ABSTRACT

PATEL, NRUPALI. Functional Analyses of Cyst Nematode Parasitism Genes. (Under the direction of Eric Davis).

Cyst nematodes in the genus *Heterodera* are microscopic worms that penetrate the roots of host plant species and become sedentary endoparasites that can cause extensive damage to a crop. The feeding of cyst nematodes within plant roots requires the transformation of selected plant root cells into elaborate feeding sites called syncytia. Syncytia are induced by proteins produced in the esophageal gland cells of infecting nematodes that are secreted into plant tissues via its stylet (oral spear). The characterization and functional analyses of selected cyst nematode parasitism genes that encode such secreted proteins was the objective of this dissertation. Homologs of four parasitism genes initially isolated from the soybean cyst nematode, *Heterodera glycines*, including *Hg4F01* (encoding an annexin-like protein), *HgSYV46* (encoding a CLAVATA3-like plant signaling peptide), *Hg4E02* and *Hg5D08* (encoding novel proteins with putative host nuclear localization) were isolated from the beet cyst nematode, *Heterodera schachtii*, which can infect the model plant species, *Arabidopsis thaliana*. Greater than 90% nucleotide and predicted amino acid identity existed between cDNA isolates of the four parasitism genes homologs between *H. glycines* and *H. schachtii*, and mRNA i*n situ* hybridization and immunolocalization confirmed the expression of each gene product exclusively within the esophageal gland cells in both nematode species. Since eukaryotic annexins affect a number of cellular processes involving calcium-dependent membrane association, the potential function of the Hs4F01 annexin-like protein secreted into plant cells was extensively analyzed. Similar to annexin mutants in Arabidopsis, expression of Hs4F01 in transformed Arabidopsis produced no observable plant phenotype,

however, a significantly greater number of *H. schachtii* females developed on roots of Arabidopsis that expressed Hs4F01. Hypersensitivity to osmotic stress in an Arabidopsis *annAt1* annexin mutant was reduced (complemented) in transformed mutants that expressed Hs4F01, suggesting a functional similarity of the nematode and plant annexins within plant cells. Expression of double-stranded RNA that was complimentary to *Hs4F01* in transformed Arabidopsis plants induced specific RNA interference (RNAi) silencing of *Hs4F01* transcripts in nematodes that infected the plants and signifcantly reduced the number of *H. schachtii* females that developed on roots of the same *Hs4F01*-RNAi plants. Expression of *Hs4E02* and *Hs5D08* in Arabidopsis produced no observable effects on plant phenotype and susceptibility to *H. schachtii* was not altered in plants that expressed *Hs4E02*. Specific RNAi silencing of *HsSYV46* and *Hs5D08* transcripts was demonstrated in nematodes extracted from roots of Arabidopsis that constitutively expressed double-stranded RNA complementary to each respective gene by the CAMV 35S promoter, but specific silencing of *Hs4E02* could not be demonstrated in 35S-*Hs4E02*-RNAi plants. A significant reduction in development of *H. schachtii* females was observed on roots of Arabidopsis that expressed double-stranded RNA to parasitism genes *Hs4F01* and *HsSYV46* driven by either the nematode-inducible *Ntcel 7* promoter or the constitutive CaMV 35S promoter. Expression of dsRNA to *Hs4e02* and *Hs5d08* in Arabidopsis behind either promoter did not significantly affect the number of females per plant when challenged with *H. schachtii*. In summary, the products of cyst nematode parasitism genes confirmed to have cellular functions similar to their plant homologs, including Hs4F01 (annexin-like protein) and HsSYV46 (CLAVATA/ESR-like peptide), were demonstrated by RNAi to have a significant biological role in cyst nematode parasitism of host plant roots.

Functional Analyses of Cyst Nematode Parasitism Genes

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Plant Pathology

Raleigh, North Carolina

2008

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BIOGRAPHY

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ACKNOWLEDGEMENTS

I would like to thank first and foremost my major advisor, Dr Eric Davis for his guidance and support throughout my graduate career at North Carolina State University. He has given me the flexibility and freedom to work on my thesis and given generously of his time when I sought assistance. I am grateful to my committee members for their guidance, encouragement and support throughout my study. I would like to thank the many funding agencies including United Soybean Board grants 5214 & 7214, USDA-NRI grant 2006-35607-16601, the NC Agricultural Research Service, and the NCSU Graduate School.

I would like to express my gratitude to the past and current members of the Eric Davis Lab, Chunying Li, Hanane Diab, Diane Meyers, Xiaohong Wang, Noureddine Hamamouch, Bingye Xue, Laura Hudson, Adrienne Smith and the many undergraduate members.

I am eternally grateful to my wonderful family who have always supported and appreciated my efforts in receiving this degree. I am blessed to have the support and love from Dr. Pranav Pandya who has always guided me through my personal and professional endeavors. Finally I would like to express my heartfelt gratitude to my wonderful husband, Manish, who even though has only been in my life for 2 years, has provided me with love, support and encouragement during the final and probably the most challenging phase of my Ph.D degree.

