Similarity and Functional Analyses of Expressed Parasitism Genes in Heterodera schachtii and Heterodera glycines

NRUPALI PATEL,1 NOUREDDINE HAMAMOUCH,1 CHUNING LI,1 RICHARD HUSSEY,2 MELISSA MITCHUM,3 THOMAS BAUM,4 XIAOHONG WANG,5 ERIC L. DAVIS1

Abstract: The secreted proteins encoded by "parasitism genes" expressed within the esophageal gland cells of cyst nematodes play important roles in plant parasitism. Homologous transcripts and encoded proteins of the Heterodera glycines pioneer parasitism genes Hgycy6, Hg4602 and Hg5408 were identified and similarly expressed within the esophageal glands cells of Heterodera schachtii. Constitutive expression of Hs5406 and Hs5408 in transgenic Arabidopsis thaliana did not produce any visible phenotypic changes in plant growth and development nor affect the number of H. schachtii females that developed on roots of these plants. Constitutive and nematode-inducible Arabidopsis expression of double-stranded RNA complementary to Hsycy6, a parasitism gene with function similar to plant CLAVATA3/ESR peptides, resulted in a significant (P < 0.05) 36% decrease in H. schachtii female numbers and significant host-derived RNA interference-mediated silencing of Hsycy6 transcript levels in nematodes excised from those roots. Significant and specific effects of Arabidopsis host-derived RNAi complementary to Hg5406 or Hg4602 on H. schachtii female development were not observed, however, up to 20% reduction in H. schachtii female numbers were observed in roots of these plants.

Key words: Arabidopsis thaliana, beet cyst nematode, esophageal gland cells, Heterodera glycines, Heterodera schachtii, host-parasite relationship, parasitism genes, RNA interference, secretions, soybean cyst nematode, syncytium.

Cyst nematodes (Heterodera and Globodera spp.) are sedentary endoparasites of plant roots that infect major crops and cause significant economic losses (Chitwood, 2003). These pathogens are obligate biotrophs that have a unique interaction with their host plants. An infective J2 penetrates host plant roots and migrates intracellularly by secreting cell wall-degrading enzymes and piercing cell walls with its stylet (Wyss and Zunke, 1986). Upon reaching the root vascular tissue, successful transformation of selected plant cells into an elaborate feeding site (a syncytium) is required for nematode feeding, growth, and subsequent molts through remaining sedentary parasitic stages to reproductive maturity (Nilback et al., 2006). Cyst nematodes use their stylets to penetrate the cell wall of an initial syncytial cell and secrete proteins to induce syncytium formation via subsequent cell wall dissolution and fusion of adjacent plant cells, resulting in a multinucleate feeding site that is highly metabolically active (Mitchum et al., 2007; Hussey and Grundler, 1998). The bioactive secretions from the nematode stylet originate within the three enlarged esophageal gland secretory cells, two located subventrally and one cell located dorsally (Hussey, 1989).

"Parasitism genes" developmentally expressed within the esophageal gland cells encode potential effector proteins that may be secreted into the host to promote the parasitic interactions with the plant (Davis et al., 2000, 2004). Over fifty candidate parasitism genes of H. glycines have been isolated from cDNA libraries constructed from mRNA derived from the microaspirated contents of the esophageal gland cells of multiple parasitic stages of the nematode (Gao et al., 2001, 2003; Wang et al., 2001). While cell wall-modifying enzymes, chorismate mutase, and a number of predicted gene homologs were identified among the H. glycines parasitism genes, approximately seventy percent of these parasitism genes encoded predicted novel "pioneer" proteins. The H. glycines parasitism gene Hgycy6 (Wang et al., 2001) encodes a novel protein whose C-terminus was predicted by domain analyses (Olsen and Skriver, 2003) to be similar to a plant peptide in the CLAVATA3/Endosperm surrounding region (CLE/ESR) family (Mitchum et al., 2008). CLAVATA3 functions as a signaling peptide involved in plant meristem differentiation and stem cell maintenance (Clark, 2001; Mitchum et al., 2008). Amazingly, expression of Hgycy6 in a transgenic Arabidopsis thaliana cko3-1 mutant background complemented the phenotype and negatively regulated expression of the Arabidopsis wuschel gene, similar to the effects of overexpression of cko3 itself in plants (Wang et al., 2003; Mitchum et al., 2008). The 16d10 parasitism gene of root-knot nematode encodes a 13-amino acid mature secreted peptide with similarity to plant CLEs, but expressed 16d10 could not complement a cko3 Arabidopsis mutant (Huang et al., 2006a). Expression of 16D10 in transgenic Arabidopsis, however, induced accelerated root growth with no observable shoot phenotype, and specific protein-protein interactions of the 16D10 peptide with the SAW domain of Arabidopsis SCARECROW-like transcription factors was confirmed (Huang et al., 2006a).

Investigations of these CLE-like parasitism genes demonstrated the utility of the model plant species,
Arabidopsis thaliana (Pang et al., 1987) for functional analyses of candidate nematode parasitism genes. The utility of RNA-mediated interference (RNAi) as a gene silencing strategy to analyze the biological significance of candidate eukaryotic genes (Fire et al., 1998) has also been demonstrated in plant-parasitic nematodes (reviewed in Lilley et al., 2007). Induced ingestion of double-stranded RNA (dsRNA) by plant nematodes from a soaking solution has resulted in specific silencing of the complementary target gene, and in some assays, effects on plant parasitism by in vitro RNAi-treated nematodes were observed (Lilley et al., 2007). Recently, plant host-derived expression of dsRNA complementary to nematode genes in transgenic plants has been reported to induce RNAi of the target nematode gene (Fairbairn et al., 2007; Gheysen and Vanholme, 2007; Huang et al., 2006b; Steeves et al., 2006; Yadav et al., 2006). The only plant host-derived RNAi report to date to target a nematode parasitism gene (Huang et al., 2006b), the root-knot nematode 16D10 gene, resulted in almost no nematode development on the RNAi plants, suggesting a fundamental and essential function of the 16D10 parasitism gene product in host parasitism.

