage juveniles during early infection, in which penetration, migration, and initiation of the feeding sites occur, and then atrophy in the subsequent sedentary developmental nematode stages. In contrast, the dorsal gland is primarily active during these latter parasitic stages, during which feeding site development and maintenance as well as the main feeding cycles take place.

The identification of complete effector panels of plant-parasitic nematode taxa followed by functional characterization of effectors is a key to understanding nematode parasitism of plants, and such an understanding is of interest for many reasons. For one, understanding nematode parasitism at the molecular level is a promising avenue for the development of sustainable control measures in agricultural systems, because many of these plant-parasitic nematodes are major pests of crops. Furthermore, it is of high practical and academic interest to not only uncover all effectors of a species but also identify the differences among populations of a species, particularly when such effector differences correlate with phenotypic traits such as nematode virulence and host range. Similarly, uncovering the effector panels of a wide range of plant-parasitic nematode taxa will be an invaluable asset in deciphering the evolution of plant parasitism and the nuances between the

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different modes of parasitism. Maybe most intriguingly, such studies would allow unparalleled high-resolution views on the intricacies of horizontal gene transfers that led to the acquisition of certain effectors (Danchin et al. 2010).

Different technical approaches have been used to identify nematode effector genes that are expressed exclusively in the esophageal gland cells of plant-parasitic nematodes (Davis et al. 2004). Initially, only a few nematode effectors were identified, mostly by comparing gene expression between specific nematode life stages or by analyzing expressed sequence tags (EST) generated from whole nematodes (Dautova et al. 2001; Jaouannet et al. 2012; Popeijus et al. 2000). However, generating EST from microaspirated cytoplasm of the esophageal gland cell region has proven to be a straightforward approach to isolate nematode effector genes (Gao et al. 2003; Huang et al. 2003).