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LITERATURE REVIEW

General Introduction

The phylum Nematoda comprises a large number of animal species including the genetic model *Caenorhabditis elegans* (Williamson and Kumar, 2006). A relatively small portion of the phylum, however, includes two of the most economically important organisms, the animal- and plant-parasitic nematodes (Williamson and Kumar, 2006). Of these, the plant-parasitic nematodes cause annual damage to most crops worldwide resulting in billions of dollars in losses (Lilley et al., 2007). The root-knot nematodes (*Meloidogyne* species) and the cyst nematodes (*Heterodera* and *Globodera* species) induce the majority of the reported crop losses (Lilley et al., 2007). These sedentary endoparasitic nematodes induce elaborate modifications for feeding within host plant roots and deplete nutritional plant resources while evading or suppressing host defenses resulting in crop damage and major yield losses.

The bulk of management practices to reduce crop damage by plant-parasitic nematodes rely on integrating chemical nematicides, nematode-resistant varieties, and cultural practices such as crop rotation. Chemical control methods are effective in controlling nematode populations among crop hosts but are limited by the costs of treatment and government regulation of the toxicity and potential environmentally harmful effects of nematicides (Lilley et al., 2005; Mitchum et al., 2007). Resistant plant varieties are useful in controlling some nematodes but can take several years to develop through breeding programs and natural sources of resistance to nematodes are not available for many crops (Roberts, 1992). Furthermore, continuous cropping of race-specific resistance can select for new

nematode races that can overcome the resistant varieties (Noel, 2007). Crop rotation is valuable for controlling nematodes that have a narrow host range (ie. *Heterodera* species) but losses in economic returns are imposed when the alternative non-host crop results in less economic value.

The continued demand for cost-effective and environmentally-friendly pest control in crops combined with contemporary molecular genetic technology has promoted the development and use of genetically engineered crops to help fill this niche. For example, genetic engineering of maize and cotton that express the *Bacillus thuringiensis* (Bt) toxin show marked resistance to specific insect pests (Cannon 2004). Transgenic plants with novel resistance to plant parasitic nematodes may be developed by targeting fundamental nematode biology. For example, plants expressing protease inhibitors as antinematode agents have shown some level of nematode resistance (Atkinson et al., 2003). The growth of *Heterodera schachtii* (beet cyst nematode) and *Meloidogyne incognita* (root knot nematode) on *Arabidopsis thaliana* expressing cysteine protease inhibitors (cystatins) was suppressed (Urwin et al., 1997). Targeting the mechanisms the nematode uses to infect and parasitize the host is also an emerging avenue to identify molecular targets to develop novel management strategies (Mitchum et al., 2007).

The Biology of *Heterodera* species

The cyst nematodes in the genus *Heterodera* are comprised of some of the most agronomically-important pathogenic nematode species (Lilley et al., 2005). *Heterodera glycines* and *H. schachtii* that infect soybean and sugar beet, respectively, are of particular

economic importance. The infection of cereals such as barley, wheat and oats by the cereal cyst nematode (*H. avenae*) is of concern to the cereal growing regions in Europe. The soybean cyst nematode (SCN), *Heterodera glycines*, is one of the most economically important nematode species causing extensive annual yield losses on its host, soybean (Davis and Tylka 2000). The fact that SCN continues to be the primary economic pathogen of soybeans grown in the U.S. has prompted extensive research on SCN from the molecular to population levels (Niblack et al., 2006).

Embryogenesis and the first molt of a cyst nematode occur within the eggshell, then infective second-stage juveniles (J2) hatch from eggs, migrate through the soil and penetrate host roots in the zone of elongation immediately behind the root tips (Fig. 1). The infective J2 migrates intracellularly in the root cortex using its stylet (a hollow, protrusible oral spear) and secreted enzymes to breach plant cell walls (Wang et al., 1999, Wyss and Zunke 1992) to reach the root vascular tissue. Once the nematode reaches the appropriate vascular tissue it becomes sedentary and secretes compounds produced in its esophageal gland cells to transform a host cell into an initial syncytial cell (Endo 1992). Incorporation of cells adjacent to the initial syncytial cell through cell wall dissolution leads to the formation of the mature multinucleate feeding site called the 'syncytium' (Fig. 2).

Although cyst nematodes secrete cell wall-modifying enzymes during migration through root tissues (Mitchum et al., 2007), endogenous plant enzymes are responsible for subsequent dissolution of cell walls within syncytia (Goellner et al., 2001; Golecki et al., 2002). The unique feeding structure is highly metabolically active and undergoes gross morphological changes including expansion of individual cells, increased cytoplasmic

density, proliferation of organelles and an enlargement and increased number of nuclei (Hussey and Grundler, 1998). Once the feeding site is initiated, the nematode starts feeding and becomes a swollen parasitic J2. Feeding continues in cycles (Sijmons et al., 1994) until they molt to the J3 stage (Fig. 1) and the initiation of sexual differentiation. After the molt to J3 stage, the males regain their vermiform shape, molt to fourth stage within the swollen J3 cuticle, and after a final molt mature males emerge from the roots into the soil and no longer feed. Feeding cyst nematode females remain pyroform through molts to J3 and J4 stages, and after the final molt the lemon-shaped *Heterodera* female protrudes from the roots allowing males to fertilize (Fig. 1). Each fertilized SCN female is capable of producing up to 600 eggs (Sipes et al., 1992) and most of the eggs remain within the female's body. Some of the eggs are deposited into a gelatinous matrix produced by the females. At maturity the female's body hardens, falls off the root into the soil, and becomes the "cyst" that contains the viable eggs that serve as inoculum for several years. Under optimum conditions, the entire lifecycle of SCN can take as little as 22 days (Lauritis et al., 1983) and multiple generations can be realized during one crop growing season.