Two different parasitism genes from H. glycines, 4e02 and 5d08 (Gao et al., 2003), encode proteins that have no known similarities with other genes present in the NCBI database. Analysis of these two genes using the PSORTII program (Nakai and Horton, 1999) indicated the presence of a putative nuclear localization signal (NLS), suggesting that these secreted parasitism proteins may localize to the nucleus of recipient host plant cells (Gao et al., 2003). Expression of tagged Hg4E02 in transformed plant cells without the endogenous signal peptide did demonstrate specific nuclear localization in both onion cells and Arabidopsis protoplasts while similar transgenic expression of tagged Hg5D08 accumulated only in the plant cell cytoplasm (Elling et al., 2007). The presence of a functional NLS in Hg4E02 suggests that this protein may function directly within the plant cell nucleus to promote parasitism of the host. Even though Hg5D08 did not show active nuclear uptake in plant cells, this parasitism protein could function as a cytoplasmic effector molecule in host cells.

In this study, we make use of the plant model Arabidopsis thaliana as a host to analyze potential roles of the H. glycines candidate parasitism genes svy46, 4e02 and 5d08. Since H. glycines cannot infect Arabidopsis but the closely-related (Subbotin et al., 2001) beet cyst nematode (BCN), Heterodera schachtii can (Sijmons et al., 1991), we first identified homologs of Hgsyv46, Hg4e02 and Hg5d08 in H. schachtii. The potential effects of expressed Hs4E02 and Hs5D08 on host plant phenotype and effects host-derived RNAi to HsSyv46, Hs4e02, and Hs5d08 on nematode parasitic success were investigated in transgenic Arabidopsis plants.

**Materials and Methods**

**Nematode culture:** Heterodera schachtii were propagated on roots of greenhouse-grown cabbage (Brassica oleracea) plants and the nematode eggs were collected from cysts as previously described (Goellner et al., 2001). The eggs were hatched over water at 28°C on a Baermann pan to collect pre-parasitic (pre-J2). Mixed parasitic stages of H. schachtii were collected by root blending and sieving (Ding et al., 1998) of inoculated plants.

**Amplification of H. schachtii parasitism gene homology:** Frozen pellets of mixed parasitic stages of H. schachtii were ground with Lysis Matrix D beads (Q-Biogene, Irvine, CA) and liquid nitrogen by placing in a mini beadbeater (BioSpec Products Inc. Bartlesville, OK). Nematode total RNA was extracted using the Micro-Midi Total RNA purification system (Invitrogen, Carlsbad, CA) following the manufacturer's instructions including digestion with DNase I to remove potential reaction contaminants. The original HgSyv46, Hg4e02 and Hg5d08 cDNA clones were identified from expressed sequence tag (EST) analyses of a cDNA library constructed from mRNA derived from esophageal gland cells of mixed parasitic stages of H. glycines (Gao et al., 2003). To obtain the full length cDNA homologs in H. schachtii, 3’ and 5’ cDNA ends were amplified from total RNA using the GeneRacer kit (Invitrogen, Carlsbad, CA). Gene specific primers used in the gene racer 5’ and 3’ amplification (Table 1) were designed from the original H. glycines cDNA of each gene. The RACE products derived from H. schachtii template were cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA) for sequencing. Based on the sequencing results

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’4e02-Gene</td>
<td>CCTTGCAACTTCTGATCGAGATGTGTT</td>
</tr>
<tr>
<td>Racer-Reverse:</td>
<td>CCTCCTGTTCCGAGAAACAA</td>
</tr>
<tr>
<td>Nested 5’s4e02-Gene</td>
<td>CCTCCCTGTTCCGAGAAACAA</td>
</tr>
<tr>
<td>Racer-Reverse:</td>
<td>CCTCCTGTTCCGAGAAACAA</td>
</tr>
<tr>
<td>Hs16D10-ORF-Forward:</td>
<td>ATGGCCCTCCAGCCGCTCTCAATCG</td>
</tr>
<tr>
<td>Hs4e02-ORF-Reverse:</td>
<td>TTAAAGTGTTGGCGCCATTCCTTCTC</td>
</tr>
<tr>
<td>5’syg546-Gene</td>
<td>CCGCGTATTTGCTCCGAGAATCTTG</td>
</tr>
<tr>
<td>Racer-Reverse:</td>
<td>CGCATTGATTTATGATCGAGATGTT</td>
</tr>
<tr>
<td>Nested 5’syg546-Gene</td>
<td>CGCATTGATTTATGATCGAGATGTT</td>
</tr>
<tr>
<td>Racer-Reverse:</td>
<td>CGCATTGATTTATGATCGAGATGTT</td>
</tr>
<tr>
<td>Hs5d08-ORF-Forward:</td>
<td>ATGGCCAAACATTCTGAAAAATC</td>
</tr>
<tr>
<td>Hs5d08-ORF-Reverse:</td>
<td>CTGAACTGAGTGGCTGGTGTTG</td>
</tr>
<tr>
<td>5’s5d08-Gene</td>
<td>TTGGCAGGCGATGGTTTGCCATTCAATA</td>
</tr>
<tr>
<td>Racer-Reverse:</td>
<td>GCGCTTTGGAGGGAGCGGCAGC</td>
</tr>
<tr>
<td>Nested 5’s5d08-Gene</td>
<td>GCGCTTTGGAGGGAGCGGCAGC</td>
</tr>
<tr>
<td>Racer-Reverse:</td>
<td>GCGCTTTGGAGGGAGCGGCAGC</td>
</tr>
<tr>
<td>Hs5d08-ORF-Reverse:</td>
<td>ATGGTCGAGTCCATGTGTTT</td>
</tr>
<tr>
<td>Hs5d08-ORF-Reverse:</td>
<td>ATGGTCGAGTCCATGTGTTT</td>
</tr>
</tbody>
</table>

