Characterization of Putative Effectors from the Cereal Cyst Nematode *Heterodera avenae*

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ABSTRACT

Few molecular details of effectors of *Heterodera avenae* parasitism are known. We performed a high-throughput sequencing analysis of the *H. avenae* transcriptome at five developmental stages. A total of 82,549 unigenes were ultimately obtained, and 747 transcripts showed best hits to genes putatively encoding carbohydrate-active enzymes in plant-parasitic nematodes that play an important role in the invasion process. A total of 1,480 unigenes were homologous to known phytonematode effectors, and 63 putative novel effectors were identified in the *H. avenae* transcriptomes. Twenty-three unigenes were analyzed by qRT-PCR and confirmed to be highly expressed

during at least one developmental stage. For in situ hybridization, 17 of the 22 tested putative effectors were specifically expressed and located in the subventral gland cells, and five putative novel effectors were specifically expressed in the dorsal gland. Furthermore, 115 transcripts were found to have putative lethal RNA interference (RNAi) phenotypes. Three target genes with lethal RNAi phenotypes and two of the four tested putative effectors were associated with a decrease in the number of cysts through in vitro RNAi technology. These transcriptomic data lay a foundation for further studies of interactions of *H. avenae* with cereal and *H. avenae* parasitic control.

Heterodera avenae is considered the most important plant-parasitic nematode (PPN) of cereal crops worldwide and causes significant economic losses (Vanstone et al. 2008). *H. avenae* was first reported in China in 1989 and was subsequently reported in 16 provinces. In fact, over 4 million hectares of the major wheat-producing regions are infested with cereal cyst nematodes (CCNs) in China (Cui et al. 2015). Cyst nematodes are biotrophic diploid sedentary endoparasites; their life cycle involves different stages, with four molts and four juvenile stages plus the adult stages (Bardou-Valette et al. 2016). Emerging infective second-stage juveniles (J2s) transfer intracellularly to the stele region and establish a sedentary feeding site in host plants through stylet punctures and the secretion of plant cell wall-modifying proteins (Seah et al. 2000). The syncytium initiates from the apoptosis of adjacent cell walls and becomes a growing and metabolically active coenocytic cell that serves as a nutrition pool for CCNs (Heinrich et al. 1998; Seah et al. 2000). Phytonematode effectors play important roles in nematode–host interactions by modifying plant cell walls, suppressing host defenses, and modulating host cell development (Hewezi and Baum 2013). The pharyngeal glands are the most important nematode organs that produce effectors. In endoparasitic nematodes, two subventral gland cells are metabolically active during the migration stage of parasitism, and the dorsal gland (DG) cell develops and becomes more energetic during feeding site development and maintenance (Davis et al. 2000). The identification of the related effector proteins will aid elucidation of the parasitic strategy of *H. avenae*.

A method for the molecular identification of *H. avenae* was recently developed, and a small-scale transcriptome project has been reported (Kumar et al. 2014; Zheng et al. 2015). However, few molecular details

are available for the interactions between *H. avenae* and plant hosts during different developmental stages. Kumar et al. (2014) previously reported one *H. avenae* transcriptome; however, these researchers only included the two nonparasitic stages of *H. avenae*, namely the J2 and cyst stages, in their assembly and did not sequence or analyze the most important parasitic stages. Yang et al. (2017) sequenced mRNA isolated from *H. avenae* at two phases: a soil stage (mixture of the egg, J1, and hatched-J2 stages) and a plant stage (mixture of the parasitic J2, J3, J4, and adult stages). Very limited studies have focused on effectors of *H. avenae*. Liu et al. (2016) cloned one predicted expansin-like protein, HaEXPB2, from *H. avenae* and transiently expressed it in *Nicotiana benthamiana*, which caused cell death in *N. benthamiana*. The results demonstrated that HaEXPB2 may play a key role in the parasitism of *H. avenae* through targeting the host cell wall (Liu et al. 2016). Qiao et al. (2016) and Le et al. (2016) identified three novel fatty acid- and retinol-binding (FAR) genes (Ha-far-1, Ha-far-2, and Hf-far-1) in the *H. avenae* and *H. filipjevi* transcriptomes. Chen et al. (2015) isolated an annexin-like gene, Ha-annexin, from *H. avenae* and found that it suppresses the programmed cell death caused by the proapoptotic mouse protein BAX and induces the expression of marker genes of PAMP-triggered immunity (PTI) in *N. benthamiana* (Chen et al. 2015). The suspected effectors that have been isolated from *H. avenae*, including Ha-expb1, Ha-eng-1a, Ha-eng-2, Ha-eng-3, and Ha-acp1, are associated with cell wall degradation (Liu et al. 2014; Long et al. 2012a, b, 2013). The investigation of changes in transcriptional levels among different developmental stages and the identification of putative effectors of *H. avenae* will provide an understanding of the molecular mechanisms of *H. avenae* parasitism. In situ hybridization is a technique that can be used to determine where certain genes are expressed, and the detection of genes expressed in nematode gland cells likely indicates that the encoded proteins are secreted in plant tissues (Haegeman et al. 2013).

RNA interference (RNAi) has been suggested to be an effective resistance mechanism against crop parasites (Runo et al. 2011). Phytonematode genes with lethal RNAi phenotypes are considered putatively valuable RNAi target genes for the breeding of phytonematode-resistant crops using RNAi technology (Alkharouf et al. 2007; Haegeman et al. 2013). The identification of such genes in *H. avenae* will help the

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development of original methods for nematode control. In this study, RNA sequencing (RNA-Seq) of *H. avenae* parasitic J2s, pJ2s, third- and fourth-stage juveniles (J3-J4s), females (Fes), and eggs was performed, and the results revealed variations in transcriptional levels among these five developmental stages. In addition, genes encoding putative effectors and carbohydrate-active enzymes (CAZymes), as well as genes with lethal RNAi phenotypes, were identified and verified in the transcriptomes.