Nematode esophageal gland cells

Penetration and migration within host roots and the initiation and maintenance of the feeding site represent fundamental and vulnerable points in the lifecycle of cyst nematodes. The parasitism of soybean roots by SCN is a multifaceted process involving an exchange of molecular information between the pathogen and the host. Evidence suggests that cyst nematodes parasitize plant roots via the secretion of multiple disease-inducing parasitism

proteins (Davis et al., 2000, 2004; Niblack et al., 2006). These secretions originate from their three elaborate esophageal gland cells, two localized subventral and one localized dorsal within the nematode body (Fig 3). The secretions are synthesized and sequestered in secretory granules within the gland cells (Hussey, 1989). The single dorsal esophageal gland cell extends anteriorly to a storage ampulla that releases the secretions through valves into the esophageal lumen at the base of the stylet (Hussey and Mims, 1990). The two subventral glands extend anteriorly to ampullae that empty secretions into the esophageal lumen immediately posterior to the metacorpal pump chamber of the alimentary canal. Proteins produced in both gland cell types can be secreted out of the nematode stylet (Davis, 2004; Hussey et al., 2002). Interestingly, the subventral gland cells are the most active glands in the migratory J2 stages, and during the transition to establishment of the feeding site, the dorsal gland cell becomes the dominant active gland (Fig 3) to produce and secrete pivotal secretions to sustain nematode parasitism (Hussey and Mim, 1990; Davis et al., 2000). These changes in esophageal gland cell activity during the nematode lifecycle suggest the importance of specific secretions in the different stages of parasitism (Davis et al., 2004; Hussey, 1989).

Esophageal gland cell secretions

Substantial evidence suggests that nematode stylet secretions play important roles in egg hatching, root penetration and migration, feeding cell formation and maintenance and in food digestion (Baum et al., 2006; Davis et al., 2004; Hussey 1989). Initial analyses of esophageal gland cell contents and stylet secretions from the root knot nematode indicate that

a mixture of proteins and not nucleic acids were present in the secretions (Hussey, 1989). Stylet secretions from cyst nematodes produced *in vitro* through stimulation by incubation with 5-methoxy DMT oxalate have been used as antigens to generate antibodies that bound specifically to esophageal gland proteins (Goverse et al., 1994). Immunoaffinity isolation of a cyst nematode subventral gland protein using gland cell-specific monoclonal antibodies (MGR48) provided amino acid sequence to subsequently isolate cDNA of the first β-1,4-endoglucanase (cellulase) gene of animal origin (Smant et al., 998). Sequence similarities to bacterial cellulases suggest that the cyst nematode cellulases may have originally been acquired via ancient horizontal gene transfer from prokaryotes (Davis et al., 2000, Smant et al., 1998, Yan et al., 1998). The approach of using antibodies generated by purifying secretory proteins to isolate specific genes proved to be a daunting and inefficient method, but identifying the many nematode genes encoding secreted nematode parasitism proteins remains and important objective. Thus, the use of contemporary molecular tools has been paramount in identifying a number of genes expressed within nematode esophageal gland cells. The genes that encode nematode secretions directly involved in the parasitism of the host are collectively termed "parasitism genes" (Davis et al., 2000). The generation of cDNA libraries and expressed sequenced tags (ESTs) from parasitic stages of nematodes is a powerful approach to discover genes involved in nematode parasitism. This can involve constructing and sequencing cDNA libraries and ESTs from whole nematodes at a specific life stage (Popeijus et al., 2000; Dautova et al., 2001; Vanholme et al., 2005). Expressed sequence tags coupled with bioinformatic tools were used to deduce potential gene identities from *Globodera roistochiensis* (potato cyst nematode) and revealed parasitism genes such as

pectate lyase (Popeijus et al., 2000). Further isolation of candidate parasitism genes has been improved by comparative analysis of gene expression in specific nematode life stages using molecular tools such as cDNA-amplified fragment length polymorphism (AFLP) and RNA fingerprinting (Qin et al., 2000; Ding et al., 2000). RNA fingerprinting of pre-parasitic and parasitic stages if the root-knot nematode, *Meloidogyne incognita,* resulted in the isolation of a parasitism gene similar to a venom allergin-like gene (Ding et al., 1998). cDNA-subtraction from the nematode tail region (esophageal glands absent) and the nematode head region (esophageal glands present) isolated genes specifically expressed within the esophageal gland cell region of the nematode (Lambert et al., 1999). Using this method the first nematode chorismate mutase parasitism gene was discovered and, interestingly shown to have high homology and functional complementarity to its bacterial homolog.