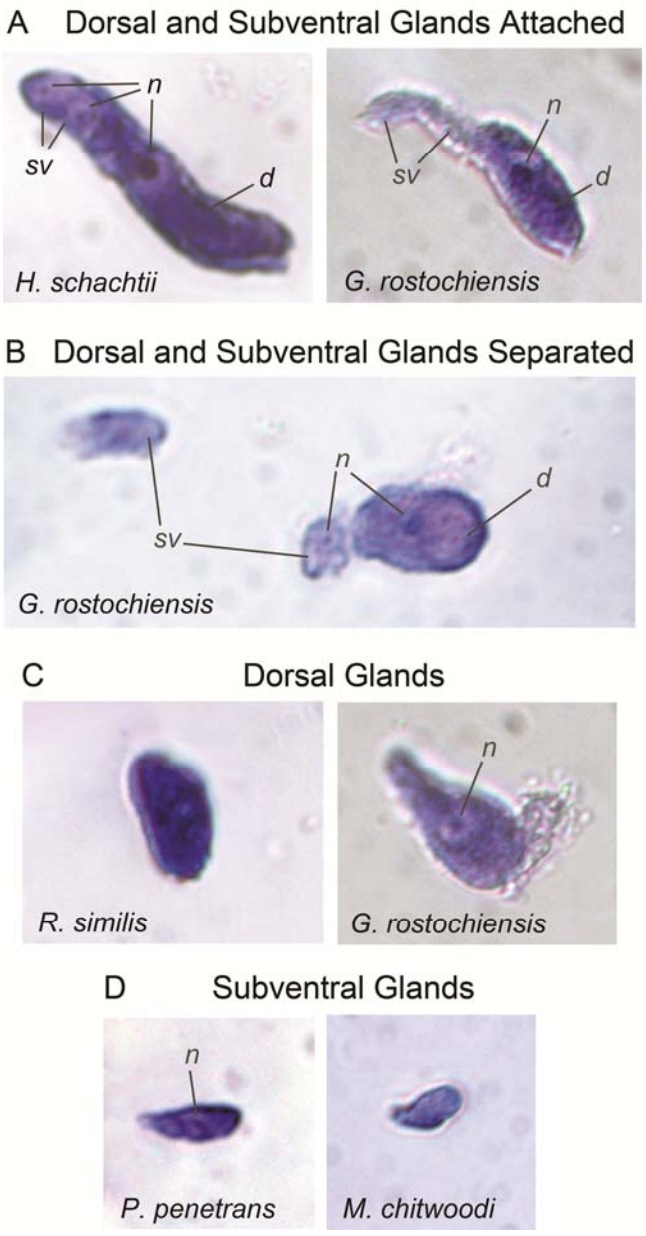

Fig. 1. Stained and purified esophageal gland cells. These cells are unambiguously identified by their shape, their large nuclei (n), and the differential purple stain. Dorsal (d) and subventral (sv) gland cells **A,** frequently are attached to each other but **B,** can easily be separated using micromanipulations. Although, in this study, we collected gland cell pools containing both cell types, our method also allows isolation of **C,** dorsal gland cells as well as **D,** subventral gland cells in different pools.

Analysis of cDNA libraries generated using this technically challenging approach resulted in the breakthrough identification of significant numbers of nematode effectors and provided the first inclusive images of nematode effector genes from *Meloidogyne incognita* and *Heterodera glycines*. Despite this method's many advantages, it is very labor intensive and subject to possible contamination with cell types surrounding the esophageal glands. Nevertheless, these advances showed that elucidation of esophageal gland transcriptomes coupled with bioinformatics mining will allow the identification of effector panels.

In this article, we report a relatively easy method that will allow the performance of esophageal gland transcriptomics of many, if not all, plant-parasitic nematodes and their life stages. We are introducing a method to directly isolate whole esophageal gland cells, extracting and amplifying their mRNA and conducting transcriptomic analyses. From our initial transcriptomic studies of three plant-parasitic nematode species, we see evidence of newly discovered transcripts with homology to previously reported plant-parasitic nematode effectors. Additionally, there are many novel effector candidates among the obtained sequences.

RESULTS AND DISCUSSION

In order to test the feasibility of our new method, we successfully subjected mixed parasitic stages of eight plant-parasitic tylenchid nematode species to our gland isolation procedure and RNA extraction: *Ditylenchus destructor, Globodera rostochiensis,* a *Helicotylenchus* sp.*, H. schachtii, M. chitwoodi, Pratylenchus penetrans, Radopholus similis*, and *Rotylenchulus reniformis*. Whereas *D. destructor*, the *Helicotylenchus* sp., *P. penetrans*, and *Radopholus similis* are migratory endoparasitic nematodes that remain vermiform throughout their life cycles, *G. rostochiensis, H. schachtii, M. chitwoodi, and Rotylenchulus reniformis* are sedentary endoparasites whose sedentary life stages grow into swollen body shapes. We did not observe any difficulties in applying our gland isolation technique to all selected nematodes, which indicates that this technique should be applicable uniformly to tylenchid nematodes. In addition, we have successfully purified glands from two members of the order Aphelenchida (A*phelenchoides fragariae* and *Bursaphelenchus xylophilus*). Although we have not yet attempted RNA extraction from these samples, it appears likely that these nematodes also can be subjected to the method introduced here.

From the eight tylenchid gland preparations of roughly 100 glands each, we extracted RNA and experimented with different RNA amplification protocols. We managed to extract good-quality RNA from six samples with a yield of 10 to 25 ng per 100 glands. Isolated glands from *H. schachtii* and the *Helicotylenchus* sp., which did not produce high-quality RNA, had been stored for 8 weeks at –20°C prior to RNA isolation, which likely was the reason for low RNA quality, because nematodes for the other six samples had been stored for considerably shorter periods (2 to 4 weeks). Furthermore, we identified an RNA amplification protocol that yielded sufficient high-quality materials for next-generation sequencing using 454 technology. This optimized approach allowed the sequence analyses of *G. rostochiensis, P. penetrans,* and *Radopholus similis* as a pilot study to validate our protocol. Individual experimental steps are outlined in the paragraphs below and described in more experimental detail below.