*Table 1.* Primer sequences used to isolate full-length Heterodera schachtii parasitism genes using the 5’ GeneRacer system (Invitrogen, Carlsbad, CA) with H. schachtii complementary DNA template. Primer design was based upon the reported sequence of the selected Heterodera glycines parasitism gene Hg4e02 (AF473826). Hg4e02 (AF473827), and Hs5d08 (AF473828).
of the 3' and 5' RACE products for each gene, forward and reverse gene specific primers of Hgyw46, Hg4e02 and Hg5d08 (Table 1) were used to amplify the full-length H. schachtii parasitism gene cDNA clones. The H. schachtii parasitism gene cDNA clones were subsequently cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA) for DNA sequence analysis.

**Sequence analyses:** Comparison of the nucleotide and predicted amino acid sequences of H. schachtii cDNA homolog to the H. glycines parasitism gene were conducted using the BLAST algorithm at National Center for Biotechnology Information (NCBI) (Tatusova and Madden, 1999). Sequence similarity searches to genes in NCBI database were conducted using the BLASTp program. Prediction of a signal peptide for secretion and the cleavage site was performed using the SIGNAL P 3.0 program (Bendtsen et al. 2004). Potential nuclear localization signal domains of the translated H. schachtii sequences were analyzed using PSORT II (Nakai and Horton, 1999).

**mRNA in situ hybridization:** Localization and developmental expression analyses of Hgyw46, Hg4e02 and Hg5d08 transcripts were performed on fixed pre-J2 and mixed parasitic stages of H. schachtii (De Boer et al., 1998) as described above. Specific forward and reverse primers for each cDNA clone were used to synthesize digoxigenin (DIG)-labeled sense (control) and antisense cDNA probes by asymmetric PCR (Gao et al., 2001). The DIG-labeled cDNA probes that hybridized to transcripts within the nematode specimens were detected colorimetrically using the method of De Boer et al. (1998) and observation of specimens under a compound photomicroscope.

**Immunolocalization of nematode parasitism proteins:** Polyclonal antibodies to H. glycines SV46, 4E02 and 5D08 proteins were produced by immunizing individual rabbits with two synthetic peptides designed to specific predicted amino acid sequence of each gene product including: SV46 - NH2-STGDKKTANDGSSNN-COOH; NH2- PNVEKRSPLPG PDPH-COOH; 5D08 - NH2-SKPNPGKPSGERRK-COOH; NH2-VNRNGWENTGTPG GR-COOH; and 4E02 NH2-RGGWPDWAGK-COOH; NH2-KQILCKTSANCKCKD-COOH (Eurogentec, Inc., Belgium). Localization of expressed gene products with each specific antiserum within nematode specimens was detected by epifluorescence compound microscopy of the treated fixed mixed parasitic stages of H. schachtii according to Geollier et al. (2000).

**Expression of Hs4E02 and Hs5d08 in Arabidopsis thaliana:** Expression assays of Hs4e02 and Hs5d08 in transformed wildtype Arabidopsis plants were conducted to assess potential affects of transgene expression on plant phenotype. The β-glucuronidase gene of the binary vector pB121 (Clonetech, Palo Alto, CA) was replaced at the BamHI and SacI sites with the coding region of Hs4e02 or Hs5d08 with and without the signal peptide sequence. The constructs were each introduced into wildtype Arabidopsis (ecotype Columbia-0) via agrobacterium-mediated floral dip transformation (Clough and Bent, 1998). Transgenic plant lines were selected on 50 μg/mL kanamycin. Total RNA from transgenic plant material was extracted using RNeasy Plant Mini Kit following manufacturer’s instructions (Qiagen, Valencia, CA). The expression of Hs4e02 and Hs5d08 transgenes were confirmed by RT-PCR on total RNA of leaf tissues on homoyzogous lines by first strand cDNA synthesis using Superscript II RT kit and PCR amplification using 4E02 primers 5'TTCTGGCCCGCTC TTGCCTGTCTCT3' and 5'TTAATGGTTGGTCCTC TCCGC3' and 5D08 primers 5'TGCCGTTTTATAT TGACCTAA3' and 5'TCATGCTGGCGTTGAG CGC3'. Four homoyzogous Hs4e02- and Hs5d08-expressing Arabidopsis lines were grown in Murashige-Skoog (MS) media plates placed vertically for root growth assays in a 24°C plant growth chamber with 16-hour light/8-hour dark cycle.