MATERIALS AND METHODS

Biological materials. Soil samples containing *H. avenae* cysts were acquired from severely infected wheat fields in Taian, China, at the end of the wheat-growing season. The population cysts were molecularly identified by PCR with species-specific SCAR primers (Qi et al. 2012) and then proliferated on susceptible wheat cultivars grown in polyvinyl chloride (PVC) tubes maintained in a greenhouse (Cui et al. 2015). Newly formed cysts were collected manually from the wheat roots and kept for proliferating for years. Then the collected cysts were sterilized with 0.5% sodium hypochlorite for 10 min, washed five times with sterile water and maintained in a freezer at 3 to 4°C until use. The *H. avenae* cysts were hatched at 14 to 16°C. Hatched J2s were used to infect wheat roots, and 3-day-old susceptible *Triticum aestivum* ‘Wenmai 19’ plants were inoculated. Thirty-six hours after inoculation, the infected roots were then washed with sterilized water to eliminate uninfected nematodes. Parasitic J2s (pJ2s), J3s, and J4s were extracted from the roots at 5, 15, and 25 days postinoculation (dpi), respectively, by manual collection using a dissecting microscope. In addition, white females (Fes) were collected 5 weeks after infection, and eggs from mature cysts were gathered and frozen with liquid nitrogen for RNA extraction.

cDNA library preparation and sequencing. The collected eggs, J2s, pJ2s, J3-J4s, and Fes of *H. avenae* were separately homogenized in 1 ml of Trizol reagent and ground in liquid nitrogen with a mortar and pestle (Wang et al. 2014a). Total RNA was isolated according to the manufacturer’s instructions (Invitrogen Trizol Reagent, Thermo Fisher Scientific Inc., CA). The quality and quantity of the RNA samples were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Shanghai, China) and Agilent 2100 Bioanalyzer (Thermo Fisher Scientific Inc., CA) prior to library construction and sequencing. RNA samples with a 260/280 ratio of 1.9 to 2.0 and an RNA integrity number (RIN) of at least 8.0 were included in the analysis. A total of 1.0 µg of RNA per sample was used as input material for cDNA library construction, and cDNA library construction and sequencing were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) with the Illumina HiSeq sequencing platform.

De novo transcriptome assembly, gene functional annotation, and analysis. The raw reads were filtered to generate clean reads prior to assembly using FASTX-Toolkit 0.0.13.2 and the riboPicker 0.4.3 software package. In this step, the clean reads were obtained by removing reads containing adapter, reads containing N ($n > 0.1\%$, N represent unknown base pair in reads) and low-quality reads (Phred score ≤ 20) from raw data. At the same time, Q20 ($>95\%$), Q30 ($>85\%$), GC-content, and sequence duplication level of the clean data were calculated. Transcriptome assembly was performed with Trinity assembly software (trinityrnaseq_r20140413) using the default parameters, specifically a K-mer of 25 and a minimum contig length of 200 and 500 bp, to obtain high-quality unigenes (Grabherr et al. 2011). Unigenes with high BLAST similarity to known protein sequences from other species were then annotated. The BLAST procedure was used to compare all the unigenes against protein sequence databases, including Nr, Swiss-Prot, and clusters of orthologous groups (COGs), with an e-value $< 1.0 \times 10^{-6}$. Gene ontology (GO) functional annotations were performed using Blast2GO software based on the best-match BLAST results (Conesa et al. 2005). The obtained results were classified and analyzed using a Perl script and WEGO (Ye et al. 2006). A reciprocal best BLAST hit (RBBH) approach was used

in the searches against the KEGG database (Kanehisa et al. 2008), and KO (reference pathway) numbers were obtained for the KEGG annotations (Kanehisa et al. 2008). Furthermore, protein domains were identified using HMMER/PFAM (Finn et al. 2011), and protein signal sequences were predicted using SignalP 4.1 (Petersen et al. 2011). Prior to differential gene expression analysis, the read counts for each sequenced library were adjusted using the edge R program package based on one scaling normalized factor. Differential expression analysis of two samples was performed using the DEGseq (2010) R package (Wang et al. 2010). The *P* value was adjusted using q value, and q value of <0.005 and $|\log_2(\text{fold change})| > 1$ were set as the thresholds for significantly differential expression.

A total of 679,480 expressed sequence tags (ESTs), including cDNA reference sequences from 62 nematode species, were downloaded from NEMBASE4 (<http://www.nematodes.org/nembase4/>) to an established local sequence database (Supplementary Table S1). The *H. avenae* transcriptome reads were mapped by searching against a local sequence database of free-living nematode (FLN), animal-parasitic nematode (APN), and PPN species using tBLASTX/BLASTN with a threshold e-value of 1.0×10^{-5} . The unigenes were also searched against the CAZy database with an e-value threshold of 1.0×10^{-5} , as previously described (Park et al. 2010).

Candidate effector identification. All cDNA sequences encoding putative effector proteins identified from among the published parasitic nematode effectors were downloaded from NCBI (up to 25 August 2016), and genomic resources of *Caenorhabditis elegans* (*C. elegans* Sequencing Consortium 1998), *Meloidogyne floridensis* (Lunt et al. 2014), *Meloidogyne incognita* (Abad et al. 2008), *Meloidogyne hapla* (Opperman et al. 2008), *Bursaphelenchus xylophilus* (Kikuchi et al. 2011), and *Globodera pallida* (Cotton et al. 2014) were obtained from the WormBase (<http://www.wormbase.org>). *Globodera rostochiensis* genome data nGr.v1.0 were downloaded from the website (Eves-van den Akker et al. 2016), and *H. avenae* transcriptomic data (Kumar et al. 2014) were downloaded from the HATdb database (<http://insilico.iari.res.in>). to the established local sequence database (Haegeman et al. 2012; Wang et al. 2014b). These sequences were then searched for matches to the *H. avenae* transcripts using BLASTX to identify potential homologs. An e-value of $\leq 1.0 \times 10^{-5}$ and a coverage of $\geq 30.0\%$ were used to identify significant matches. To identify putative novel effectors of *H. avenae*, sequences with a putative signal peptide and without a transmembrane region were selected (Jones et al. 2009). Sequences showing differential expression among the different developmental stages of *H. avenae*, transcripts that were up-regulated in the J2s compared with the eggs or in the pJ2s compared with the J2s, and sequences without matches to *C. elegans* sequences (e-value $\leq 1.0 \times 10^{-6}$) were considered putative novel effectors (Haegeman et al. 2013).