To enrich nematode samples for the presence of expressed parasitism genes, a microaspiration technique was used to obtain the cytoplasm of the esophageal gland cells to supply mRNA to generate gland cell-specific cDNA libraries from multiple parasitic stages of cyst and root-knot nematodes (Gao et al., 2001, 2003; Wang et al., 2001; Huang et al., 2003, 2004). One of the comprehensive parasitism gene profiles from SCN (Gao et al., 2003) identified 51 *H. glycines* gland expressed candidate parasitism genes (Table 1). Each candidate parasitism gene was predicted by Signal P algorithm (Bendtsen et al. 2004) to encode a N-terminal signal peptide to direct the proteins into the secretory pathway. Further, the genes were expressed exclusively within the esophageal gland cells as confirmed by mRNA *in-situ* hybridization (Gao et al., 2003). Interestingly, the parasitism gene candidates from *Meloidogyne incognita* (Huang et al., 2003, 2004) had few sequence similarities to

parasitism genes obtained from the cyst and root knot nematodes, indicating that the two species have unique molecular strategies to parasitize the host. The parasitism gene profiles of the two species indicate the complexity and elaborate picture of host cellular events specifically controlled by the phytonematode.

Parasitism gene function

With the discovery of multiple candidate parasitism genes specifically in SCN, the development of tools to confirm the function of putative parasitism genes remains a critical and essential step in understanding molecular plant-nematode interactions (Baum et al., 2006; Davis et al., 2004; Mitchum et al., 2007). BLAST analyses identifies sequence similarity in other organisms for approximately thirty percent of the candidate SCN parasitism genes and provides some indication into the function of the protein in SCN-host interactions — the remainder of the SCN parasitism gene candidates appear to encode novel "pioneer" proteins (Baum et al., 2006; Gao et al., 2001, 2003; Wang, 2001). The expression of specific parasitism genes in the subventral esophageal glands in early infective J2 stages and differential expression of other parasitism genes in the dorsal gland of later parasitic stages provides an added measure of functional significance in the parasitic interaction (Baum et al., 2006; Davis et al., 2004; Mitchum et al., 2007). Genes that encode cell-wall degrading enzymes were expressed in the subventral glands of motile J2 suggesting their roles in penetration and migration within host roots (Davis et al., 2000; Wang et al., 1999). These hydrolytic enzymes aid in the digestion of cell wall polymers which are barriers in nematode penetration and migration (Wang et al., 1999). Some of these hydrolytic enzymes

expressed within the esophageal glands include pectate lyases and polyglucturonase which degrade pectic polysaccharides (Popeijus et al., 2000, Gao et al., 2003) and endoglucanases and xylanases which degrade celluloses and hemicelluloses cell wall structural components respectively (Baum et al., 2006; Hussey et al., 2002; Davis et al., 2000, de Boer et al., 1999, Gao et al., 2003). The first non-plant expansin was also isolated as a parasitism gene of cyst nematodes and thought to aid in cell wall loosening allowing the nematode to migrate within host root tissues (Qin et al., 2004).

Beyond cell wall-modifying enzymes expressed within subventral glands in the migratory stages of the nematode, many other parasitism genes are expressed in the esophageal gland cells that may be involved in other processes such as induction of the elaborate feeding sites (Baum et al., 2006; Mitchum et al., 2007). The size and major activity of the dorsal esophageal gland cell during feeding and feeding site formation suggests that dorsal gland secretions may be provide the majority of nematode signals involved in these processes (Hussey et al, 1989). While some of the genes expressed in the dorsal gland have predicted functions based on sequence similarities in other organisms (Mitchum et. al., 2007; Gao et al., 2003), a large portion of these parasitism genes are novel (Table 1). A few genes have similarities to plant genes (Gao et al., 2003) and may be indicative of the nematode's close parasitic association with its host by mimicking plant process to promote a compatible interaction. Relatively few candidate cyst nematode parasitism genes have homologs in *C. elegans* suggesting that this model nematode may be limited in its usefulness to study parasitism by nematodes (Mitchum et al., 2007). In addition to sequence similarities of the candidate parasitism genes, computational analyses using PSORT II predicted several

secreted parasitism gene products that have putative nuclear localization signals (NLS). Confirmation that two SCN parasitism gene products (4E02, 6E07) have functional NLS in plant cells suggests that the activity of some secreted parasitism proteins may be localized to the host nucleus, perhaps for transcriptional regulation of host cell biology for parasitism (Elling et al., 2007).

Candidate parasitism genes similar to chorismate mutase (CM) have been identified in a number of plant-parasitic nematode species (summarized in Mitchum et al., 2007). This enzyme is essential in the plant shikimic acid pathway and is involved in synthesis of cellular aromatic amino acids, plant defense compounds, phytohormones, and a number of secondary metabolites (Doyle and Lambert, 2003). To date, CM is has not been identified in animals other than root-knot and cyst plant-parasitic nematode species. Since the shikimate pathway does not appear essential for endogenous animal cell metabolism, it is thought that nematode CM secreted into the host may modulate plant shikimic acid pathway and aid the nematode parasitism (Doyle and Lambert, 2003). Expression of the nematode CM in soybean root tissue was shown to alter vascular differentiation, and a model for cellular portioning of IAA in host cells was supported by response to exogenous auxin appication (Doyle and Lambert, 2003). Further, studies on SCN *chorismate mutase* alleles demonstrate that SCN *chorismate mutate-1A* is preferentially selected on resistant soybean PI88788, and it is hypothesized that these alleles may act as virulence genes by a suppression of chorismate derived compounds involved in plant defense (Bekal et al., 2003; Lambert et al., 2005).