**Host derived-RNAi - vector construction and analyses of transgenic lines:** Full-length Hs4e02, Hs5d08 and partial Hgyw46 cDNA sequences (70bp-240bp) were cloned in the sense and antisense orientation at the Xhol – KpnI and BamHI – HindIII restriction sites respectively of the pHANNIBAL RNAi vector containing the CaMV 35S promoter (Wesley et al., 2001). A control RNAi vector that contained cDNA of complementary strands of the green fluorescent protein (GFP; U87974) was also constructed in 35S::pHANNIBAL. A nematode-inducible RNAi vector was constructed by replacing the CaMV 35S promoter in pHANNIBAL with the 1.5 kb promoter of the Nicotiana tabacum cellulase 7 (Ncel7) gene that has demonstrated upregulation within cyst and root knot nematode feeding cells (Goeillner et al., 2001; Wang et al., 2007). All pHANNIBAL clones containing the sense and antisense gene of interest were subcloned as NotI fragments into the binary vector pART27 (Gleave, 1992). The constructs were introduced into wildtype Arabidopsis (ecotype Columbia-0) via agrobacterium-mediated floral dip transformation. Transformant (T1) lines designed to express dsRNA via the Ncel 7 promoter were selected on kanamycin for 10 days after which some seedlings were transferred to soil for T2 seed collection. Confirmation of the presence of the transgene within the plant genome was conducted using plant DNA template and parasitism gene-specific primers in PCR. Leaf samples from transformed Arabidopsis lines were used to analyze transgene expression of the PDK intron of each hairpin dsRNA (Wesley et al., 2001) using RT-PCR. Total RNA from plant material was extracted using the RNeasy Plant Mini Kit following manufacturer’s instructions (Qiagen, Valencia, CA). RT-PCR analysis for PDK intron expression was conducted using the First-Strand cDNA Synthesis kit using Superscript II RT (Invitrogen, Carlsbad, CA). Gene-specific primer PDK-RT-R: 5’ATCAATGAT AACACAA TGGCATTAA3' was used to make first strand cDNA
which was used as a template for amplification of a 300bp amplicon using primers pltronF: 5’GACGGAAGAAHATAAAAGTTGAGAG3' and pltronR: 5’TTGATAAATTCAAGCAGATTGG A3’. Products of RT-PCR were separated by agarose gel electrophoresis to assess expression of the RNAi construct in transgenic Arabidopsis lines.

H. schachtii infection assays: Twenty-four seedlings (5 days post germination) from each transgenic Arabidopsis line that were selected on MS media supplemented with 50 µg/ml kanamycin were aseptically transferred as one seedling per well in six-well culture plates (Falcon, Lincoln Park, NJ) containing 6 ml of sterile modified Knops medium (Sijmons, et al., 1991) solidified with 0.8% Daishin agar (Brunschwig Chemie BV, Amsterdam, Netherlands). The plates were sealed twice with parafilm and placed in a 24°C plant growth chamber with 16-hour light/8-hour dark cycle for 7 days before nematode inoculation. Hatched H. schachtii pre-J2 were collected as described above and surface-sterilized by incubating the pre-J2 for 10min in 0.004% mercuric chloride, 0.004% sodium azide, and 0.002% Triton X and subsequently rinsed three times with sterile distilled water. The surface-sterilized pre-J2 were suspended at a concentration of 10 J2/10 µ1 in 35°C 1.5% low melting point agarose (LMA) to allow even distribution of nematodes to each plate and to facilitate the penetration of the J2 into the solid growth medium. Each plant was inoculated with approximately 60 J2 on Nicotiana tabacum 7-RNAi plants lines and 100 J2 on the 35S-RNAi plants lines after which the plates were re-sealed with parafilm and placed back in the plant growth chamber. Statistical differences in number of H. schachtii females observed per root system at 21 days post-inoculation were determined by the paired t-test with an alpha level of 0.05 using the SAS (Cary, NC) statistical software package.

Quantitative RT-PCR (qRT-PCR) of parasitism gene expression in H. schachtii upon infection of host-derived RNAi plants: Transgenic Arabidopsis plants confirmed to express hairpin dsRNA to target nematode genes via the 35S::pHANNIBAL construct were grown on modified Knops medium (Sijmons, et al., 1991) with 0.8% Daishin agar (Brunschwig Chemie BV, Amsterdam, Netherlands). The plates were grown in a growth chamber at 24°C with 16-hour light/8-hour dark cycle. Each plate was grown vertically for 10 days before being inoculated with sterilized pre-parasitic H. schachtii J2. Plates were viewed under a dissecting microscope and any parasitic J3 nematodes observed were hand-dissected out of the roots using sterilized forceps. The mRNA from parasitic J3 H. schachtii excised individually from dsRNA-expressing Arabidopsis lines was isolated using Dynabeads mRNA DIRECT micro kit (Invitrogen, Carlsbad, CA) and DNase treated with Turbo DNA-free kit (Ambion, Austin, TX) according to the manufacturer’s instructions. First strand cDNA synthesis was made from 10ng of parasitic nematode mRNA using SuperScript II RT (Invitrogen, Carlsbad, CA). The cDNA was used as a template for qRT-PCR which was performed in the DNA Engine Opticon2 (Biorad, Hercules, CA). A single 20 µl PCR reaction included 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 3 µl cDNA template and 5 µM each forward and reverse primers (Table 2). The PCR cycling parameters were set at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C. After the completion of the cycling parameters, dissociation melt curve analyses (60-90°C every 0.5°C for 1 sec) was conducted to discount the effects of primer dimmer formation and contaminations. The qRT-PCR reactions were performed in triplicate and the negative controls included mRNA extracted from the nematodes to check for DNA contamination in the samples and a no DNA control. Each sample was normalized against the actin gene control (AY443352). The fold change relative to the nematodes infecting the control lines was calculated according to the 2-ΔΔCT method (Livak and Schmittgen, 2001). A qRT-PCR assay of a non-target parasitism gene in each reaction was also included to verify potential specificity of RNAi effects. A paired t-test with an alpha level of 0.05 was used to compare relative transcript level means using the SAS statistical software package.