Identification of genes with lethal RNAi phenotypes. Protein sequences of Embryophyta (txid3193), Craniata (txid89593), and Insecta (txid50557) were downloaded from the NCBI database, and *C. elegans* (Wormpep V.234) protein sequences were downloaded from WormBase (<http://www.wormbase.org>). All of the unigenes from the five *H. avenae* transcriptomes were searched against homologous sequences in the local database using BLASTX (e-value $\leq 1.0 \times 10^{-10}$). The *H. avenae* transcripts that matched sequences of *C. elegans* but showed no homology to sequences of Embryophyta, Craniata, or Insecta according to the BLAST results were used in the subsequent RNAi phenotype analyses. The RNAi phenotypes of these *H. avenae* transcripts were obtained from WormBase (<http://www.wormbase.org>) using the WormBase IDs of their homologous *C. elegans* sequences (e-value $\leq 1.0 \times 10^{-40}$) (Alkharouf et al. 2007). Genes with lethal phenotypes and without hits against Embryophyta, Craniata, or Insecta sequences were retained as possible RNAi targets.

Quantitative real-time PCR analysis of putative effector proteins. First-strand synthesis of cDNA from each sample was conducted using a SuperScript III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Thermo Fisher Scientific Inc., CA), according to the manufacturer’s instructions. Quantitative real-time

PCR (qRT-PCR) was then conducted using SYBR Premix Ex Taq II and ROX Reference Dye II (Applied Biosystems 7500 Fast Real-Time PCR System) according to the manufacturer's instructions. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an endogenous control gene. The relative changes in gene expression were quantified using the $2^{-\Delta\Delta CT}$ method using actin as an endogenous control gene. Three independent experiments with three technical replicates of each reaction were performed.

In situ hybridization. The spatial expression patterns of the putative effector proteins in J2s were determined by in situ hybridization, as described by Long et al. (2012b) with minor modifications. Freshly hatched J2s were washed three times with DEPC water, fixed in 4% paraformaldehyde at room temperature for 36 h, and incubated at 4°C for 8 h. Specific primers (Supplementary Table S2) were used to synthesize digoxigenin (DIG)-labeled sense and antisense cDNA probes (Roche Diagnostics GmbH, Mannheim, Germany) by asymmetric PCR. In situ hybridization was performed overnight at 43°C.

RNAi soaking and infection test. RNAi soaking was performed as described by Long et al. (2013) with minor adaptations. Double-stranded RNA (dsRNA) against the target genes was synthesized with a T7 *In Vitro* Transcription Kit (New England BioLabs) according to the manufacturer's instructions using the primers, and the concentration of dsRNA was 2 mg/ml. Newly hatched J2s were incubated in 1.5-ml tubes containing 100 µl of dsRNA soaking buffer for 24 h on a shaker at 16°C. For the infection assay, the susceptible host Wenmai 19 was used, and surface-sterilized seeds were germinated for 36 h in the dark at 30°C and inoculated as described by Cui et al. (2015). The same experiment was performed three times, and 10 replicates were performed each time.

RESULTS

Illumina paired-end sequencing and assembly. After filtering the data, the reads with sequence length longer than 125 bp were obtained from the eggs, J2s, pJ2s, J3-J4s, and Fes were assembled using Trinity software, and the mean of GC content were 47.9, 46.9, 49.0, 47.6, and 49.2%, more than 48, 49, 54, 46, and 47 million high-quality reads, respectively, were mapped to the Trinity assembly. Subsequently, 104,409 transcripts, with a mean length of 667 bp, a maximum length of 15,536 bp, and an N50 of 1,033 bp, were acquired. In addition, 64.8% of the transcripts were 200 to 500 bp in length, and 35.2% were longer than 500 bp. Moreover, 82,549 unigenes with a mean length of 514 bp and an N50 of 615 bp were acquired. In addition, 72.8% of the unigenes were 200 to 500 bp in length, and 27.2% were longer than 500 bp (Table 1).

Gene functional annotation and classification. The 82,549 unigenes from the *H. avenae* transcriptomes were successfully annotated using seven public nucleic acid and protein databases (Nr, Nt, Pfam, GO, KO, KOG/COG, and Swiss-Prot). A total of 30,559 homologous sequences were identified in the Nr database, and 28,863 and 28,474 were detected in the GO and Pfam databases, respectively. Among all the unigenes, 42,884 were annotated in at least one database, and 581 were annotated in all

seven databases (Supplementary Fig. S1). The results of the GO functional classification analysis of the unigenes from the *H. avenae* transcriptomes are shown in Supplementary Fig. S2. A total of 12,718 genes were annotated with KOG functional classifications, and these were classified into 26 different functional categories (Supplementary Fig. S3). In addition, 9,294 genes were annotated using the KEGG database, as shown in Supplementary Fig. S4. Furthermore, the following numbers of unigenes were found to show specific expression in the transcriptomes at the different stages: 4,406 in eggs, 68 in J2s, 47,700 in pJ2s, 117 in J3-J4s, and 797 in Fes (Supplementary Table S3). Analysis of the obtained *H. avenae* transcriptomes showed that unigenes specifically expressed at the pJ2 stage constituted the largest proportion (57.78%) of the total annotated unigenes, and the J2 stage was associated with the expression of the lowest number of unigenes.

Comparative analysis of transcriptomes. Searches were performed to identify transcripts in the *H. avenae* transcriptomes that might be orthologous or functionally equivalent to published parasitism genes or gene products of PPNs, APNs, or FLNs. A total of 29,547, 26,319, and 22,598 unigenes showed the highest homology to PPN, APN, and FLN sequences, respectively. Moreover, 18,567 unigenes showed the highest homology or functional equivalence to all three APN, PPN, and FLN sequence databases (Fig. 1). The unigenes were blasted to known nematode genomes with predicted proteins, including the *C. elegans* (*C. elegans* Sequencing Consortium 1998), *Meloidogyne floridensis* (Lunt et al. 2014), *M. incognita* (Abad et al. 2008), *M. hapla* (Opperman et al. 2008), *Globodera pallida* (Cotton et al. 2014), *G. rostochiensis* (Eves-van den Akker et al. 2016), and *Bursaphelenchus xylophilus* (Kikuchi et al. 2011) genomes and one transcriptome of *H. avenae* from India (Kumar et al. 2014) (Fig. 2). The alignment rates were 53% for *C. elegans* (14,639/27,876), 38% for *M. floridensis* (19,057/49,941), 59% for *M. incognita* (12,592/21,232), 61% for *M. hapla* (8,848/14,420), 72% for *G. pallida* (11,819/16,417), 75% for *G. rostochiensis* (10,785/14,309), 58% for *B. xylophilus* (10,407/18,074), and 96% for *H. avenae* from India (26,784/27,765) (Fig. 2).

