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Assessment of *Globodera pallida* RNA Extracted from *Solanum* Roots

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Abstract: The introduction of high-throughput sequencing technologies has made transcriptome analyses of plant–pathogen interactions almost routine. Nevertheless, it is still challenging to obtain RNA from populations made up of two species. An RNA extraction method that worked well on free-living *Caenorhabditis elegans* failed when applied to isolated *Globodera pallida* J2 larva. Furthermore, alternative protocols that extracted RNA from free-living J2 larva produced less satisfactory results once the animals entered their hosts' roots. We have compared several extraction procedures to ascertain whether a single protocol was capable of recovering high-quality, high-molecular-weight RNA from newly hatched J2 larva as well as from larva embedded in roots of both potatoes (*Solanum tuberosum* L. cv. Desiree) and a very distantly related species, *Solanum sisymbriifolium*. Although it was possible to recover large amounts of RNA from J2 larvae using Proteinase K treatments, this protocol failed to yield high-quality nematode RNA from infected roots. By comparison, mechanical disruption procedures yielded lower amounts of RNA from infected roots, but what was recovered was of higher quality. We conclude that different extraction protocols need to be developed to sample mixed populations of organisms.

Key words: Globodera pallida, molecular biology, RNA extraction, *Solanum tuberosum*, technique.

Improved sequencing technologies have provided researchers with a means to monitor genome-wide changes in the transcriptomes of many previously intractable biological systems. However, while high-throughput sequencing and sequence annotation have now become more routine, it can still be challenging to extract RNA from species living closely intertwined in plant–parasite associations.

One example of the kind of problems researchers face when working with mixed systems is the isolation of RNA from parasitic nematodes such as *Globodera* spp. after they have invaded host plant roots. These nematodes emerge from their eggs as a J2 larva with an unbroken cuticle (Forrest et al., 1989) built up from three differently organized layers of collagen and other extracellular matrix proteins (Spiegel and McClure, 1995; Gray et al., 2001). This cuticle is in turn enclosed in a glycocalix composed of mucins and glycolipids (Davies and Curtis, 2011). On the other hand, the walls of their plant hosts are porous matrices of crystalline cellulose embedded in an interwoven framework of lignins, pectins, hemicellulose, and glycosylated structural proteins (Baron-Epel et al., 1988; Hansen et al., 2011). Cell disruption procedures that are sufficient against isolated animals release RNA inefficiently when parts of the nematodes are completely surrounded by their plant host.

The host–parasite symbiosis presents a secondary problem, because the number of J2 larvae infecting roots is generally low under natural conditions (estimated at 19.4–38.6 animals/m root. http://www.iaea.org/inis/collection/NCLCollectionStore/_Public/30/023/30023912.pdf) so that the amount of nematode RNA is greatly diluted by extracted plant RNA unless tedious microdissection procedures are applied to separate the two (Cotton et al., 2014). A single analysis depending on 100 ng RNA would require carefully isolating 0.13 to 0.17 mg of nematodes (Elling et al., 2007), or approximately 250 animals (http://plpnemweb.ucdavis.edu/nemaplex/Ecology/nematode_weights.htm). Labor costs would then increase with each treatment and replicate that the transcriptome would require to be truly representative of messenger RNA in the animal.

Rather than focusing on new ways to separate nematodes from roots, we have compared several different RNA extraction procedures and evolved a consensus protocol that is able to efficiently and reliably disrupt *Globodera* larvae in situ. Increasing the yield of high-molecular-weight RNA from both the parasite and the host compensated for the low abundance of nematode messenger RNA in the combined transcript population. To verify the robustness of the protocol, we tested its use on nematode-infected roots from two different plants, the common potato, *Solanum tuberosum*, and a distantly related species, *Solanum sisymbriifolium*, also known as Litchi Tomato or Sticky Nightshade. Both attract *Globodera pallida*; however, the parasite's life cycle proceeds only in *S. tuberosum* (Sasaki-Crawley et al., 2010; Sasaki-Crawley et al., 2012). We were able to detect *G. pallida* actin sequences from as little as 7.2 ng cDNA prepared from infected roots.

Materials and Methods

Plant cultivation: Sterile *S. tuberosum* and *S. sisymbriifolium* were vegetatively propagated in 120-ml baby food jars containing standard Murishige and Skoog (MS) salts, pH 5.6, 3% sucrose, 0.7% agar, 100 μg/ml myo-inositol, 2.0 μg/ml glycine, 1 μg/ml thiamine, 0.5 μg/ml pyridoxine, and 0.5 μg/ml nicotinic acid. Plants were maintained at 25°C in light for 16 hr, and subcultured vegetatively every 4 wk. The roots grown in this environment

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were used for initial evaluation of RNA extraction protocols; however, experiments showed that this concentration of MS salts and agar reduced nematode infection. To circumvent this, plants were removed from this medium 10 to 14 d prior to the experiment and roots were trimmed before plants were transferred into infection medium prepared with 0.4% Phytogel and half-strength rice hydroponic medium (Yoshida et al., 1976), referred to here as Fake Field, (1× strength: 12 mM H_2SO_4, 1.4 mM NH_4NO_3, 325 mM $NaH_2PO_4.2H_2O$, 513 μM K_2SO_4, 1 μM $CaCl_2$, 1.6 mM $MgSO_4.7H_2O$, 10 μM $MnCl_2.4H_2O$, 75 nM $(NH_4)_6.Mo_7O_{24}.4H_2O$, 19 μM H_3BO_3, 150 nM $ZnSO_4.7H_2O$, 150 nM $CuSo_4.5H_2O$, 36 nM $FeCl_3.6H_2O$, and 71 μM citric acid monohydrate) at pH 6.4.