RESULTS

Isolation of selected parasitism gene homologs in H. schachtii: A cDNA (Hs4e02) of 279 bp encoding a predicted 93 amino acid protein was amplified from mRNA of H. schachtii using PCR primers designed from the Hs4e02 (AF473826) parasitism gene (Gao et al., 2003). Blastp analysis of Hs4E02 did not result in any significant homology to any known proteins within the NCBI

---

Table 2. Forward and reverse primers used for quantitative real-time RT-PCR to evaluate the differential expression of selected parasitism genes from parasitic stages of Heterodera schachtii that were excised from roots of transgenic Arabidopsis thaliana plants.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSActin</td>
<td>CAGTGACTGACTGACCTACCT</td>
<td>CGTGACCAAGACTTGCTTCTTG</td>
</tr>
<tr>
<td>Hs4e02</td>
<td>ATTCGCGGCGAGCAACACTG</td>
<td>GGGCGTAGCGCTCCTGGTCATT</td>
</tr>
<tr>
<td>Hs5d08</td>
<td>TCGAGGCGCGGAGAGCCCTTTGGA</td>
<td>GGTAGAGCGCGCGGCTTTTTC</td>
</tr>
<tr>
<td>Hsaly46</td>
<td>ACCGCTGGCTGTCTTCA</td>
<td>CCGCATTCTCCGCGAGCAA</td>
</tr>
</tbody>
</table>

---
Alignment of the full-length complimentary DNA of parasitism gene 4E02 of Heterodera schachtii (Hs-4E02) and Heterodera glycines (Hg-4E02; AF473826) demonstrate a 99% nucleotide sequence identity.

The gene Hg5D08 (AF473828) is a candidate parasitism gene (Gao et al., 2003) that also does not have significant homology to any known sequence in the NCBI database. The Hs5D08 cDNA (411 bp) was amplified from H. schachtii mRNA using Hg5D08 PCR primers and showed a 98% nucleotide identity (Fig. 2) and a 96% predicted amino acid identity to the Hg5D08 sequence. Hs5D08 also possessed a predicted secretion signal peptide that is cleaved between position 18 (Alanine) and 19 (Valine) to derive the mature secreted protein. PSORT II analyses indicated that, like in H. glycines, both Hs4E02 and Hs5D08 possess a predicted nuclear localization signal (NLS) that suggests that the secreted proteins are potentially targeted for import into the host cell nucleus. The Hgsyv46 parasitism gene product is unusual (Mitchum et al., 2008; Wang et al., 2001, 2005) in that the C-terminal domain contains a motif similar to that of the CLAVATA3/ESRrelated (CLE) family in Arabidopsis thaliana. An Hgsyv46 cDNA was amplified from H. schachtii mRNA using Hgsyv46 PCR primers that had a 95% nucleotide identity (Fig. 3) and a 92% predicted amino acid identity to Hgsyv46 (AF473827). HsSYV46 possessed a predicted secretion signal peptide that would be cleaved between position 24 (Thrreonine) and 25 (Aspartic acid) of the protein.

Parasitism gene expression and translation in H. schachtii: The localization of expression of selected parasitism genes within developmental stages of H. schachtii was analyzed by mRNA in situ hybridization (Deboer

Fig. 1. The nucleotide alignment of full-length complimentary DNA of parasitism gene 4E02 of Heterodera schachtii (Hs-4E02) and Heterodera glycines (Hg-4E02; AF473826) demonstrate a 99% nucleotide sequence identity.

Fig. 2. The nucleotide alignment of full-length complimentary DNA of parasitism gene 5D08 of Heterodera schachtii (Hs-5D08) and Heterodera glycines (Hg-5D08; AF473828) demonstrate a 98% nucleotide sequence identity.
et al., 1998). The digoxigenin-(DIG) labeled antisense cDNA probes of Hs4e02, Hs5d08 and Hssyv46 hybridized exclusively within the esophageal gland cells of the nematodes. Specifically, the 4e02 DIG-probes hybridized to transcripts expressed within the two subventral esophageal gland cells of pre-parasitic J2 and parasitic J2, J3 and J4 of H. schachtii (Fig. 4A and 4B). The Hssyv46 and Hs5d08 DIG-probes hybridized to transcripts expressed within the single dorsal esophageal gland cell of parasitic J2, J3 and J4 (Fig 4C and 4D) of H. schachtii, but not within pre-J2.

Immunolocalization of the parasitism gene products within the esophageal gland cells and the gland cell extension was observed for parasitism gene products SYV46 and 5D08. Binding of the anti-HgSYV46 sera (Wang et al., 2005) to translated HsSYV46 within the dorsal gland cell of H. schachtii was observed (Fig. 5A). The anti-Hg5D08 sera bound to the Hs5D08 protein within secretory granules that migrated to the dorsal gland extension connecting to the ampulla (collecting reservoir) at the base of the stylet (Fig 5B). No binding of the anti-Hg4E02 sera was observed within nematode specimens of any life stage of H. glycines or H. schachtii.