Plant-parasitic nematode disruption and gland cell liberation.

The method to quickly isolate the esophageal gland cells from a wide range of plant-parasitic nematode species at various developmental stages relies on the quick disruption of the

nematode cuticle and release of nematode tissues and cells into an anhydrous environment. We established that 100% ethanol works well for this purpose and protects the RNA of the esophageal gland cells from degradation. We established a nematode disruption method similar to the one we described for in situ hybridization (de Boer et al. 1998) (i.e., via mechanical cutting with a single-edge razor blade). However, unlike with in situ hybridization, nematodes were cut unfixed in phosphate-buffered saline supplemented with an RNAse inhibitor to protect cellular RNA during the cutting and prior to the transfer to ethanol. Cutting efficiency was improved by agitating the slide carrying the nematodes with a conventional laboratory vortex while moving the razor blade across the surface of the slide. Additionally, we found that a greater number of individual gland cells could be obtained when a vortexing step was added after transferring cut nematodes to ethanol. Optimally, cut nematode tissues were vortexed for 3 min in 100% ethanol before storage at –80°C for at least 24 h. The resulting nematode-ethanol mixture is referred to as a nematode homogenate.

Esophageal gland cell isolation.

A critical step in the process was being able to differentiate esophageal gland cells from other cell types in the homogenate. We used several distinct characteristics of esophageal gland cells to achieve this goal. First, esophageal gland cells are among the largest individual cells in the developing nematodes and possess a large, distinct nucleus in a teardrop-shaped cellular body. Second, the esophageal gland cells are cytoplasmically very dense. We exploited this latter trait by differentially staining these cells using a histochemical stain (HistoGene). The physical characteristics of the gland cells combined with their differential staining are essential for the unambiguous detection of esophageal gland cells in a mixture of cell types in the nematode homogenate.

HistoGene stain was originally designed to stain tissues being prepared for laser-capture microdissection that are subsequently used for RNA isolation (Mikulowska-Mennis et al. 2002; Pietersen et al. 2009). Although HistoGene stain yields results similar to the histochemical stains hematoxylin and eosin, the advantages of using HistoGene are the ability to quickly penetrate tissues and to protect nucleic acids from degradation. A variety of purified esophageal gland cells stained with HistoGene are shown in Figure 1, where teardrop-shaped cells containing purple cytoplasm and large, darkly stained nuclei can be seen. The hematoxylin-like component of the stain is largely responsible for the coloration, because it is a basic dye that binds to the highly acidic nucleic acid-enriched cytoplasm of the esophageal gland cells. This staining is a common pattern observed with the esophageal gland cells of all plant-parasitic nematode species studied. Both dorsal and subventral glands stain similarly and can often be seen attached to each other. Interestingly, both gland cell types can be routinely separated via gentle vibration of the microaspiration needle that is used to isolate the gland cells (Fig. 1).

Stained nematode homogenates were washed once with 100% ethanol and then mixed with halocarbon oil, a nonaqueous substrate commonly used in *Caenorhabditis elegans* microinjection to stabilize the tissue and protect RNA from degradation during collection of esophageal gland cells using micromanipulation equipment (discussed below). At least 100 dorsal and subventral esophageal gland cell combinations for each species were successfully collected.

RNA isolation from minimal quantities of starting material.

Total RNA was isolated from each of the collected gland cell pools using the Arcturus PicoPure RNA isolation kit (Applied Biosystems, Carlsbad, CA, U.S.A.) and quantified. The PicoPure kit was chosen because it was developed for use with very low-input quantities of tissues or cells, and works in conjunction with the Arcturus HistoGene stain, which originally was sold as part of a kit for staining frozen, sectioned tissue prepped for laser-capture microdissection (HistoGene LCM Frozen section staining kit). As expected, initial total RNA yield was low, ranging between 10 and 25 ng for a pool of approximately 100 gland cells.