Preparation of Globodera stocks: Nematode cysts were collected from greenhouse grown *S. tuberosum* cv Russet Burbank. Two populations of cysts were extracted from the soil 16 wk postinoculation. Once dried, they were placed at 4°C in November and December of 2013 for 1 yr postharvest to provide time to complete diapause (Hominick et al., 1985). Between 200 and 300 cysts were hydrated for 3 d in a six-well tissue-culture plate in 3 ml of sterile water augmented with 0.2 mg/ml nystatin. Cysts were then transferred into 96-well plates with each well containing between 1 and 10 cysts and 150 μl of potato root diffusate augmented with 0.05 mg/ml nystatin and left to hatch for 7 to 21 d at 4°C in the dark (Clarke and Perry, 1977).

Potato root diffusate was made by growing *S. tuberosum* for 4 wk in the greenhouse in 6-inch pots with 1,500 g of soil made to a ratio of 2:1 sand to loam. Each pot was fertilized with All Purpose 20–20–20 fertilizer (JR Peters Inc, Allentown, PA) three times per week for 3 wk followed by a 4th wk without fertilizer. After the 4th wk, the soil was allowed to dry out for 14 d. The diffusate was then made by adding 200 ml of water to each pot and the flow-through was collected. This was filter sterilized and stored for up to 1.5 yr at −20°C.

Caenorhabditis elegans culture: Caenorhabditis elegans were grown for 6 to 12 d on Nematode Growth Medium (NGM) lite agar (EJ Lambie, personnel communication, as described in Schweinsberg and Grant, 2005) that had been seeded 24 hr previously with a culture of OP50 *Escherichia coli*. The nematodes were washed from the plates with distilled water, collected in micro centrifuge tubes, centrifuged at 5,000 rpm for 5 min, and then washed twice more with sterile distilled water. The animals were resuspended in 1 ml sterile distilled water and the number of animals was determined by microscopy. The nematodes needed for each RNA extraction were withdrawn, pelleted, and residual liquid was aspirated.

Preparation of juvenile G.pallida for plant infections and RNA extraction: The majority of *G. pallida* eggs hatched after 10- to 14-d treatment with potato root diffusate. Hatched juveniles were collected into micro centrifuge tubes and centrifuged at 5,000 rpm for 5 min. The pellet was rinsed twice with sterile distilled water. After the final wash, animals were suspended in sterile water and an aliquot was removed and examined microscopically to determine concentration. These juveniles were either directly used for inoculation or pelleted again for RNA extraction.

Plant infection with Globodera: Solanum tuberosum and *S. sisymbriifolium* with sufficient root masses at the end of the 14-d period of growth in Phytogel medium were inoculated with 500 to 550 infective juveniles delivered into the medium of each box via a 200-μl pipet tip. The infected plants were incubated for 3 d at 25°C in light for 16 hr. The roots were then extricated from the boxes by inverting the plant over a container of water and carefully dislodging the gel with a spatula. The gelling material was gently removed from the roots without damaging the delicate root tips. The roots were dipped into the water several times to rinse off any remaining gelling material and gently blotted with paper towels. Once blotted, the mass of roots from each plant was cut from the stem into individual lengths and distributed into groups of 100 to 150 mg. Prior to extractions, several plants were set aside to assay for infection (Bybd et al., 1983). The roots from these plants were washed to release any of the gelling material, and then incubated in 5% sodium hypochlorite (NaOCl) for 5 min. After rinsing three times in distilled water, they were drained and covered with a solution consisting of 4.2 ml acid Fuchsin stain (3 g Fuchsin in 1 liter of 25% acetic acid) dissolved in 145.8 ml distilled water, and brought to a boil. Once cooled to room temperature, the roots were drained of the stain, covered with acidified glycerol (10 mM HCl and 70% glycerol), and examined microscopically for attached or embedded nematodes.

RNA extraction from G. pallida: Nematodes to be used for RNA extraction were pelleted from potato diffusate solution and washed twice with sterile water. After the remaining liquid was aspirated, 200 μl of Proteinase K solution (100 μg/ml Proteinase K in 1% s*odium dodecyl sulfate* (SDS), 50 mM Tris pH 7.5, and 10 mM ethylenediaminetetraacetic acid (EDTA) pH8 brought to 5% β-mercaptoethanol immediately prior to use) was added to the pellet. The tube was incubated at 50°C for 12 min followed by visual confirmation of worm lysis. Five hundred microliters of RNAzolRT (Molecular Research Center, Inc. (MRC), Cincinnati, OH) was added and incubated for 15 min at room temperature followed by centrifugation at 13,000*g* for 12 min. Approximately 660 μl was transferred to a new tube and precipitated by mixing sufficient freshly made 75% ethanol to bring the final solution to 30% ethanol. Tubes were then incubated at room temperature for 45 min. The tube was centrifuged at maximum speed (21,000*g*) for 8 min and washed twice with 75% ethanol. After a final centrifugation and aspiration to remove any residual liquid, 15 μl nuclease-free water (Ambion Thermo-Fisher,

Waltham, MA) was added. The nucleic acid concentration was first determined using a Thermo NanoDrop 2000C (Thermo-Fisher) spectrophotometer and then the quality was assessed using an Agilent 2200 Bioanalyzer TapeStation (Agilent, Santa Clara, CA). The correlation between the number of J2 larvae and RNA yield (ng) was analyzed using XLSTAT linear regression (version 2015.5; Addinsoft, New York, NY). During the course of this work, we found that the quality of RNA extracted from nematodes stored at −80°C declined relative to the RNA extracted from fresh tissues.