CAZyme analysis. BLASTX or BLASTN searches of the CAZy database revealed a total of 747 *H. avenae* unigenes that matched sequences of carbohydrate esterases (CEs), glycosyl transferases (GTs), glycoside hydrolases (GHs), polysaccharide lyases (PLs), or auxiliary activity (AA) proteins from other organisms. The numbers of matching CAZymes in the different CAZy families were as follows: eight AAs (41 unigenes), eight CEs (84 unigenes), 50 GHs (608 unigenes), and three PLs (14 unigenes). Most of these sequences were from other nematodes, specifically *G. rostochiensis*, *H. glycines*, *Radolophus similis*, *Pratylenchus coffeae*, *Ditylenchus africanus*, *Rotylenchulus reniformis*, *Aphelenchoides fragariae*, and *Aphelenchus avenae*. Eight transcripts matched PL3 family proteins, and six of these were from PPNs, including pectate lyases from *H. avenae* (ADD82848.1), *H. glycines* (ADW77533.1), and *A. avenae* (BAI44497.1 and BAI44499) (Karim et al. 2009). Moreover, three transcripts matched glycosyl hydrolase family 1 proteins, including the putative β -1,4-endoglucanase from *P. vulnus* (AER27786.1). Cellulase, β -1,4-endoglucanases or GH family 5 (GHF5) proteins, which are secreted from the glands, are

TABLE 1. Summary of the assembled unigenes from the *Heterodera avenae* transcriptomes^a

	Eggs	J2s	pJ2	J3-J4	Females
Raw reads	51,760,974	49,532,552	58,083,284	46,759,702	47,668,102
Cleanreads	48,439,740	47,348,811	54,415,262	45,186,071	45,260,659
Transcripts 200 to 500 (bp)	33,173	18,322	205,705	17,255	26,677
Transcripts 500 to 1,000 (bp)	10,073	12,639	51,272	10,365	13,944
Transcripts >1,000 (bp)	25,535	35,988	45,184	16,315	22,059
N50 length (bp)	2,870	2,473	995	1,668	1,867
Average transcript length (bp)	1,328	1,546	630	1,029	1,068
Total number of transcripts	68,781	66,962	302,161	43,953	62,697
Total number of unigenes	41,895	43,489	251,100	43,953	51,575
Total nucleotides in transcripts (nt)	91,360,698	103,537,600	190,437,665	45,245,950	66,961,015

^a The raw data (raw reads) in the fastq format were first processed through in-house Perl scripts. The Q20, Q30, GC-content, and sequence duplication level of the clean data were calculated. All downstream analyses were based on clean data with high quality.

present mainly in most PPNs and are speculated to soften cell walls, accelerating the intracellular migration of nematode infective juveniles as they traverse host tissues. Five transcripts homologous to GHF5 proteins matched sequences in archaeobacteria or eubacteria. In addition, transcripts homologous to the GHF5 proteins were annotated to the PPN protein database (Table 2). Carbohydrate-binding modules (CBMs) are noncatalytic domains of GH enzymes that degrade polysaccharides (Arantes and Saddler 2010), and among the CBM families present in the CAZy database, 19 families associated with 128 transcripts were identified in the *H. avenae* transcriptomes (Supplementary Table S4).

Fig. 1. Venn diagram showing the number of sequences among the *Heterodera avenae* transcripts homologous or functional equivalence to animal-parasitic nematodes (APN), plant-parasitic nematodes (PPN), and free-living nematodes (FLN) sequences. Classification of contigs with significant similarity to known nematode expressed sequence tags according to the nematode’s lifestyle based on the Venn diagram.

Homology effector candidate analysis. Numerous effector proteins with various functions in plant cells are secreted into plants by phytonematodes. The primary aim of this study was to identify *H. avenae* effectors at certain development stages. According to the blast results of *H. avenae* transcriptome and phytonematodes effector proteins database, a total of 1,480 *H. avenae* transcripts were detected to be homologous to 176 reported effector proteins from 20 known phytonematodes, including previously characterized effectors from *H. avenae* and more frequently characterized effectors from *H. glycines*, *G. pallida*, and *G. rostochiensis*. The best hits of the homology effector candidates for 752 transcripts were known effectors secreted from esophageal gland cells. Seventy of the encoded secreted plant cell wall-modifying proteins were detected, such as pectate lyase, cellulases, chitinase, expansin and endoglucanase. Effectors secreted from the phasmids and amphids of PPNs have not been well studied, and in the present study, we detected four unigenes with best hits to a putative amphid protein from *G. rostochiensis* (CAB66341.1 and AHW98768.1). Sixty-six FAR proteins from *G. mexicana* and *G. pallida*, 47 polyprotein allergen Hgg proteins from *H. glycines*, 16 secreted SPRY domain proteins from *G. rostochiensis*, 21 venom allergen-like proteins unigenes, 10 annexin proteins, six ubiquitin extension proteins, and five “14-3-3” effector proteins were also identified from the obtained *H. avenae* transcriptomes. Hypervariable apoplastic (HYP) effectors, which are secreted by amphids of *G. pallida* and contain hypervariable regions among individuals of the nematode population, have been characterized (Eves-van den Akker et al. 2014), and we identified seven transcripts with a best hit to an HYP effector from *G. pallida* (AIT18718) in the obtained transcriptomes (Supplementary Table S5).

Of the 1,480 homology effector candidates, 793 were found to be specifically expressed in the pJ2s, 24 were specifically expressed in the eggs, and three were specifically expressed in the Fes. Five hundred and one homology effector candidates were found to be expressed in the later growth stages, namely J3-J4s, and no effectors were specifically expressed in the J3-J4s. Nevertheless, analysis of the numbers of fragments per kilobase of transcript sequence per million base pairs sequenced (FPKMs) revealed that 167 of the 501 homology effector candidates were found at a higher level in the J3-J4s than in the pJ2s, and 41 homology effector candidates showed 10-fold higher expression in the J3-J4s than in the pJ2s. The homology effector candidates were clustered using a hierarchical clustering method, which resulted in the formation of eight groups,

Fig. 2. Venn diagram showing the number of homologous sequences from known phytonematode genomes or transcriptomes. Venn diagram showing the number of homologous sequences identified between the *Heterodera avenae* unigenes and the predicted proteins from the free-living (*Caenorhabditis elegans*) and plant-parasitic (*Meloidogyne incognita*, *M. hapla*, *M. floridensis*, *Globodera pallida*, *G. rostochiensis*, *Bursaphelenchus xylophilus*, and *H. avenae* from India) nematodes. The numbers represent the common sequences.