Expression of Hs4E02 and Hs5D08 in transgenic Arabidopsis thaliana: The effects of expressing the HgSYV46 parasitism gene in transgenic Arabidopsis has been previously reported as similar to plant CLV3 (Wang et al., 2005). To assess whether the novel 93-amino acid Hs4e02 protein, which possesses a putative nuclear localization signal (NLS), affects plant phenotype, the Hs4e02 cDNA was constitutively expressed (with and without the nematode signal peptide) in transformed Arabidopsis. The exclusion of the signal peptide should target the protein within the cytosol of the transformed plant cells and the presence of the signal peptide should target the protein to the host cell (extracellular) secretory pathway if the nematode signal peptide has similar activity in plant cells. The expression of Hs4e02 in three independent transgenic Arabidopsis lines was confirmed by RT-PCR (data not shown) of the full-length transcript. No visible differences in root or shoot growth were observed in plants that expressed Hs4e02 with or without the signal peptide compared to the

---

**Fig. 3.** The nucleotide alignment of full-length complimentary DNA of parasitism gene *syv46* of *Heterodera schachtii* (Hs-SYV46) and *H. glycines* (Hg-SYV46; AF473827) demonstrate a 93% nucleotide sequence identity.

**Fig. 4.** In situ hybridization of digoxigenin-labeled antisense complementary DNA (cDNA) probes (dark staining) of selected parasitism genes to transcripts specifically expressed within the subventral (SvG) or dorsal (DG) esophageal gland cells of *Heterodera schachtii*. A. cDNA probe of Hs4e02 binding in the SvG of a pre-parasitic second-stage juvenile (J2). B. cDNA probe of Hs4e02 binding in the SvG of a parasitic J3 excised from wild-type host roots. C. cDNA probe of Hssyv46 binding in the DG of a parasitic J3. D. cDNA probe of Hs5d08 binding in the DG of a parasitic J4.
control transgenic Arabidopsis plant that expressed GUS (data not shown). No significant difference in the number of developed nematode females was observed three weeks post inoculation when compared to the control GUS lines in infection assays with *H. schachtii* of transgenic Arabidopsis lines that were confirmed to express Hs4E02 without the signal peptide (data not shown).

The *Hs5d08* parasitism gene encodes a novel protein with a NLS sequence predicted by PSORT II. Similar to Hs4E02, although Hs5D08 expression was confirmed by RT-PCR in transgenic Arabidopsis lines, there were no visible effects of Hs5D08 expression on plant phenotype compared to control plants (data not shown).

**Host-derived RNA-interference of the Hssyv46, Hs4e02 and Hs5d08 parasitism genes:** Plant host-derived RNAi (Gheysen and Vanholme, 2007) was used as a method to potentially silence the expression of the target parasitism genes within the nematode and observe the consequential effects on parasitism of its host. Two pHANNIBAL (Wesley et al, 2001) constructs (Fig 6A) were generated with different promoters to express the hairpin dsRNA in transformed Arabidopsis. The two promoters included the CaMV 35S (constitutive) promoter and the tobacco *Cel7* gene promoter (*Ncel7*) that has limited tissue-specific expression in plants and has been demonstrated to be upregulated in cyst and root-knot nematode feeding sites in heterologous plants species (Wang et al., 2007). After kanamycin selection of transgenic Arabidopsis lines possessing the sense and antisense strands of the nematode target gene, two or three recovered independently-transformed homogenous Arabidopsis lines with either the *Ncel7* promoter or the 35S promoter to drive hairpin dsRNA expression of *Hssyv46*, *Hs4e02*, and *Hs5d08* were confirmed for dsRNA expression by RT-PCR of the single-stranded hairpin loop (Wesley et al., 2001) of the PDK intron (Fig 6A). No visible differences in root or shoot phenotype of all the RNAi plants lines were observed compared to the control plants. The lines confirmed to express dsRNA complementary to the target parasitism gene were used to assess potential effects on *H. schachtii* by counting the number of established females on each root system at 21 days post inoculation.

Homogenous Arabidopsis L8 and L10 transgenic lines that expressed *Ncel7* promoter-driven dsRNA complementary to *Hssyv46* showed a significant (*P ≤ 0.05*) 36% reduction in the number of females compared to the control *Ncel7* empty vector RNAi plant lines (Fig 6B). Two T2 lines expressing CaMV 35S-driven dsRNA of *Hssyv46* showed a significant (*P ≤ 0.05*) 32% reduction in the number of established females when compared to the control lines that expressed 35S-driven hairpin dsRNA of the non-target green fluorescent protein (GFP) gene (Fig. 6C). Quantitative real-time PCR of nematodes excised from the Arabidopsis *Hssyv46* RNAi lines indicated that transcript levels of endogenous *Hssyv46* were significantly (*P ≤ 0.05*) reduced compared to nematodes infecting control plants expressing dsGFP (Table 3). The transcript levels of a non-target
endogenous parasitism gene, Hs4e02, in H. schachtii excised from Hssyv46-RNAi plants were not significantly decreased as compared to nematodes excised from dsGFP control plants, indicating that the observed RNAi-silencing of Hssyv46 was specific.

Statistically significant differences in H. schachtii female development were not demonstrated in Arabidopsis lines confirmed to express dsRNA to either Hs4e02 or Hs5d08, even though up to 20% reduction in female numbers compared to controls were observed on some plant host-derived RNAi lines driven by the Ntce7 or CaMV 35S promoter (Figs. 7 and 8). Significant (P ≤ 0.05) silencing of endogenous nematode Hs4e02 transcript levels were observed compared to controls in Hs4e02-RNAi plants (Table 3) while a significant fold increase of non-target Hssyv46 transcripts were observed in Hs4e02-RNAi plants. No significant silencing of endogenous nematode Hs5d08 transcript levels were observed compared to controls in Hs5d08-RNAi plants (Table 3), however, silencing of endogenous Hs5d08 transcripts were approximately one and a half-fold greater than silencing of endogenous non-target Hssyv46.