RNA extraction from C. elegans: This extraction was performed according to MRC using RNAzolRT with the following modifications. Four hundred microliters of RNAzol was added to the *C. elegans* pellet and vortexed for 20 min. (groups.molbiosci.northwestern.edu). One hundred and sixty microliters of sterile distilled water were added, incubated for 15 min, and then centrifuged at 13,000*g* for 15 min. Approximately 75% of the volume was transferred to a new tube, 75% ethanol was added to bring the solution to 30% ethanol and incubated for 30 min at room temperature. After 30 min, the tube was centrifuged at 12,000*g* for 8 min followed by aspiration and two washes with 75% ethanol. After a final centrifugation and aspiration to remove any residual liquid, 15 µl nuclease-free water was added. The nucleic acid concentration was determined using a Thermo NanoDrop 2000C spectrophotometer and then quality was assessed using an Agilent 2200 Bioanalyzer TapeStation.

RNA extraction directly from frozen roots: Roots from *S. tuberosum* or *S. sisymbriifolium* were divided into 100 to 150 mg batches, wrapped in aluminum foil, and frozen in liquid nitrogen. Uninfected roots were extracted directly from agar media while infected roots had been transferred for 10 to 14 d in infection media as described in Materials and Methods. The root mass was held in a prechilled 15-ml Ultra Tissue Grinder (VWR, Randor, PA), and ground while still frozen. Samples were kept on dry ice until all samples were processed. The powder was then thawed in the presence of 500 µl of RNAzolRT and mixed with 200 µl cetyl trimethylammonium bromide (CTAB) solution (1% CTAB, 1 M NaCl, 0.05 M Tris (pH 8), 0.01 M EDTA, 2% PVP-40, and 5% β-mercaptoethanol added immediately prior to use). The tubes were shaken for 15 sec and incubated at room temperature for 15 min. The tubes were then centrifuged for 15 min at 13,000*g*. The upper 75% of the supernatant was removed and mixed vigorously with equal volumes of a chloroform and isoamyl alcohol solution (24:1). After centrifuging at 5,000*g* for 5 min, the supernatant was removed carefully to avoid taking the interphase. Nucleic acids were precipitated by adding ethanol to a final concentration of 30% and incubating the mixture at room temperature for 30 min. The tube was centrifuged at full speed (21,000*g*) for 8 min and washed twice with 75% ethanol. After a final centrfugation and aspiration to remove any residual liquid, 20 µl nuclease-free water was added. The nucleic acid concentration was determined using a Thermo NanoDrop 2000C spectrophotometer and the quantity was confirmed and quality assessed using an Agilent Bioanlayzer 2200 TapeStation.

RNA extraction roots using Proteinase K: Roots from *S. tuberosum* or *S. sisymbriifolium* (grown as described above) were divided into 100 to 150 mg batches. The root mass was placed into a 15-ml Ultra Tissue Grinder with 500 µl Proteinase K (100 µg/ml Proteinase K in 1% SDS, 50 mM Tris pH 7.5, and 10 mM EDTA pH 8 with 5% β-mercaptoethanol added immediately prior to use) and ground at room temperature. Prior to use, pestles were first dipped into another tube of Proteinase K solution. Once ground, the tube was incubated at 50°C for 30 min, after which the slurry was transferred into a micro centrifuge tube mixed with 1 ml of RNAzolRT, shaken vigorously, and incubated at room temperature for 15 min. The tubes were centrifuged at 13,000*g* for 12 min. The aqueous phase was then removed and extracted with an equal volume of chloroform and isoamyl alcohol (24:1). The final aqueous phase was transferred to a new tube and precipitated by bringing it to 30% with ethanol. After 30 min at room temperature, the tube was centrifuged at 21,000*g* for 8 min and the pellet was washed twice with 75% ethanol. After a final centrifugation and aspiration to remove any residual liquid, 20 µl nuclease-free water was added. The nucleic acid concentration was determined using a Thermo NanoDrop 2000C spectrophotometer and the quantity confirmed and the quality assessed using an Agilent Bioanalyzer 2200 TapeStation.

Transcription into cDNA and amplification by PCR: Approximately 0.8 µg RNA was reverse transcribed into cDNA according to instructions (Invitrogen Superscript III First Strand; Invitrogen, Carlsbad, CA) and the final product was stored immediately at −20°C until used for amplification by PCR. Amplification by PCR was performed in 20 µl reactions containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM primers, 1 unit Taq polymerase (Invitrogen), and 1 µl undiluted cDNA using β-actin (CV579373.1) primers (Forward 5′-CCGAGAAAAGATGACCCAAA-3′ and Reverse 5′-AAGGAGTAACCACGCTCAGTG-3′; Dalzell et al., 2009) or equal micromolar amounts of other primers as indicated in Figs. S1–S3. Unless indicated otherwise, amplification conditions were 95°C for 1.5 min followed by 25 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Ten microliters of the PCR products were run on 1.5% agarose gels to assess the reaction.