Other cyst nematode parasitism gene products thought to play a role in feeding cell formation by altering its host metabolism include potential secreted members of the

proteasome (Baum et al., 2006). Predicted secreted Skp-1 (8H07), RING-H2 zinc finger (10A06), and ubiquitin extension (3H07) parasitism proteins (Table 1) of SCN (Gao et al., 2003) have significant similarity to plant genes and may modulate selective protein degradation in host cells. Interestingly, SCN ubiquitins are unique in that they possess signal peptides for secretion and have a novel 19 amino acid extension at the C-terminus in contrast to similar plant ubiquitins that are not secretory proteins and have ribosomal extension proteins (Tytgat et al., 2004; Gao et al., 2003).

The theme of molecular mimicry of host proteins by secreted products of nematode parasitism genes extends to signaling peptides (Baum et al., 2006; Mitchum et al., 2007). The SYV46 (2B10/4G12) gene is one of the most abundantly expressed parasitism gene in SCN and limited to the dorsal gland cell of parasitic life stages (Bakhetia et al., 2007a; Wang et al., 2001; Gao et al., 2003) A computational peptide domain search discovered strong similarity of the SYV46 C-terminal domain with the consensus sequence of the CLAVATA-ESR-like (CLE) peptide in *Arabidopsis thaliana* (Olsen and Shriver, 2003). In plants, CLAVATA3 (CLV3) signaling peptide ligand binds to a membrane receptor kinase CLAVATA1/CLAVATA2 complex to shoot meristem stem cell differentiation (Clark, 2001). Interestingly, the 4G12 member (Gao et al., 2003) of the SYV46 family has been experimentally shown to complement an *Arabidopsis clv3* mutant and expression of this parasitism gene in *Arabidopsis* resulted in premature bolting without differentiation of the shoot apical meristem and inflorescence, the distinct phenotype observed by overexpression of plant CLV3 (Wang et al., 2005; Hobe et al., 2003). Recent observations that distantly related CLV3-like genes (CLE40) can complement *clv3* mutant in plants and that CLV3

comprises a large gene family (Cock and McCormick, 2001; Hobe et al., 2003) suggest that unraveling the exact role of nematode SYV46 in nematode parasitism of host plants will be a challenging task. Although it is unclear how SYV46 plays a role in host parasitism, it is postulated that SYV46 from the nematode is a CLE mimic that functions in redirecting and maintaining the differentiation of root vascular cells into nematode feeding cells, potentially via a CLV1-like receptor complex (Wang et al., 2005; Mitchum et al., 2008).

The investigations of CLE-like nematode parasitism genes (Huang et al., 2006; Wang et al 2005) demonstrate the utility of the model plant species *Arabidopsis thaliana* (Pang and Meyerowitz, 1987; Sijmons et al., 1991) for analyses of nematode parasitism gene function. However, infection studies using this model are limited since SCN cannot infect Arabidopsis. Fortunately, Arabidopsis is a good host for the beet cyst nematode (BCN), *H. schachtii* (Sijmons et al., 1991). The infection process including the formation of the syncytia appears to be identical among cyst species (Hussey and Grundler 1998) and the relatively close phylogenetic relationship of BCN to SCN estimated from rDNA to the internal transcribed spacers sequences (Subbotin et al., 2001) suggest that BCN can be used as a model for cyst nematode parasitism gene function. Pilot assays for this dissertation using genomic DNA of BCN as template and primers designed to short regions of selected SCN parasitism gene clones obtained products with nearly identical sequence that provided further evidence that a number of parasitism genes are common between BCN and SCN. This commonality suggests that fundamental signals from the two species are likely similar, allowing the use of BCN-Arabidopsis pathosysm for parasitism gene function analyses.

An Arabidopsis-BCN pathosystem may serve as a good model to investigate SCN parasitism gene (*Hg4f01*) that encodes a predicted annexin-like protein (Gao et al., 2003). Annexins are calcium dependant phospholipid binding proteins that are found in both animals and plants (Morgan and Fernandez, 1997; Clark et al., 2001). Typical annexin structure has four conserved domains where the calcium and phospholipid-binding sites are located (Fioretti et al., 2001; Clark et al., 2001). *Hg4f01* contains four conserved domains similar to annexins found in *C. elegans* and *Arabidopsis*. The specific functions of annexins in plants and animals remain unclear but it is evident that annexins play a diverse role in various cellular processes such as membrane channel activity and phospholipid metabolism (Clark and Roux, 1995). Specifically, celery plant annexins are vacuolar specific and may be involved in vacuole biogenesis (Clark et al., 2001) and spinach annexins bind to choloroplasts in a calcium dependant manner. In another study, annexins were upregulated in response to nodulation in *Medicago truncatula* (Niebel et al., 1998). In more recent findings, annexins in *Arabidopsis thaliana* have been implicated in abiotic stress responses such as drought, osmotic and salinity stress (Cantero et al. 2006; Lee et al., 2004). Available annexin Arabidopsis T-DNA insertion lines provided evidence for its role in salt stress response (Lee et al., 2004). Since the role of annexins is diverse it would be interesting to understand the potential role of secreted nematode 4F01 in parasitism of a plant host. Could secreted nematode annexin (4F01) play a role in mimicking plant annexin functions? How would such a potential function promote a compatible plant-nematode interaction? If cyst nematodes require annexins for infection, the potential exists that plants mutated in annexins may compromise infection. Using the available T-DNA mutant lines, the assessment of

annexins in response to cyst nematode infection can be easily conducted with the Arabidopsis-BCN model system.