RNA amplification for sequence analysis.

To generate sufficient RNA for next-generation sequencing, we experimented with two different downstream procedures, one including a gel purification step and the other omitting this step. Only the inclusion of the gel purification allowed meaningful sequence generation, and this protocol is described here. The three RNA pools that were used for this successful procedure (*P. penetrans, G. rostochiensis*, and *R. similis*) were amplified using Epicentre's RiboMultiplier Sense-RNA amplification kit. This kit was chosen because it is described as providing an excellent 3′/5′ ratio and less 3′ bias when compared with other RNA amplification kits. Because nematode effectors contain N-terminal signal peptides that direct the proteins into the secretory pathway, obtaining sequence reads with 5′ ends will facilitate the identification of effector gene candidates. After amplification, we obtained up to 10 µg of total RNA for each of the three species subjected to this approach. The amplified RNA was then purified on a 5% polyacrylamide gel, and the RNA fraction larger than 300 nucleotides was eluted overnight, precipitated, and quantified. Final approximate total RNA yield was between 1.5 and 4 µg after purification. RNA pools were assessed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA, U.S.A.) using an RNA 6000 Pico chip and found to be of good quality (Supplementary Fig. S1).

Gland library sequencing and analysis.

Amplified gland RNA from each of the three plant-parasitic nematodes was submitted for sequencing library construction and sequencing via the Roche Genome Sequencer FLX, using Titanium chemistry at the University of Iowa Sequencing Facility. The sequencing run yielded 456,801 total reads, with an average read length of 409 bp. Total reads were broken down by nematode library, and local blast databases were generated from the single-pass sequences of each library. Previously identified effectors from *H. glycines* and *M. incognita* were blasted against each of the three newly generated data sets to give an estimation of coverage of the new gland cell sequence pools. Results of this search are shown in Table 1. We identified homologs of effector genes coding for plant cell-wall-degrading enzymes, including cellulase genes, which have been found in a relatively large number of plant-parasitic nematode species to date. These effectors are used by plant-parasitic nematodes to facilitate penetration and migration through the root system during the early stages of infection (Davis et al. 2004). The presence of these common effector transcripts in our sequence reads provided an indication that our sequence data set is valid and representative of a library generated from esophageal gland cells. Also, the presence of the known nematode effector venom allergen-like proteins and chitinases in all three studied species further validated our methodology and also suggested that these proteins are likely to play a general role in parasitism in plant-parasitic nematodes. Other nematode effector candidates were found in one or more species, suggesting the presence of a common suite of genes involved in parasitism in multiple genera. Continued analysis is underway to

identify potential novel effectors in our sequence data set but the rediscovery of a number of known nematode effector types in our sequence pools proves the feasibility of this method to unravel esophageal gland transcriptomes.

Conclusion.

In this study, we have demonstrated the ability to isolate, quickly identify, and collect individual esophageal gland cells from various plant-parasitic nematodes in a simple and efficient manner. This approach provided sufficient RNA to construct cDNA pools for high-throughput sequencing. Homologs of previously known nematode effectors were detected in these libraries and more detailed sequence analyses are expected to establish the entire set of effector molecules in these nematodes. The new method described here is easy and will facilitate access to a new set of sequence data that will provide unique opportunities for understanding the mechanisms of parasitism types, host range, and, ultimately, the evolution of parasitism in Aphelenchida and Tylenchida and, potentially, all plant-parasitic nematodes.

MATERIALS AND METHODS

Nematode purification.