Densitometry on PCR products: The gels were photographed and densitometry readings were performed on an Alpha Imager version 3.4.0.0 (Protein Simple, San Jose, CA). The densitometry was performed with the accompanying software using the analysis tool, "Multiple Band Analysis". The measurement square was made

around each band only to the width and length of the band. The background was determined using local background which picks the 10 lowest pixels in the band region following the manufacturer's preset conditions. A spread sheet was prepared from a single gel which contained amplifications from two separate experiments each containing either duplicate or triplicate RNA extractions for cDNA synthesis. The averages and standard deviations were determined by the manufacturer's software.

Results

RNA extraction from nematodes: Initial attempts to produce high-quality RNA from *Globodera pallida* using protocols established for similar systems proved unreliable in our hands. We were able to obtain 54 ng RNA with an RNA Integrity Number (RIN) score (Schroeder et al., 2006) or RIN^e (TapeStation RIN equivalent) of 9.8 characteristic of intact, undegraded, high-quality nucleic acid from 1,000 L4 and adult *C. elegans* simply by vortexing the biological material for 20 min in the presence of RNAzolRT. However, this basic approach failed when applied to *G. pallida*: two attempts with greater than 1000 individuals generated no quantifiable RNA. One possible explanation was that *G. pallida* had a tougher cuticle than *C. elegans*, and needed more effective disruption procedures. The first protocol modification that we made was to use a BeadBeater (Retsch MM300) with 3-mm metal beads set for a frequency of 30 vibrations/sec for 1.5 min in the presence of RNAzolRT followed by ethanol precipitation. Significantly less than 5 ng of RNA was obtained from 400 *Globodera* J2 larva on two different attempts. Increasing the starting material to 2,800 animals gave no significant increase in quantifiable RNA. Three attempts using 100-μm glass beads and between 800 and 3,000 J2 larvae failed as well, while three further attempts using 800 larvae yielded 180 ng of very low quality RNA with a RIN^e score <4.1. From these results, we concluded that the applied mechanical methods either did not consistently disrupt the tough nematode cuticle, or, that the chemical extraction solutions did not adequately inhibit released RNases prior to subsequent purification steps.

Having tried the above mentioned mechanical disruption techniques without great success, we turned to digesting the cuticle enzymatically by use of Proteinase K (Holterman et al., 2008; Ly et al., 2015). Microscopic examination revealed that a 12-min exposure to 100 μg/μl Proteinase K at 50°C left no larvae intact. By comparison, mechanical disruption techniques in which animals were shaken at a frequency of 30 vibrations/sec for 4 min with 100-μm glass beads in the presence of RNAzolRT left corpses transparent but intact. As illustrated in Fig. 1, treating nematodes with Proteinase K reproducibly yielded 0.3 to 0.5 ng RNA/nematode from as few as 88 J2 larvae per sample. However, at the same time, we found that reproducibility was low when fewer than 175 larvae were used, most likely because small amounts of nucleic acid failed to precipitate efficiently. We attempted to increase recovery using Precipitation Carrier (an acyl polymer from MRC) but found this decreased RNA yields even further. No further attempts with carriers were made.

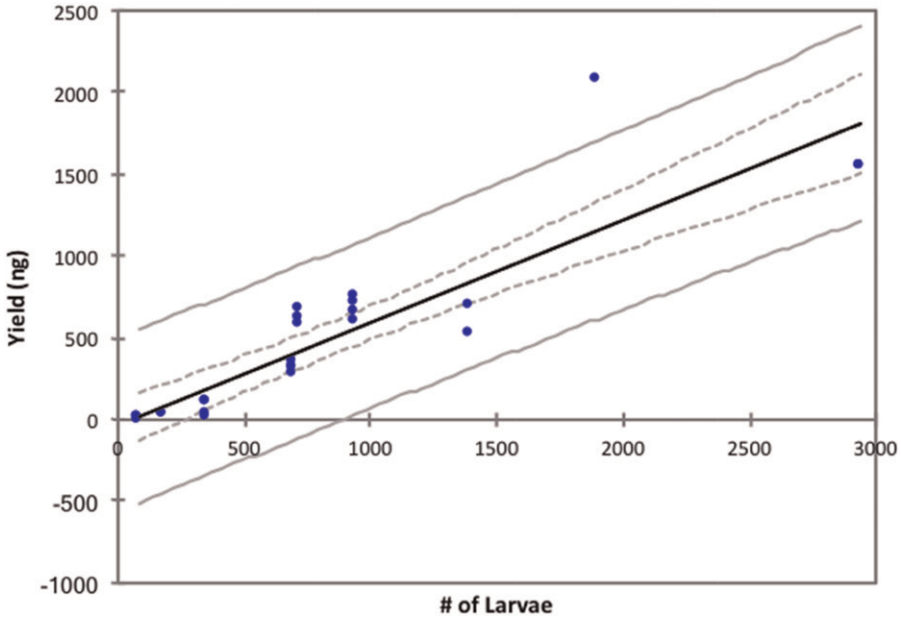

Fig. 1. Association between the number of J2 larvae used to extract RNA and the RNA yield (nanograms) recovered after Proteinase K treatments. Dots indicate independent extraction experiments. Solid black line indicates the predicted regression value (R^2 = 0.798). Dashed grey line represents the 95% confidence interval for the predicted line. Solid grey line represents the 95% confidence interval around the observations. Based on these values, approximately 0.4 ng of RNA could be extracted from each larva.