and most of the effectors were specifically or highly expressed in the pJ2 stages (sub2 and sub4). The other important effectors identified in the *H. avenae* transcriptomes included two D406 proteins (AHW98772) and one CLE-related protein (CL4B2_GLORO) from *Globodera rostochiensis*; two calreticulin proteins (AIW66697) from *Pratylenchus goodeyi*; one 19C07 protein (ADM21466), one 10A07 protein (AJR22224) and one chorismate mutase protein (ABA06539) from *H. schachtii*; and one mucin-like protein (AAC62109), one putative glutathione reductase (AAK94486), and one putative pyruvate dehydrogenase protein (AF345797_1) from *H. glycines*. Notably, *H. glycines* was the most closely related species, with 680 homologous putative effectors and annotated putative proteins. *H. avenae* was the second most closely related species, with 226 unigenes successfully mapped to annotated effector proteins, and *B. xylophilus* was the third most closely related species (Supplementary Fig. S5).

Novel effector candidate analysis. The transcripts were screened for gene-coding proteins with the signal peptide and without the transmembrane domain and for unigenes that were up-regulated in the pJ2s compared with the J2s and in the J2s compared with the eggs, which could include novel effectors. Furthermore, 42 of the 63 *H. avenae* unigenes were found to have no matches in the effector databases (Supplementary Table S6). Of the 63 putative novel effectors, 17 were specifically expressed in the pJ2s. The putative novel effectors were clustered using a hierarchical clustering method, which resulted in the formation of eight groups. Five unigenes were highly up-regulated in the pJ2s and J2s (sub1 and sub2), and eight additional unigenes showed increasing up-regulation with advancing stages (sub3 and sub7). Interestingly, 13 unigenes grouped in the up- and down-regulated clusters displayed opposite expression patterns at different time points, e.g., some of these genes were transiently up-regulated during the early stage, showing peak expression in the pJ2s, and down-regulated later (J3-J4 and Fe stages) in the experiment (sub4 and sub6) (Supplementary Fig. S6).

Functional verification. The expression patterns of the putative novel effectors genes were examined by qRT-PCR and in

situ hybridization. Twenty-three of the putative novel effectors were randomly selected for subsequent analysis of their read data, annotations and existence during at least one of the five developmental stages of *H. avenae*. Among these unigenes, three (c83831_g1, c110667_g1, and c69587_g2) were highly expressed in the pJ2 stage, seven (c52184_g1, c67581_g1, c71908_g1, c27846_g1, c32721_g1, c43589_g1, and c71335_g1) were highly expressed in the J2 stage, eight (c60169_g1, c5222_g1, c83614_g1, c38219_g1, c69246_g1, c35506_g1, c5882_g1, and c23404_g1) showed high expression in the J3-J4 stage, two (c19158_g1 and c71012) were highly expressed in the Fe stage, and one (c28113_g1) was highly expressed in the egg stage. The c96797_g1 and c59403_g1 unigenes showed slight differences in expression in the J2 stage, and the c83614_g1 unigene showed a slight difference in expression in the J3-J4 and Fe stages (Fig. 3). The qRT-PCR results verified the accuracy and credibility of the transcriptome analysis.

In the present study, we also localized the gene expression of 11 homology effector candidates and 11 putative novel effectors that had been verified by qRT-PCR. The 11 homology effector candidates were expressed specifically in the subventral gland cells, and the results of the present in situ hybridization were completely consistent with the corresponding reports (Fig. 4). These effectors included expansin Hg-expB1-1 (ADL29728.1), expansin protein (ADY2960.1), hypothetical protein Hgg-18 (AAL78227.1) and putative gland effector protein G20E03 (AAO85459.1) from *H. glycines*; expansin B1 Ha-expb1 (AEP19215.1), β -1,4-endoglucanase Ha-eng-1a (ACO55952.1), beta-1,4-endoglucanase 3 (AFQ55682.1), and cellulose-binding protein CBP1 (ADD62691.1) from *H. avenae*; beta-1,4-endoglucanase precursor GR-eng-1 (AAC63988.1) from *G. rostochiensis*; a GHF5 endo-1,4- β -glucanase precursor (ABV54448.1) from *R. similis*; and a putative esophageal gland cell secretory protein 2 (AAQ10016) from *M. incognita*.

Eleven putative novel effector genes, as well as c5882_g1 (Fig. 5A), c69246_g1 (Fig. 5B), c83831_g1 (Fig. 5D), c52184_g1 (Fig. 5J), and c71335_g1 (Fig. 5K), were expressed specifically in the DG. In addition, c67581_g1 (Fig. 5C), c110667_g1 (Fig. 5E),

TABLE 2. Summary of the putative homologs of all currently known CAZymes in phytonematodes from the *Heterodera avenae* transcriptomes