The tested nematode species were isolated using common plant-parasitic nematode extraction procedures. For the migratory endoparasitic species, we employed the use of a Baermann funnel to isolate mixed parasitic stages from infected plant materials (Hooper et al. 2005). For the sedentary endoparasitic species, mixed parasitic stages were isolated by macerating infected roots in a blender followed by sieving and separation on a sucrose gradient (de Boer et al. 1996).

Plant-parasitic nematode gland cell isolation.

Pooled nematodes were suspended in phosphate-buffered saline (pH 7.4) and mixed with SUPERase-In (Life Technologies, Grand Island, NY, U.S.A.) at a final concentration of 1 U/µl to inactivate native RNase activity upon disruption of nematode bodies. Small volumes of each pool (approximately 150 µl each) were aliquotted directly onto baked glass slides and mechanically disrupted by cutting with a sterile razor blade. Cutting was assisted by resting the slide on top of the rubber head of a bench-top vortex (Vortex Genie 2; Thermo Fisher, Waltham, MA, U.S.A.) and allowing the blade to contact the slide as the vortex speed was slowly increased to medium speed. Cutting progress was monitored with a dissecting microscope and, when nematodes were cut into approximate eighths, the cut nematodes were washed into a 50-ml centrifuge tube with RNA-grade 100% ethanol. The process was repeated until each pool was exhausted. Ethanol was added to 25 ml and each tube was vortexed for 3 min at full speed to further disrupt nematode tissues and to dislodge esophageal gland cells. Centrifuge tubes were transferred to a –80°C freezer and nematode homogenates were fixed in ethanol for at least 24 h before proceeding.

Esophageal gland cell staining and micromanipulation.

Fixed nematode homogenates stored at –80°C were centrifuged in a swinging bucket rotor for 3 min at 600 × *g* to pellet the nematode tissue. The ethanol was decanted and the tissue pellet with residual ethanol was transferred to a 1.5-ml microfuge tube by pipetting. The tissue was centrifuged again in a swinging bucket rotor to remove the remainder of ethanol. Then, 100 µl of HistoGene stain (Applied Biosystems), supplemented with Superase-In at 1 U/µl, was added and the pellet resuspended. Staining was allowed to progress for 1 min at room temperature, and then 1 ml of 100% ethanol was added to stop the staining. Tubes were centrifuged in a swinging bucket rotor for 1 min at 600 × *g* to pellet the stained tissue. Supernatants were removed and pellets washed once with 100% ethanol. A final centrifugation to pellet the stained tissues was performed, the supernatants were removed, and 300 µl of Halocarbon Oil 700 (Sigma-Aldrich, St. Louis) was added. The tissues suspended in halocarbon oil were transferred onto glass slides of coverslip thickness previously baked at 180°C for 4 h. Slides were mounted onto the stage of a Zeiss Axiovert 100 (Carl Zeiss, Thornwood, NY, U.S.A.) inverted compound microscope. A glass needle holder attached to a micromanipulator, in line with a Cell Tram (Eppendorf, Hauppauge, NY, U.S.A.) oil-filled piston, was affixed to the microscope. A borosilicate glass needle, previously pulled to an opening of approximately 40 to 50 µm with a needle puller, was inserted into the needle holder. The Cell Tram was used to generate a

Table 1. Sequence verification and validation of gland pools[a]