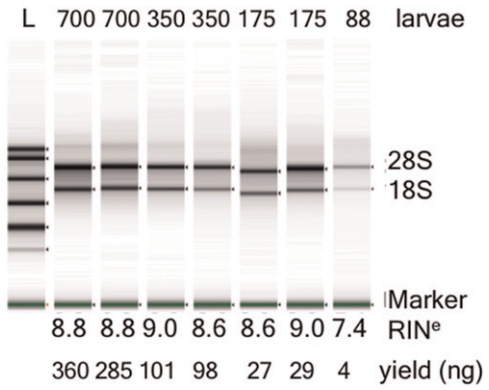

FIG. 2. Profile of RNA extracted from juvenile *Globodera pallida* demonstrated on a gel processed by the Agilent TapeStation. The prominent high-molecular-weight bands correspond to 28S and 18S rRNA while the lowest band corresponds to a marker included with the loading buffer and used as an internal control by the instrumental program. RIN^e scores are noted underneath each lane. With the exception of the sample in the last lane, the remaining samples were diluted to equal the least concentrated sample and run using the high-sensitivity buffer and gel tape. Lane L was a ladder provided by the bioanalyzer software. The number of J2 larvae used in each extraction is shown at the top of the figure, and the yield of RNA in nanograms is shown at the bottom.

Figures 2 and 3 illustrate the quality of the RNA extracted from the nematodes. The gels in Fig. 2 and the eletropherograms in Fig. 3 corresponding to individual lanes in Fig. 2 revealed RIN^e scores obtained from four different sample sizes ranging from as few as 88 to as many as 700 individuals per sample. The RIN^e scores were typically greater than 8.5 with a range of 7.4 to 9.1. Although the concentration of the RNA extracted from the sample containing 88 larvae was below the manufacturer's recommendations, there was sufficient RNA to visualize the 18S and 28S bands (Figs. 2,3). Figures 2 and 3 also show that the majority of the RNA was distributed above the 18S species with very little below. The 25-bp band includes an internal standard in the manufacturer's sample buffer. It is possible that larger amounts of RNA could be recovered if higher amounts of ethanol were used in the final precipitation steps, but we found that using lower amounts of alcohol reduced the recovery of 5S and tRNA (data not shown) and allowed the preferential recovery of large molecules. We saw no reduction in quality or increase in yield when we lengthened the Proteinase K treatments to 1 hr. Samples treated for 12 or 60 min produced RNA with RIN^e scores of 7.8 and 8.3, respectively, with no notable effect on yield (data not shown).

RNA extraction from roots: To develop a protocol that could be used to monitor gene expression in both nematodes and their hosts, we developed a protocol that could reliably produce high-quality RNA from less than 200 mg of nematode-infected roots. To test the robustness of the extraction protocols, we proceeded to use two *Solanum* species susceptible to infection by *G. pallida*. Table 1 illustrates the three protocols that were developed and the differences among the three. In Protocol 1 (Tables 1 and 2), frozen tissue was manually ground then thawed and incubated in Proteinase K solution, and finally treated with RNAzolRT. In Protocol 2, frozen tissue was manually ground then thawed in RNAzolRT followed by incubation with CTAB and PVP to eliminate contaminating carbohydrates commonly found in roots of *Solanum* as well as other plant species. Including CTAB and PVP with the Proteinase K buffer caused significant foaming and so CTAB and

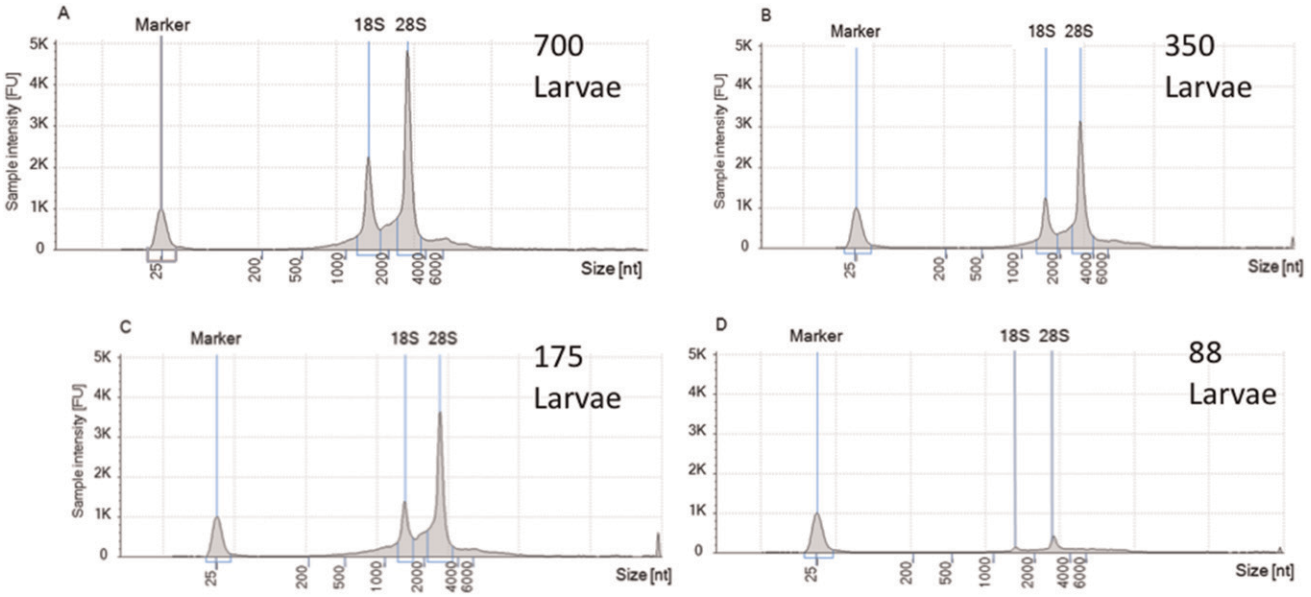

FIG. 3. Electropherograms showing the integrity of RNA from lanes in Figure 2. The Y axis is the intensity of the signal and the X axis is the size in nucleotides. The marker located at 25 nt is included with the manufacturer's loading buffer and used for internal controls by the instrument. A. Electropherogram of Fig. 2 lane 2. B. Electropherogram of Fig. 2 lane 4. C. Electropherogram of Fig. 2 lane 6. D. Electropherogram of Fig. 2 lane 8.