RNA interference (RNAi)

From the above observations of understanding parasitism gene functions, the potential to clone and express putative parasitism genes within host tissue and assess phenotypes will provide some indication of gene function. An exciting and emerging alternative strategy is the use of RNA-mediated interference (RNAi) to silence gene expression (Fire et al., 1998) and assess its potential effects on parasitism (Baum et al., 2006; Bhaketia et al., 2005; Davis et al., 2004; Lilly et al., 2007). RNAi is a natural mechanism in eukaryotes that is triggered by cellular double-stranded RNA (dsRNA) and specifically suppresses the expression of the corresponding genes within the organism (Fire et al., 1998). This phenomenon involves the cleavage of the dsRNA into small interfering RNA (siRNAs) of 21-23 nucleotides (Elbashir et al., 2001) which, when combined with the RNA-inducing silencing complex (RISC), guide the recognition of their complementary mRNAs for degradation (Bakhetia et al., 2005).

In *C. elegans,* RNAi can be achieved by feeding the nematodes bacteria that express dsRNA complementary to a gene present within the nematode (Timmons et al., 2001), by microinjection of dsRNA into the nematode body (Mello and Conte, 2004), and by nematode oral uptake of the dsRNA from a soaking solution (Tabara et al., 1998). Applying the mechanism of *C. elegans* dsRNA oral uptake to plant parasitic nematodes has resulted in the silencing of nematode genes (reviewed in Lilley et al., 2007). Since plant-parasitic

nematodes do not naturally ingest until they are within the host and have established the feeding site, a chemical neuroactive stimulant (octopamine) is used to stimulate the ingestion of dsRNA *in vitro* by hatched pre-parasitic J2 nematodes incubated in soaking solution (Urwin et al., 2002). In cyst nematode species, the *in vitro* uptake of dsRNA to different nematode genes has resulted in distinct RNAi phenotypes in the nematode life cycle. Targeted silencing of cysteine proteinas genes in SCN by RNAi soaking resulted in the development of more males than females during the infection process, and silencing of C-type lectin showed a reduction in established nematode infection (Urwin et al., 2002). Further, the silencing of major sperm protein (MSP) gene expression in cyst nematode males indicated that the RNAi effect from dsRNA ingestion can be achieved for several days after the dsRNA soaking treatment and therefore has the potential to be used to silence genes expressed in later parasitic life stages (Urwin et al., 2002). A recent study on the SCN gland cell expressed parasitism genes using *in vitro* dsRNA ingestion resulted in gene silencing that had potential value for studying early parasitic interaction with the host (Bakhetia et al., 2007b). RNAi soaking targeting the cellulase gene (*ENG-1*) in SCN and *Globodera rostoschiensis* resulted in a decrease in the number of established females (Bhaketia et al., 2007b; Chen et al., 2005). *In vitro* RNAi of choristmate mutase gene and pectate lyase individually resulted in the increased number of males but no effect on female numbers (Bhaketia et al., 2007b). Interestingly, targeted RNAi soaking disruption of the HgSYV46 gene, which was potentially shown to functionally mimic CLAVATA3 in *Arabidopsis* (Wang et al., 2005) resulted in both phenotypic effects of favoring male production and decreased number of females (Bakhetia et al., 2007b). *In vitro* RNAi of root knot nematode

parasitism genes calreticulin and polygalacturonase expressed within the subventral glands resulted in transcript reduction (Rosso et al., 2005), but the silencing effect was short lived (<72 hours) compared to the 14 days observed after silencing of major sperm protein gene in cyst nematode J2 (Urwin et al., 2002). The variability in the efficiency of RNAi using the *in vitro* soaking method with preparasitic J2 stage neamtodes may be attributed to the variability in transcript levels of each parasitism gene at different life stages of the nematode. The effect of RNAi on genes expressed during different stages of development can be difficult to study if the effect of the RNAi is short-lived.