Effector accession number	Highest hit annotation	*Pratylenchus penetrans*	*Globodera rostochiensis*	*Radopholus similis*
AY028639	Venom allergen-like protein	5.00E-26	2.00E-31	6.00E-34
HQ123260	Ran-binding protein	…	2.00E-13	…
AF468679	Chitinase	2.00E-09	4.00E-25	6.00E-22
AF473826	Pioneer	…	…	1.00E-09
AF469059	Annexin	…	1.00E-18	…
AY043224	β-1,4-endoglucanase-4	1.00E-13	2.00E-64	2.00E-64
AF500024	SKP1-like protein	9.00E-26	…	…
AF502391	Zinc finger, C3HC4 type	…	4.00E-06	…
AF500017	Pioneer	…	…	8.00E-12
AF490244	Pioneer	…	7.00E-17	…
AF469055	β-1,4-endoglucanase-5	5.00E-09	5.00E-32	2.00E-49
AF520566	Pectate lyase	9.00E-24	1.00E-61	4.00E-26
AF490251	Pioneer	…	5.00E-81	1.00E-15
AY101191	β-1,4-endoglucanase-2	1.00E-14	3.00E-23	8.00E-83
AF520565	Chorismate mutase	9.00E-25	1.00E-41	…
AF531161	Pioneer	…	…	9.00E-09
AF531170	Pioneer	9.00E-13	…	…
AY134436	Pioneer	…	4.00E-101	…
AY134440	Histidine acid phosphatase	4.00E-13	…	6.00E-41
AF527788	Pectate lyase	1.00E-27	4.00E-10	1.00E-33
AF049139	Cellulose binding protein	…	2.00E-07	2.00E-11

[a] E-value scores for each species represent the highest significant alignment to the specified effector, when a hit was present. Scores were calculated by a TBLASTN search against 96 known effector sequences previously isolated from *Heterodera glycines* and *Meloidogyne incognita* gland cell content, and directed toward local BLAST databases created from single-pass sequences for each of the three plant-parasitic nematode species indicated.

negative pressure on the needle which, in turn, was used to remove individual gland cells from the halocarbon oil into the glass needle. Pools of collected cells for each nematode species were deposited into 1.5-ml microcentrifuge tubes and stored at –80°C until RNA isolation.

Esophageal gland cell RNA isolation and amplification.

Total RNA was extracted using the PicoPure RNA isolation kit (Applied Biosystems) with slight modifications. Individual halocarbon oil-containing gland cell preparations were mixed with mineral oil (Sigma-Aldrich) at a 1:5 ratio and centrifuged for 1 min at 10,600 × *g* to decrease the density of the gland-cell-containing solution below that of the extraction buffer (XB). A higher ratio of halocarbon oil could be used to prevent the separation of the mixture into two phases. Then, 100 µl of XB was added to the mixture, and gland cells were resuspended by gently pipetting and finally centrifuged at 10,600 × *g* for 1 min to separate the oil and the XB containing the RNA into two phases. The tubes were then incubated at 42°C for 30 min and the XB containing RNA (lower phase) was transferred to a new tube. The samples were centrifuged at 3,000 × *g* for 2 min and the supernatant was transferred into a new tube. Samples were stored at –80°C and then used for the subsequent RNA isolation step, as described in the kit. Isolated total RNA was amplified using the RiboMultiplier Sense-RNA amplification kit (Epicentre, Madison, WI, U.S.A.) following the manufacturer's instructions. We obtained up to 10 µg of amplified RNA, using between 10 and 25 ng of total RNA as input. The amplified RNAs were then fractionated on a 5% polyacrylamide gel to select for RNA molecules larger than 300 nucleotides. Gel slices containing RNA molecules larger than 300 bp were eluted overnight in 0.3M NaCl, purified using 0.2-µm filters (Thermo Fisher), precipitated in 3× volume of 100% ethanol for at least 4 h, and resuspended in 20 µl of RNAse-free water. The quality and concentration of RNA were verified using an Agilent Bioanalyzer 2100 RNA 6000 Pico chip.

ACKNOWLEDGMENTS

We thank P. Agudelo of Clemson University, Clemson, SC, U.S.A. for kindly hosting T. Maier and providing access to her plant-parasitic nematode cultures; and X. Wang of Cornell University, Ithaca, NY, U.S.A., M. Wubben of the Agricultural Research Service at Mississippi State University, Mississippi State, and A. Elling of Washington State University, Pullman for providing access to and preparing nematode homogenate from *G. rostochiensis*, *R. reniformis*, and *M. chitwoodi*, respectively.

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