CAZy family ^a	Number of transcripts	Function	Accession number	Organism origin	Identity (%)	E-value	Reference
GH5, CBM2	3	Cellulase, partial	AFD33557.1	<i>Aphelenchoides fragariae</i>	89.10	9.25E-59	Fu et al. 2012
GH5	2	Cellulase	AFD33558.1	<i>Aphelenchoides fragariae</i>	93.57	9.18E-103	Fu et al. 2012
GH5	5	Cellulase, partial	AFI63769.1	<i>Aphelenchoides fragariae</i>	96.89	2.11E-132	Fu et al. 2012
GH5, CBM2	3	Endo-1,4-beta-glucanase precursor	ABV54446.1	<i>Radolphus similis</i>	77.93	3.90E-132	Haegeman et al. 2008
GH5, CBM2	1	Endo-1,4-beta-glucanase precursor	ABV54447.1	<i>Radolphus similis</i>	56.88	4.73E-31	Haegeman et al. 2008
GH5	2	Endo-1,4-beta-glucanase precursor	ABV54448.1	<i>Radolphus similis</i>	78.21	7.64E-28	Haegeman et al. 2008
GH5, CBM2	4	Endoglucanase precursor	ABX79356.1	<i>Pratylenchus coffeae</i>	85.92	3.78E-30	Kyndt et al. 2008
GH5, CBM2	1	Endoglucanase	ABY52965.1	<i>Ditylenchus africanus</i>	59.34	7.93E-10	Kyndt et al. 2008
GH5, CBM2	2	Beta-1,4-endoglucanase	ACO55952.1	<i>H. avenae</i>	100.00	0	Long et al. 2012b
GH5	8	Beta-1,4-endoglucanase	ADM72857.1	<i>Rotylenchulus reniformis</i>	88.46	7.09E-20	Unpublished
GH5, CBM2	5	Beta-1,4-endoglucanase	ADW77528.1	<i>D. destructor</i>	63.37	1.04E-14	Unpublished
GH5	3	Beta-1,4-endoglucanase, partial	AER27786.1	<i>P. vulnus</i>	85.71	1.63E-36	Rybarczyk et al. 2012
GH5	1	Beta-1,4-endoglucanase, partial	AER27792.1	<i>P. vulnus</i>	62.01	2.50E-22	Rybarczyk et al. 2012
GH5, CBM2	1	Beta-1,4-endoglucanase precursor	AAC63988.1	<i>Globodera rostochiensis</i>	66.46	1.36E-167	Unpublished
GH5, CBM2	1	Beta-1,4-endoglucanase 1 precursor	AAD56392.1	<i>G. tabacum</i>	75.32	4.05E-19	Unpublished
GH5	1	Cellulase ENG-5	AAN32884.1	<i>H. glycines</i>	79.46	2.37E-159	Gao et al. 2003
GH5	1	Beta-1,4-endoglucanase 2	AFQ55680.1	<i>H. avenae</i>	100.00	1.09E-54	Unpublished
GH5	1	Beta-1,4-endoglucanase 3	AFQ55682.1	<i>H. avenae</i>	100.00	0	Unpublished
GH5	4	Beta-1,4-endoglucanase	BAI44493.1	<i>Aphelenchus avenae</i>	98.77	6.90E-167	Karim et al. 2009
PL3	3	Pectate lyase	BAI44497.1	<i>Aphelenchus avenae</i>	100.00	2.47E-45	Karim et al. 2009
PL3	1	Pectate lyase	BAI44499.1	<i>Aphelenchus avenae</i>	84.39	1.45E-86	Karim et al. 2009
PL3	1	Pectate lyase 1	ADD82848.1	<i>H. avenae</i>	99.22	0	Unpublished
PL3		Pectate lyase	ADW77533.1	<i>H. glycines</i>	86.45	4.70E-133	Unpublished

^a CBMs, carbohydrate-binding modules; GH, glycoside hydrolases; PL, polysaccharide lyases.

Fig. 3. Results of the expression analysis of unigenes performed by qRT-PCR. The relative expression levels of putative novel effector genes at five different *Heterodera avenae* stages were quantified by qRT-PCR. GAPDH was used as an internal control gene. The fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method and are presented as the changes in the mRNA level in various nematode developmental stages relative to that of egg. Each column represents the mean and standard deviation of three independent assays. J2, second-stage juveniles; pJ2, parasitic second-stage juveniles; J3-J4, third- and fourth-stage juveniles; and Fe, females. The main Y-axis with a scale mark represents the log2 fold change values, and the auxiliary Y-axis represents the number of read counts from the transcriptome.

c71908_g1 (Fig. 5F), c73057_g1 (Fig. 5G), c84585_g1 (Fig. 5H), and c35506_g1 (Fig. 5I) were expressed specifically in the subventral gland cells. In situ hybridization showed that the previously reported effectors or software predicted that novel effectors mainly accumulated specifically in the esophageal glands of *H. avenae* and that these secretory proteins play a key role in the process of *H. avenae* interaction with plant host. Two homology effector candidates (c12062_g1 and c33309_g1) and two novel effector candidates

(c51707_g1 and c70067_g1) were soaked with dsRNA, and c51707_g1 and c12062_g1 resulted in markedly decreased cyst reproduction by up to 70.3 and 32.5%, respectively. In addition, soaking with dsRNA of c70067_g1 and c33309_g1 led to reduced nematode infectivity but no significant decrease in cyst production compared with the GFP control groups. All of the treatments with RNAi technology significantly reduced nematode infectivity and reproduction compared with the control group (Fig. 6).

In situ hybridization	Results ^a	Query ID	Expression stage ^b	Reads ^c	Highest protein similarity	Highest similarity Organism	Gene ID	E-value
SvG	c109973_g1	pJ2	9035	expansin Hg-expB1-1	<i>H. glycines</i>	ADL29728.1	3.92E-27
SvG	c60169_g1	J3-J4	4765	hypothetical protein Hgg-18	<i>H. glycines</i>	AAL78227.1	1.96E-10
SvG	c1687_g1	pJ2	2204	putative gland protein G20E03	<i>H. glycines</i>	AAO85459.1	1.89E-86
SvG	c27846_g1	J2	7346	expansin	<i>H. glycines</i>	ADY2960.1	1.64E-44
SvG	c61490_g1	J2	20037	expansin B1 Ha-expb1	<i>H. avenae</i>	AEP19215.1	4.48E-180
SvG	c32721_g1	J2	20328	beta-1,4-endoglucanase 3	<i>H. avenae</i>	AFQ55682.1	0
SvG	c83669_g1	J2	74791	beta-1,4-endoglucanase	<i>H. avenae</i>	ACOS5952.1	0
SvG	c5222_g1	J3-J4	8575	Cellulose binding protein CBP1	<i>H. avenae</i>	ADD62691.1	1.20E-08
SvG	c83617_g1	J2	13957	beta-1,4-endoglucanase precursor	<i>G. rostochiensis</i>	AAC63988.1	5.74E-166
SvG	c7535_g1	J2	4710	GHF5 endo-1,4-beta-glucanase precursor	<i>R. similis</i>	ABV54448.1	5.11E-89
SvG	c60615_g1	pJ2	797	putative esophageal gland cell secretory protein 2	<i>M. incognita</i>	AAQ10016.1	1.80E-23

Fig. 4. In situ hybridization of digoxigenin-labeled antisense DNA probes (dark staining) of the homology effector candidates of *Heterodera avenae* J2 nematodes. ^a Indicates location of the signal (DG, dorsal gland cells; SvG, subventral gland cells); ^b indicates the highest expression stage of the annotated effectors given: second-stage juvenile (J2), parasitic J2 (pJ2), third- and fourth-stage juveniles (J3-J4s); and ^c indicates the number of the highest expression stage reads.