**DISCUSSION**

The striking inter-species sequence identity between the H. schachtii homologs of the H. glycines syv46, 5d08 and 4e02 pioneer parasitism genes suggests that the two nematode species share common parasitic mechanisms even though their host plant species vary. This comparison was further supported by similar developmental expression of the H. schachtii parasitism genes within the esophageal glands cells as confirmed by mRNA in
situ hybridization and immunolocalization of the translated parasitism gene products with polyclonal sera raised to the peptides predicted from the *H. glycines* parasitism genes. These data suggest the potential utility of *H. schachtii* and *Arabidopsis thaliana* as a model system for functional analyses of *H. glycines* parasitism genes in soybean.

The *syr46* parasitism gene (Wang et al., 2001) is unique in that it contains a biologically functional C-terminal domain of the CLAVATA/ESR (CLE) family in *Arabidopsis thaliana* (Mitchum et al., 2008; Olsen and Skriver, 2003; Wang et al., 2005). The exclusion of the nucleotide sequence encoding the C-terminal CLE-like domain of *HsSYV46* in the RNAi constructs used here to transform Arabidopsis proved to be a useful strategy to avoid potential off-target effects on plant growth and development. The host-derived RNAi assays that targeted *Hssyv46* resulted in 32-36% reduction in *H. schachtii* females on roots of transgenic plants, supporting a significant role of *HsSYV46* in the parasitic interaction. Similarly, hatched *H. glycines* J2 induced to ingest *Hssyv46* dsRNA from a soaking solution produced a lower percentage of females (40%) and a higher percentage of males on host roots compared to control lines that supported approximately 78% females (Bakhetia et al., 2007). Although the number of males was not assessed in this study, the reason for the reduction in established females could be a result of a

---

**TABLE 3.** Relative endogenous expression levels of parasitism genes within *Heterodera schachtii* specimens that were excised from roots of transgenic RNA interference (RNAi) *Arabidopsis thaliana* plant lines that constitutively (CaMV 35S promoter) expressed double-stranded RNA targeted to individual parasitism genes. Expression of a non-target *H. schachtii* parasitism gene was also monitored in each Arabidopsis line to assess target gene specificity of any observed RNAi effect.

<table>
<thead>
<tr>
<th>Arabidopsis RNAi Lines</th>
<th>Parasitism Gene Analyzed</th>
<th>Relative fold Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S- <em>Hs4e02L1</em></td>
<td><em>Hs4e02</em></td>
<td>-2.18 ± 0.22*</td>
</tr>
<tr>
<td>35S- <em>Hs4e02L1</em></td>
<td><em>Hssyv46</em> (non-target gene)</td>
<td>2.8 ± 0.76*</td>
</tr>
<tr>
<td>35S- <em>Hssyv46L1</em></td>
<td><em>HsSyV46</em></td>
<td>-2.2 ± 0.04*</td>
</tr>
<tr>
<td>35S- <em>Hs4e02L1</em></td>
<td><em>HsSyV46</em> (non-target gene)</td>
<td>-0.5 ± 0.4</td>
</tr>
<tr>
<td>35S- <em>Hs5d08L7</em></td>
<td><em>Hs5d08</em></td>
<td>-1.2 ± 0.4</td>
</tr>
<tr>
<td>35S- <em>Hs5d08L7</em></td>
<td><em>Hssyv46</em> (non-target gene)</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

* A significant (*P < 0.05) fold difference in parasitism gene expression when compared to the same parasitism gene expression in *H. schachtii* excised from roots of transgenic 35S-GFP-RNAi Arabidopsis used as a non-nematode gene (negative) control.