TABLE 1. Comparison of three different protocols for extracting RNA from root tissue.

	Protocol 1	Protocol 2	Protocol 3
Conditions for tissue grinding	Frozen	Frozen	Room temperature, Proteinase K
Thawing solution	Proteinase K	RNAzolRT	not frozen
Proteinase K incubation	30 min	n/a	30 min
RNA extraction solution	RNAzolRT-Proteinase K	RNAzolRT-CTAB	RNAzolRT-Proteinase K
Second extraction	Chloroform–isoamyl alcohol	Chloroform–isoamyl alcohol	Chloroform–isoamyl alcohol
Ethanol precipitation	30 min	30 min	30 min

PVP were omitted whenever Proteinase K was used. After RNAzolRT treatments, the aqueous phases were extracted with chloroform:isoamyl alchohol prior to ethanol precipitation. The disparity in quality of the RNA produced by the two protocols is shown in Table 2. When thawed in the presence of Proteinase K (Protocol 1), the RNA extracted was very low quality with an average RIN[e] score of 6 or less (Table 2). However, when Protocol 2 was applied, the RNA extracted was of a much higher quality, with an average RIN[e] score over 8 for both *Solanum* species (Table 2).

Protocol 3 (Table 1) was developed to determine if grinding at room temperature in the presence of Proteinase K increased RNA quality compared to freezing and thawing in the Proteinase K solution. The improvement was more notable in *S. tuberosum* than in *S. sisymbriifolium*. While Protocol 1 produced RNA with an average RIN[e] score of 5, Protocol 3 produced RNA with an average RIN[e] score of 7.3 ± 0.3. The yields for *S. tuberosum* although improved when Protocol 3 was applied, were highly variable based on standard deviations. For *S. Sisymbriifolium*, there was no statistical improvement either in quantity or quality. Although there was significant improvement with Protocol 3 relative to Protocol 1 when applied to *S. tuberosum*, the highest quality of RNA for either *S. sisymbriifolium* or *S. tuberosum* came from the application of Protocol 2: ground frozen tissue thawed in the presence of RNAzolRT (Table 2). To ensure that this protocol provided nearly full-length RNA of the sort required for constructing de novo transcriptomes, we extracted sequence information from a *S. sisymbriifolium* transcriptome database (Wixom et al, in preparation) for a gene similar to *RPP13* of *Arabidopsis thaliana* (Bittner-Eddy, el al., 1999) and used this to amplify the predicted 2.38-kb open reading frame (Fig. S1). To ensure we could also obtain moderate-to-low copy nematode sequences, we compared our ability to amplify a 274-bp portion of the 3′ end of a *G. pallida* acetylcholinesterase gene (*ace-2*; FJ499505.1), as well as a larger, 2.02 kb portion of its open reading frame, from cDNA prepared from RNA isolated using Protocol 3. It is thought that this gene is expressed exclusively in anterior chemosensory and mechanosensory neurons (Costa et al., 2009) and so provides a stringent standard for the recovery of low-abundance transcripts. As shown in Fig. S2, both the 3′ and full-length sequences were recovered to similar, although not identical degrees. The recovery of large plant and nematode transcripts illustrates the potential usefulness that the two extraction protocols offer.

Inoculation, infection, and extraction of Solanum species: The next step was to assess whether our protocols were efficient enough to extract RNA from the low numbers of nematodes associated with roots in an average infection. Potato and *S. sisymbriifolium* plants were replanted with one plant per container in sterile inoculation medium to encourage new root growth. After 2 wk, the roots of several plants were each inoculated with approximately 500 newly hatched

TABLE 2. Comparison of extracted RNA from roots using three different protocols.

	Extraction method					
	Protocol 1		Protocol 2		Protocol 3	
Tissue	RNA yield (μg)/root (mg)	RIN	RNA yield (μg)/root (mg)	RIN	RNA yield (μg)/root (mg)	RIN
Uninfected						
Solanum tuberosum[a]	0.049 ± 0.010	5.0 ± 0.0	0.094 ± 0.027	8.3 ± 0.4	0.104 ± 0.052	7.3 ± 0.3
Solanum sisymbriifolium[b]	0.041 ± 0.033	6.0 ± 1.0	0.026 ± 0.017	9.0 ± 0.4	0.028 ± 0.022	6.8 ± 1.0
Infected						
Solanum tuberosum[c]			0.075 ± 0.030	7.6 ± 1.2	0.035 ± 0.01	6.7 ± 1.3
Solanum sisymbriifolium[d]			0.033 ± 0.015	9.0 ± 0.7	0.024 ± 0.017	6.0 ± 1.1

[a] RNA extracted from uninfected roots of *S. tuberosum* Protocol 1 average and standard deviation for triplicates over one experiment; Protocol 2 average and standard deviation for six replicates over two experiments; Protocol 3 average and standard deviation for eight replicates over three experiments.