Fig. 5. In situ hybridization of digoxigenin-labeled antisense DNA probes (dark staining) of the putative novel effectors expressed exclusively within the subventral or dorsal esophageal gland cells of *Heterodera avenae*. **A**, (c5882_g1), expression at J3-J4 and located in the dorsal gland. **B**, (c69246_g1), expression at J3-J4 and located in the dorsal gland. **C**, (c67581_g1), expression at J2 and located in the subventral gland. **D**, (c83831_g1), expression at J2 and located in the dorsal gland. **E**, (c110667_g1), expression at J2 and located in the subventral gland. **F**, (c71908_g1), expression at pJ2 and located in the subventral gland. **G**, (c73057_g1), expression at J2 and located in the subventral gland. **H**, (c84585_g1), expression at Fe and located in the subventral gland. **I**, (c35506_g1), expression at J3-J4 and located in the subventral gland. **J**, (c52184_g1), expression at J2 and located in the dorsal gland. **K**, (c71335_g1), expression at pJ2 and located in the dorsal gland. DG = dorsal gland cells, SvG = subventral gland cells, M = median bulb.

RNAi phenotype. We sought to identify conserved genes that were potentially more specific and whose RNAi-mediated knock-down was lethal to *H. avenae* at all stages. And the identified RNAi phenotypes genes were thus considered potential candidates for *H. avenae* parasitic control. First, to identify nematode-specific genes in *H. avenae*, a local tBLASTX search was performed between transcripts of *H. avenae* and those of selected plants and animals. Second, the intersection of the transcripts from *H. avenae* with those of selected sequences was taken, resulting in transcripts of *H. avenae* without homologous sequences in plants and animals. In addition, the results yielded 3,924 *H. avenae* transcripts with homologous sequences in *C. elegans* but no homologous sequences in any plants or animals. Finally, according to WormBase (<http://www.wormbase.org>), 115 of the 3,924 transcripts had RNAi phenotypes, and 84 with reported lethal phenotypes were selected (Supplementary Table S7).

RNAi can be used to reduce the crop destruction caused by PPN. Based on the RNAi phenotypes identified results, the transcripts of *H. avenae* with match sequence in *C. elegans* and without homology in plants and animals were used to carried out the following RNAi soaking experiments. The RNAi phenotypes of three of the tested genes significantly decreased cyst number compared with the controls (Fig. 6). The target lethal phenotypes genes c59821_g1, c69968_g1, and c45915_g1 were soaked with dsRNA and reduced number of *H. avenae* female cysts by 67.1, 38.1, and 52.8% compared with the GFP control groups, respectively (Fig. 6).

DISCUSSION

The present study constitutes the first de novo analysis of the transcriptomes of all of the life stages of *H. avenae*, and the results greatly enrich the available information regarding *H. avenae* parasitism and further confirm the function and expression of different

effectors at different developmental stages. PPNs can soften and/or degrade the host cell walls, suppress or prevent host defense responses and manipulate host signaling pathways to generate complex feeding structures with the effectors (Davis et al. 2000; Haegeman et al. 2012). In this study, we first assembled transcriptomes for five different developmental stages, namely, the egg, J2, pJ2, J3-J4, and Fe stages, of the sedentary parasitic CCN *H. avenae*. Following assembly of the transcriptomes and functional annotation, 82,549 unigenes were successfully annotated by at least one of the databases of Nr, Nt, Pfam, GO, KO, KOG/COG, or Swiss-Prot. Kumar et al. (2014) obtained the first profile of *H. avenae* transcriptomes, i.e., the set of genes from J2s and females stages, that might be important in CCN parasitism. Unfortunately, Kumar and colleagues identified only 13 transcripts that belonged to known effectors of *Heterodera* spp. The present transcriptome covered almost all of the sequences annotated by Kumar et al. (2014), and the analysis revealed that the best hits for the unigenes were sequences from *H. glycines* (Supplementary Fig. S7). Nevertheless, Kumar and colleagues reported that the *H. avenae* transcriptome contains genes that are highly similar to those hypothesized to be related to nematode parasitism in *G. pallida*. This finding is inconsistent with the results reported by van Megen et al. (2009), who showed that *H. avenae* is much more closely related to *H. glycines* than to *G. pallida*. These differences among previous studies might be due to the use of different methods and databases, and most importantly, the *H. avenae* transcriptomes obtained in the present study contain a markedly higher number of complete gene sequences and include more stages than the transcriptome reported by Kumar et al. (2014).

Furthermore, Xu et al. (2012) and Zheng et al. (2015) reported 839 parasitic genes in *H. avenae* that are expressed during interactions with resistant hosts with high homology to genes from other nematode species. The studies conducted by Xu et al. (2012) and Zheng et al. (2015) add the list of parasitic genes from *H. avenae*

Fig. 6. Number of female cysts collected from wheat of RNAi of target genes on *Heterodera avenae*. The number of nematodes in wheat roots at 40 days postinoculation. The column with asterisks represents the mean of three independent assays with standard deviation. Treatments that are significantly different ($P < 0.01$) from the green fluorescent protein (GFP) treatment are indicated by **.

that are expressed in the sedentary stages, but these studies focused mainly on the host (*A. variabilis*)-resistant genes, and their findings were not expanded in a further study. Recently, Yang et al. (2017) reported transcriptome data for *H. avenae* at preparasitic mix-stages and postparasitic mix-stages. Nevertheless, the transcripts, unigenes or putative secretory proteins reported by Yang et al. (2017) were significantly less than those identified in the present study (Table 3). The present study adds two new transcriptomes from the sequenced samples to the literature and yielded a substantially higher number of transcripts compared with those reported by Kumar et al. (2014) and Yang et al. (2017) (Table 3). We had markedly stricter standards for the identification of putative secretory proteins: in addition to sequences with a putative signal peptide and without a transmembrane region, sequences showing differential expression among the different developmental stages of *H. avenae*, transcripts that were up-regulated in the J2s versus the eggs or in the pJ2s versus the J2s, and sequences without matches to *C. elegans* sequences (e-value $\leq 1.0 \times 10^{-6}$) were considered putative novel effectors (Haegeman et al. 2013; Nicol et al. 2012). We obtained 5,058 putative secretory proteins, whereas Kumar et al. (2014) and Yang et al. (2017) reported 1,471 and 4,229 putative secretory proteins, respectively. The number of annotated effectors from phytonematodes obtained in the present study was clearly higher than the numbers reported by Kumar et al. (2014) and Yang et al. (2017). We ultimately identified 63 putative novel effector proteins for further study and functional verification (Table 3).