Plant host-derived RNAi using vectors designed to express dsRNA in transformed plants (Wesley et al., 2001) can provide optimum opportunity for dsRNA ingestion by parasitic nematodes at the stages when target nematode genes are expressed. Since cyst and root-knot nematodes appear to only start feeding after establishing elaborate feeding sites in the host (Hussey and Grundler, 1998), the dsRNA must be present within the host for potential ingestion. RNAi silencing of housekeeping genes in *Meloidogyne incognita* encoding a splicing factor or an integrase of root knot nematode prevented visible gall formation when infected on the host (Yadav et al., 2006). Parasitism gene 16D10 of *Meloidogyne incognita* encodes a peptide that stimulates root growth when expressed in *Arabidopsis thaliana* and functions as a ligand for a putative plant transcription factor (Huang et al., 2006a). The expression of 16D10-dsRNA in *Arabidopsis* resulted in almost 90% reduction in nematode reproduction after infection and was similarly effective against four major *Meloidogyne* species (Huang et al., 2006b). The efficiency of the *in planta* RNAi strategy reported in both papers is extraordinary and implies that the target genes are

essential to sustain nematode development. These data support the hypothesis that plant host-derived RNAi is a viable method to assess the function and significance of candidate cyst nematode parasitism genes.

This dissertation is based upon the hypothesis that secretions from the esophageal gland cells of cyst nematodes play critical functional roles in parasitism of host plant roots. The beet cyst nematode (BCN), *Heterodera schachtii* and *Arabidopsis thaliana* were chosen to provide a model pathosystem to explore the biological significance of parasitism genes that are common in SCN (*H. glycines*) and BCN. Investigations of several selected cyst nematode parasitism genes comprise this dissertation, including an extensive functional analysis of a putative annexin-like parasitism gene of cyst nematodes.

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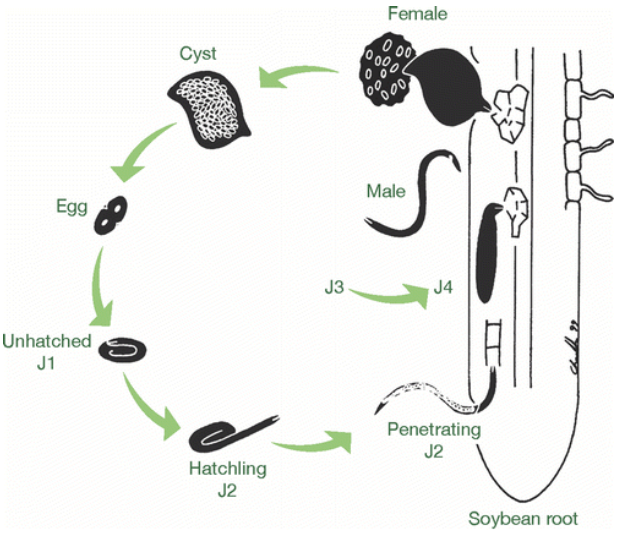

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Figure 1. Life cycle of *Heterodera glycines*. A developed first-stage juvenile (J1) eventually forms in the egg. The J1 molts within the egg, becoming a second-stage juvenile (J2) that hatches from the egg. The J2 penetrates the host root and develops through the third and fourth juvenile stages (J3 and J4, respectively). Vermiform, adult males fertilize lemon-shaped, adult females and the adult females produce eggs externally, in an egg mass, which then fill up internally with eggs (Niblack et al., 2006).

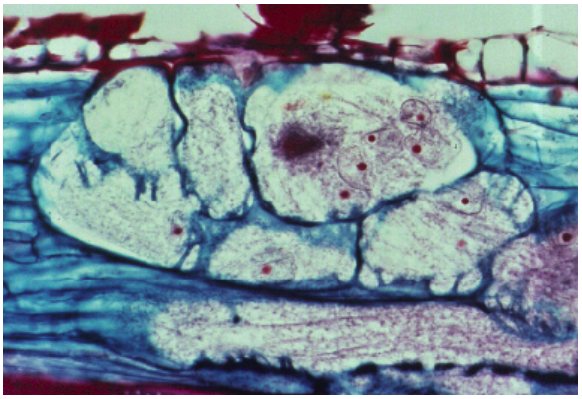

Figure 2. Micrograph of a cyst nematode feeding site (syncytium). Longitudinal section of the vascular cells of a soybean root modified into a multinucleate syncytium by a soybean cyst nematode (tip of nematode head in red at center of top). Photo courtesy of B.Y. Endo.

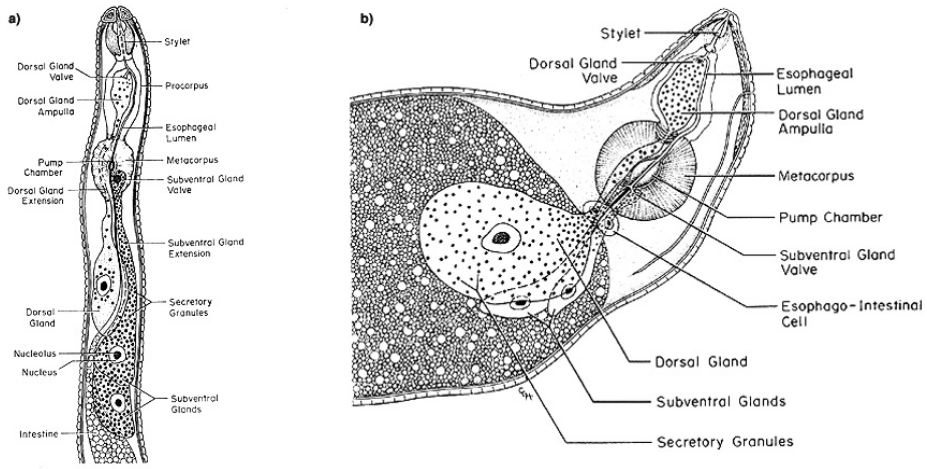

Figure 3. Illustration of the esophageal gland secretory cells. Gland cells connected to the stylet within **a**) a preparasitic second-stage juvenile **b**) a parasitic adult female of a soybean cyst nematode (from Hussey, 1989).