[b] RNA extracted from uninfected roots of *S. sisymbriifolium* for Protocol 1 average and standard deviation for triplicate over one experiment; Protocol 2 average and standard deviation for six replicates over two experiments; Protocol 3 average and standard deviation for eight replicates over three experiments.

[c] RNA extracted from infected *S. tuberosum* Protocol 2 average and standard deviation for six replicates over three experiments; Protocol 3 average and standard deviation for five replicates over three experiments.

[d] RNA extracted from infected *S. sisymbriifolium* Protocol 2 average and standard deviation for seven replicates over three different experiments; Protocol 3 average and standard deviation for five different replicates over two experiments.

G. pallida juveniles. A fraction of these larvae was found embedded in the roots within 3 d post-inoculation. Prior to RNA extraction, the roots from each of the plants used in a single infection were distributed into batches of between 100 and 135 mg each to ensure that each extraction protocol would not be done on the same source material. Infected roots were extracted using Protocols 2 and 3 since they yielded the most similar quality nucleic acid (Table 2). As shown with the uninfected roots, Protocol 2 produced higher quality RNA on average from both *Solanum* species with *S. sisymbriifolium* showing the most pronounced difference. When Protocol 3 was applied to infected *S. sisymbriifolium* roots, the RNA had an average RIN^e score of 6.0 ± 1.1. However, when Protocol 2 was applied, the average RIN^e score increased to 9.0 ± 0.7. When Protocols 2 and 3 were applied to *S. Tuberosum*, the results were less extreme than observed with *S. sisymbriifolium*, but still showed an average increase from a RIN^e score of 6.7 ± 1.3 when Protocol 3 was used to an average RIN^e score of 7.6 ± 1.2 when Protocol 2 was used. The large standard deviation suggests a less robust extraction particularly pertaining to Protocol 2 for potato compared to *S. sisymbriifolium* and for both when Protocol 3 was applied. Whether the roots were grown in agar or Phytogel, the quality of the RNA obtained was specific to the extraction method, not to the gelling material. Roots ground and treated with Proteinase K produced lower quality RNA than roots frozen and thawed in the presence of RNAzolRT irrespective of the *Solanum* species (Table 2).

To determine whether some of the extracted RNA came from *Globodera*, aliquots from four representative infections of *S. sisymbriifolium* and of *S. tuberosum* were extracted in two separate experiments using each of the two protocols (Protocols 2 and 3). Where possible (see Fig. 4) 800 ng RNA from each extraction was then reverse transcribed into cDNA and 40 ng of this product was assayed using primers directed against a β-actin gene of *Globodera*. The number of cycles/PCR reaction was intentionally low (25), so that we could detect differences in the final levels of amplification. The sample in lane 16 was derived from 7.2 ng RNA, nevertheless it, as well as the samples produced from 40 ng of cDNA, assayed positive for the nematode gene (Fig. 4) and no amplification occurred when the template came from uninfected plants or when no template was provided (Fig 4, lanes UN and NT, respectively). Based on densitometry measurements (Table 3), more material was converted into cDNA and amplified when RNA was extracted using Protocol 3 than when using Protocol 2.

Discussion

We have examined several protocols for efficiently and effectively extracting quality RNA from *Globodera pallida* J2 larvae and from the roots of two *Solanum* species, *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* that they infect. Our effort concentrated on recovering high-quality RNA based on RIN^e score values (Schroeder et al., 2006). This algorithm compares the fraction of the sample comigrating as 18S and 28S RNA to the entire spectrum of size classes. We used a lower-than-usual percentage of alcohol to deplete the samples of potential degradation products, tRNAs, and microRNAs; however, other RNA sources could have artifactually lowered the RIN^e scores. For example, the current program assesses the abundant RNA species of the chloroplasts as degradation products. On the other hand, nematode RNA probably did not bias RIN^e score calculations significantly because our infection levels (<50 J2 larvae/100 mg root material) were so low.

The *Globodera* cuticle proved to be more difficult to disrupt than that of the free-living nematode, *C. elegans*. Although mechanical disruption methods produced