Effectors were defined as all pathogen or pest proteins and small molecules that modify the host cell structure or function; launch defense responses caused by elicitors, avirulence factors, microbial-associated molecular patterns, pathogen-associated molecular patterns, and herbivore-associated molecular patterns; and/or promote infection (Hogenhout et al. 2009). The pattern of the secretion of phytonematode effectors from esophageal gland cells is the classical secretory pathway. These esophageal gland cell secretory proteins can induce and suppress host defenses or establish a sedentary feeding site (syncytium) in host plants (Davis et al. 2000; 2004). The putative effectors that have been isolated from *H. avenae* to date, which include Ha-expb1, Ha-eng-1a, Ha-eng-2, Ha-eng-3, Ha-acp1, and Ha-cbp-1, are specifically located in the two subventral gland cells of J2s. The knockdown of these genes using RNAi resulted in a reduction in nematode infectivity and the suppression of nematode cyst development (Gu et al. 2011; Liu et al. 2014; Long et al. 2012a, b; 2013). In addition, our analysis of the *H. avenae* transcriptomes obtained in this study also showed secreted plant cell wall-modifying proteins with homologous sequences to putative effectors, and in situ hybridization revealed that their expression was localized to the two subventral gland cells of *H. avenae* (Fig. 4). Three novel FAR genes

(Ha-far-1, Ha-far-2, and Hf-far-1) in *H. avenae* and *H. filipjevi* and one annexin-like gene, Ha-annexin, from *H. avenae* were also identified in the *H. avenae* transcriptomes obtained in the present study (Chen et al. 2015; Qiao et al. 2016). Sixteen SPRYSEC effectors/genes from *G. rostochiensis* and *G. pallida* encode a secreted protein that induces effector-triggered immunity (Carpentier et al. 2012; Rehman et al. 2009), and this finding helped us understand the interaction between *H. avenae* and plants. One crucial unigene identified in the *H. avenae* transcriptomes was found to be homologous to the effector 10A06 from *H. schachtii*, which might suppress host defense responses (Hewezi et al. 2010). Venom allergen-like proteins from *G. rostochiensis* are also thought to suppress plant defense systems (Lozano-Torres et al. 2012). Thirteen annotated unigenes in the *H. avenae* transcriptomes encode proteins with ubiquitination activity that play a crucial role in the degradation of target proteins (Craig et al. 2009). Furthermore, 10 glutathione- and glutathione peroxidase-encoding unigenes were detected in the *H. avenae* transcriptomes, and these might play a key role in the detoxification of host-derived nematicidal compounds.

It has been widely assumed that genes associated with cyst nematode parasitism have well-defined roles in the early and late stages of the infection process (Elling et al. 2009). In the present study, we obtained 63 putative novel effectors and found that these novel effectors are up-regulated in the J2s compared with the eggs and/or in the pJ2s compared with the J2s. We verified 23 novel effectors through RT-PCR, and most of the effectors were highly expressed in the pJ2 and J3 to J4 stages. We also performed 22 effectors for in situ hybridization analyses, 11 of the putative novel effectors and 11 of the homology effector candidates, and in situ hybridization showed that their expression was localized in esophageal gland cells. Subventral gland secretory proteins consist of cell wall-degrading enzymes that facilitate the invasion of plants, and DG cell secretory proteins play a role in feeding site induction and regulation of feeding cells (Hussey 1989). Other parasitism-related proteins synthesized in the subventral gland cells might play other roles in the induction of feeding cells (Davis et al. 2004). We also soaked four putative effector proteins with dsRNA, which resulted in highly variable decreases in cyst number (Fig. 6).

Genes in phytonematodes with lethal RNAi phenotypes are putative important candidate genes for parasitic control (Alkharouf et al. 2007; Haegeman et al. 2013). Alkharouf et al. (2007) first identified these types of genes showing putative lethal mutations or RNAi phenotypes in *H. glycines*. Expression of a dsRNA directed against housekeeping or parasitism genes resulted in *A. thaliana* plants with a 69 to 93% reduction in the number of root knot nematode eggs after infection compared with the empty-vector control (Huang et al. 2006). According to our RNAi results, soaking

TABLE 3. *Heterodera avenae* transcriptome sequencing data according to the present study and earlier reports

	Present study					Kumar et al. (2014)		Yang et al. (2017)	
	Illumina HiSeq					Illumina GAIIx		Roche FLX+	
	Eggs	J2s	pJ2	J3-J4	Females	J2s	Females	Preparasitic	Postparasitic
Number of reads	51,760,974	49,532,552	58,083,284	46,759,702	47,668,102	65,264,470	66,168,842	551,935	514,784
Average reads length	2 × 100					2 × 100		488	
Number of transcripts/contigs	104,409					27,765		10,811	
Number of unigenes	82,549					10,452		7,687	
Number of putative secretory proteins ^a	5,058					1,471		4,229	
Number of putative secretory proteins of phytonematodes	1,480					13 ^b		59	
Number of putative novel effectors	63					/		/	

^a To identify putative secretory proteins of *H. avenae*, sequences with a putative signal peptide and without a transmembrane region were selected in the present study. In addition, sequences with differential expression among the different developmental stages of *H. avenae*, transcripts that were up-regulated in the J2s compared with the eggs or in the pJ2s compared with the J2s, and sequences without matches to *Caenorhabditis elegans* sequences (e-value $\leq 1.0 \times 10^{-6}$) were also considered.

^b The statistical analysis performed by Kumar et al. (2014) only covers the known effectors of *Heterodera* spp.

with dsRNA of genes with embryonic lethal, lethal and larval lethal RNAi phenotypes resulted in reduced cyst formation compared with the control group (Fig. 6). Bioengineered crops expressing dsRNAs of phytonematode genes can disrupt the life cycles of parasitic nematodes; therefore, this strategy holds great promise for the development of PPN-resistant crops (Rehman et al. 2016). Notably, the targeting of genes involved in early parasitism using dsRNA-expressing transgenic plants appears less feasible because these genes are expressed before or soon after hatching; thus, the encoded proteins are present before the nematode enters the plant and initiates syncytium formation (Makunde 2012). It was not easy to allot notes to the transcripts and infer putative effector functions from transcriptomic data due to the lack of available reference genomes. The putative novel effectors without functional annotations are likely specific to *H. avenae* and play important roles in *H. avenae*-cereal interactions. These putative novel effectors of *H. avenae* are of interest for further study.

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