---

**Fig. 7.** Number of *Heterodera schachtii* females that developed on roots of transgenic *Arabidopsis thaliana* lines that expressed double-stranded RNA (dsRNA) to induce RNA interference (RNAi) to the target nematode parasitism gene *Hs4e02*. A. RT-PCR indicates expression of the PDK intron region of the *Hs4e02* hairpin dsRNA in *Ntce7* RNAi lines (construct design detailed in Fig. 6A) that were each inoculated with sixty *H. schachtii* infective juveniles. No significant reduction in female numbers on *Ntce7-Hs4e02* RNAi lines compared to Arabidopsis containing the empty vector construct (control) were determined by paired t-test (*p* < 0.05). B. RT-PCR indicates expression of the PDK intron region of the *Hs4e02* hairpin dsRNA in 35S RNAi lines (construct design detailed in Fig. 6A) that were each inoculated with one hundred *H. schachtii* infective juveniles. No significant reduction in female numbers on 35S-*Hs4e02* RNAi lines compared to Arabidopsis that expressed hairpin dsRNA complementary to the green fluorescent protein (GFP) transcript (a non-nematode target gene negative control) were determined by paired t-test (*p* < 0.05). Bars indicate ± standard error; *n* = 24.
shift to male development that is a characteristic of nematode stress environment (Lilley et al., 2005). The partial resistance observed on the H. schachtii females that developed on roots of transgenic Arabidopsis thaliana lines that expressed double-stranded RNA (dsRNA) to induce RNA interference (RNAi) to the target nematode parasitism gene Hs5d08 (Bakhetia et al., 2007) could not provide transcript levels after the dsRNA soaking treatment and attributed this to the very low levels of Hs5d08 transcript levels throughout the lifecycle in a quantitative manner, as observed with H. glycines throughout the life cycle, Bakhetia et al. (2007) could not provide transcript levels after the dsRNA soaking treatment and attributed this to the very low levels of Hs5d08 before root invasion making it difficult to detect any further reduction following the RNAi effect. Time-course assessment of Hs5d08 transcript levels throughout the H. glycines lifecycle (up to 21 day post invasion) showed a significant increase in mRNA abundance by 2 days post invasion, however later stages in the time-course Hs5d08 remained stable at relatively high levels (Bakhetia et al., 2007). Incomplete silencing of this transcript abundance by RNAi could potentially affect the nematode fate during the lifecycle in a quantitative manner, as observed with female development in H. schachtii RNAi plants. The seemingly minimal reduction in Hs5d08 expression determined from viable nematodes excised from RNAi plants could also be an artifact of obtaining only nematodes that survived to the J3 stage, masking any potential complete disruption of parasitism (and death) by nematodes within the same root system. The ability for some nematodes to survive to later parasitic stages in RNAi plant lines could also be an artifact of obtaining only nematodes that developed on roots of transgenic Arabidopsis thaliana lines that expressed double-stranded RNA (dsRNA) to induce RNA interference (RNAi) to the target nematode parasitism gene Hs5d08 (Bakhetia et al., 2007, A. RT-PCR indicates expression of the PDK intron region of the Hs5d08 hairpin dsRNA in NtCel7 RNAi lines (construct design detailed in Fig. 6A) that were each inoculated with sixty H. schachtii infective juveniles. No significant reduction in female numbers on NtCel7 Hs5d08 RNAi lines compared to Arabidopsis containing the empty vector construct (control) were determined by paired t-test (p < 0.05). B. RT-PCR indicates expression of the PDK intron region of the Hs5d08 hairpin dsRNA in 35S RNAi lines (construct design detailed in Fig. 6A) that were each inoculated with one hundred H. schachtii infective juveniles. No significant reduction in female numbers on 35S-Hs5d08 RNAi lines compared to Arabidopsis that expressed hairpin dsRNA complementary to the green fluorescent protein (GFP) transcript (a non-nematode target gene negative control) were determined by paired t-test (p < 0.05). Bars indicate ± standard error; n = 24.
stages of cyst nematode parasitism (Bertioli et al., 1999; Wang et al., 2007).

The confirmation of a functional NLS to import the 4E02 (but not 5D08) cyst nematode parasitism gene product to a plant nucleus, has suggested a potential functional role of secreted 4E02 within a recipient host cell nucleus (Elling et al., 2007). Constitutive expression of nematode parasitism genes Hs4e02 and Hs5d08 within Arabidopsis, however, did not result in visible phenotypic changes in plant root and shoot development, nor any significant affect on the development of H. schachtii females in roots of these Arabidopsis lines. Host-derived RNAi of Hs4e02 and Hs5d08 did not result in significant suppression in development of H. schachtii females on roots as compared to the GFP-RNAi control plants, however up to 20% reduction in female development was observed in some Hs4e02-RNAi and Hs5d08-RNAi plant lines.

The lack of confirmed specific and significant silencing of endogenous Hs4e02 and Hs5d08 within H. schachtii excised from RNAi plant roots is consistent with the minimal effects observed on nematode development. As with Hs4e02-RNAi above, including DNA gel blots that suggest multigene families of Hs4e02 and Hs5d08 within H. schachtii (Mitchum, M.G., unpublished), sufficient silencing of either parasitism gene to induce complete suppression of parasitism could not be achieved by targeting a single cyst nematode parasitism gene with the host-derived RNAi constructs used in the current study. In contrast, targeting the single 16D10 parasitism gene of root-knot nematodes via host-derived RNAi resulted in almost complete disruption of nematode parasitism of host roots (Huang et al., 2006b), suggesting the essential nature of the 16D10 gene product in the parasitic interaction. Since multiple parasitism genes also exist in root-knot nematodes (Huang et al., 2003, 2004), the choice of parasitism gene targeted by RNAi may be critical to the effect observed on parasitism. The parasitism genes and ontogenies of the parasitic interactions of root-knot and cyst nematodes differ substantially, so it remains unknown if a single cyst nematode parasitism gene is essential for the interaction that could be targeted for silencing and complete parasitic disruption. Future host-derived RNAi experiments could potentially target multiple cyst nematode parasitism genes to assess potential effects on nematode infection of plant roots. The range in observations on the effect of RNAi on parasitism genes observed here substantiates potential effects on nematode infection of plant roots. The parasitism genes and ontogenies of the parasitic interactions of root-knot and cyst nematodes differ substantially, so it remains unknown if a single cyst nematode parasitism gene is essential for the interaction that could be targeted for silencing and complete parasitic disruption. Future host-derived RNAi experiments could potentially target multiple cyst nematode parasitism genes to assess potential effects on nematode infection of plant roots. The range in observations on the effect of RNAi on parasitism genes observed here substantiates potential effects on nematode infection of plant roots.

**Literature Cited**


Huang, G. Z., Dong, R. H., Maier, T., Davis, E. L., Baum, T. J., and Hussey, R. S. 2004. Use of solid-phase subtractive hybridization for the identification of parasitism gene candidates from the root knot...
nematode *Meloidogyne incognita*. Molecular Plant Microbe Interactions 5:217-222.


Molecular and Plant Biology 33:991-999.


Yadav, B. C., Veluthambi, K., and Subramaniam, K. 2006. Host generated double-stranded RNA induces RNAi in plant parasitic nematodes and protects the host from infection. Molecular and Biochemical Parasitology 148:219-222.