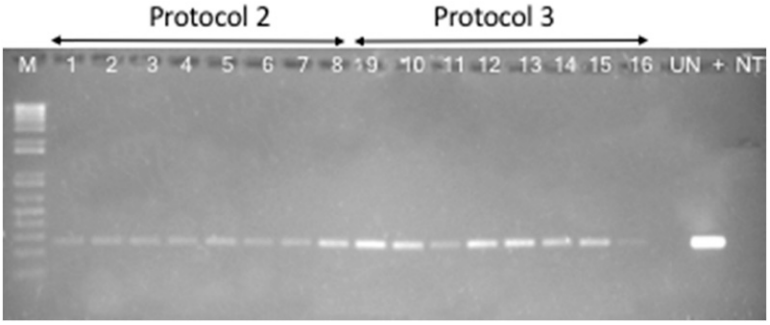

Fig. 4. Amplification products using *Globodera pallida* β-actin primers applied to cDNA prepared from independent samples of infected roots. Eight-hundred nanograms RNA (save for 144 ng in lane 16) was extracted using Protocols 2 and 3, and converted into cDNA. In all but the experiment in lane 16, 40 ng of cDNA was amplified with β-actin primers; 7.2 ng cDNA was assayed in lane 16. Finally, half of each reaction mix was loaded onto the gels. Samples on gel are M = 1 Kb plus ladder; lanes 1 and 2 = *Solanum tuberosum*; lanes 3 to 5 = *Solanum sisymbriifolium*; lanes 6 and 7 = *S. tuberosum*; lane 8 = *S. sisymbriifolium;* lanes 9 and 10 = *S. tuberosum*; lanes 11–13 = *S. sisymbriifolium*; lanes 14 and 15 = *S. tuberosum*; lane 16 = *S. sisymbriifolium*. UN = uninfected root; + = worm control; and NT = no Template. Each pair of samples represents replicates or independent experiments. The amplified products in lanes 1 to 5 were extracted and reverse transcribed in the same experiment as lanes 9 to 13, and similarly, lanes 6 to 8 were extracted and reverse transcribed in the same experiment as lanes 14 to 16.

TABLE 3. Densitometry averages for Figure 3.

Protocol	BC average for the respective protocol[a]	Standard deviation for BC average	BC sum average for the respective protocol[b]	Standard deviation for the BC sum
Protocol 2	1,188	304	943,827	393,213
Protocol 3	2,054	442	2,113,237	735,574

[a] BC average = Band region average − Band background average.
[b] BC sum = Background corrected region average × Region area.

high-quality RNA from *Globodera* eggs (data not shown) and from *C. elegans* juveniles (primarily L3 and L4), they failed to release adequate amounts of RNA from infective *Globodera* juveniles. Other researchers extracting RNA from other nematodes have reported greater success with this approach, but yields were extremely variable (Elling et al., 2007). Based on the low RIN[e] scores we measured, it is possible that the extraction buffer used failed to completely inactivate nucleases released in this manner.

Our results demonstrated that extractions using Proteinase K more reliably produced RNA with high RIN[e] scores than the alternative methods tested for "nematode-only" samples. Whereas mechanical treatments left the outer layers of many *Globodera* translucent but intact, bodies completely disappeared after Proteinase K treatment. Moreover, this protocol worked equally well on eggs and juveniles suggesting that the RNA recovered from a sample would be representative of all the life stages present in the mixture. The application of the Proteinase K on root-embedded nematodes increased the availability of the *G. pallida* RNA but at the expense of quality when compared to roots frozen and thawed in the presence of the RNAzolRT. Our studies showed that better quality RNA could be obtained if either the infected or uninfected roots were first frozen and then thawed in the presence of RNAzolRT. Consequently, when applied to infected roots, one protocol extracted superior quality RNA but reduced yields of nematode RNA compared to a second protocol that extracted lower quality RNA with increased yields of nematode RNA.

Further experiments showed that Protocol 2 worked reliably on roots of both *S. tuberosum* and *S. sisymbriifolium*. RNA was extracted from uninfected roots taken from older plants that had been grown for 1 to 2 months in cultivation media, as well as from 2- to 3-wk-old outgrowths of trimmed roots that had been infected with *G. pallida*. Whether the plants were infected or uninfected, older or younger, the cultivated potato produced larger yields of RNA than the wild *S. sisymbriifolium*.

We also assessed the efficiency of the extraction protocols by amplifying nematode β-actin from cDNA prepared from infected roots. Based on acid fuchsin staining of comparable root masses taken from the same plants that we extracted, we estimated there were 40 nematodes per sample which would be equivalent to approximately 24 μg of nematodes/100 mg infected potato tissue (and less than this in *S. sisymbriifolium*) and yet we were able to amplify the nematode transcript consistently with only 25 cycles. When we increased the reactions to 27 cycles, we were able to detect actin sequences coming from as few as 10 larvae diluted with RNA extracted from 150 mg root tissue. When we consider the amount of the sample that was analyzed, all of the lanes in Fig. S3 contained less nematode RNA than would be extracted from a single larva. However, these amounts were too low to detect a tissue-specific acetylcholinesterase (data not shown) with the primers used in Fig. S2. While this indicates low-abundence nematode genes could be overlooked in transcriptome studies of infected tissues, a preliminary analysis of two MiSeq (Illumina, San Diego CA) data sets found 138,693 out of 37,749,597 reads, and 121,124 out of 32,494,546 reads, mapped unambiguously to *Globodera* spp. open reading frames (data not shown). Furthermore, despite the low levels of infection in these experiments, these reads within our two-species "meta-transcriptome" mapped to 50.4% and 47.9%, respectively, of the *G. pallida* coding sequences in the Sanger database (ftp.sanger.ac.uk/pub/project/pathogens/Globodera/pallida/Gene_Predictions). We have also amplified a number of full-length *S. sisymbriifolium* genes ranging between 2.0 and 3.6 kb from the same RNA samples that were analyzed above. These two sets of preliminary results demonstrate that this extraction protocol can provide templates for a variety of multispecies gene expression studies.

Because we could not produce as much nematode-infected material as other protocols required (Soares et al., 1994), we set out to evolve an alternative nucleic acid extraction procedure that could reliably produce high-quality RNA suitable for future use in RNAseq analyses of infected plants. We have not determined whether our protocols for tissue disruption are compatible with any of the commercially available RNA extraction kits. We have also not determined the lower limits of our method but were able to show that we needed no more than 40 nematodes within a root mass of 100 mg to detect a variety of *Globodera* transcripts. These protocols worked reproducibly in the plant–nematode systems we tested, and should be directly applicable to a variety of other situations where

RNA needs to be extracted from small, very heterogeneous samples.

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