Table 1. Summary of 51 candidate parasitism genes encoding proteins for secretion and expressed exclusively within the esophageal gland cells of *Heterodera glycines* (Gao et al., 2003).

Clone[a]	Accession no.[b]	FL/ORF (bp)[c]	Highest protein similarity	BLASTP score/E value	Gland expression[d]		
					Pre-J2	Par-J2	J3-A
2A05	AY028639	683/439	MI-MSP-1—*Meloidogyne incognita*	114/1e^{-24}	SvG	SvG	SvG
2B10	AF273728	607/420	Gland cell protein—*H. glycines*	0	–[f]	DG	DG
2D01	AF469057	711/558	Pioneer[e]		–	DG	DG
3B05	AF469058	585/423	CBP - *H. glycines*	35/.19	–	SvG	SvG
3D11	AF468679	1120/10533	Chitinase - *Caenorhabditis elegans*	274/2.7e^{-21}	–	SvG	SvG
3H07	AF473831	571/318	Ubiquitin extension—*Nicotiana tobacco*	136/5e^{-32}	DG	DG	DG
4D06	AF469063	750/615	Pioneer		–	DG	DG
4D09	AF469061	738/501	Pioneer		DG	DG	DG
4E02*	AF473826	449/279	Pioneer		SvG	SvG	SvG
4F01	AF469059	1174/1026	Annexin—*C. elegans*	242/4e^{-63}	–	DG	DG
4G05	AF473830	928/765	Pioneer		–	DG	DG
4G06	AF469060	613/360	Hexaubiquitin—*Helianthus annuus* (85% identity to 3H07)[g]	151/1e^{-36}	DG	DG	DG
4G12	AF473827	621/417	Pioneer (91% identity to 2B10)		–	DG	DG
5D06*	AF469062	1937/1470	Pioneer		–	DG	DG
5D08*	AF473828	693/441	Pioneer		–	DG	DG
6E07*	AF473829	1046/645	Pioneer		–	DG	DG
6F06	AY043224	1333/1059	Cellulase—*H. glycines*	601/1e^{-170}	SvG	SvG	
7E05	AF500023	518/330	Pioneer		–	DG	DG
8H07*	AF500024	1457/1197	SKP1-like protein—*Arabidopsis thaliana*	94/3e^{-18}	–	DG	DG
10A06*	AF502391	1239/927	RING-H2 zinc finger protein—*A. thaliana*	50/3e^{-05}	–	DG	DG
10A07*	AF500021	837/729	Pioneer		–	DG	DG
10C02*	AF500017	449/279	Pioneer (92% identity to 4E02)		–	SvG	SvG
11A06	AF500015	673/561	Pioneer (91% identity to 2D01)		–	DG	DG
12H04	AF490244	1908/1614	Pioneer		DG	DG	DG
13A06*	AF500020	899/669	Pioneer (95% identity to 6E07)		–	DG	DG
13C08	AF469055	1101/1002	Cellulase—*H. glycines*	270/1e^{-71}	SvG	SvG	
16B09	AF490246	676/555	Pioneer		–	DG	DG
17C07	AF520566	957/792	Pectate lyase—*H. glycines*	461/e^{-129}	SvG	SvG	
17G06*	AF490247	600/300	Pioneer		–	SvG	
18H08	AF490248	632/399	Pioneer		–	DG	DG
19B10	AF490249	782/666	Pioneer		–	DG	DG
19C07	AF490250	660/333	Pioneer		–	DG	DG
20E03	AF490251	654/579	Pioneer		–	SvG	SvG
20G04*	AF500022	816/648	Pioneer (95% identity to 10A07)		–	DG	DG
21E12*	AF500028	439/354	Pioneer		–	DG	DG
22C12	AF500029	676/549	Pioneer (92% identity to 16B09)		–	DG	DG
23G12	AF500033	605/321	Pioneer		DG	DG	DG
24A12	AF500034	598/441	Pioneer		–	DG	DG
25A01	AF500019	750/528	Pioneer		–	DG	DG
25G01	AF006052	1600/1428	*Hg-eng-1—H. glycines*	0	SvG	SvG	
26D05	AY101191	1125/1008	Cellulase—*Pratylenchus penetrans*	263/2e^{-69}	SvG	SvG	SvG
27D09*	AY101190	851/708	Pioneer (86% identity to 10A07)		–	DG	DG
28B03	AF500025	1500/1302	Pioneer		DG	DG	DG
29D09	AF500016	757/615	Pioneer (95% identity to 4D06)		–	DG	DG
30C02	AF502393	537/492	Pioneer		–	DG	DG
30D08*	AF500027	443/384	Pioneer (82% identity to 21E12)		–	DG	DG
30E03	AF500035	675/558	Pioneer (98% identity to 16B09)		–	DG	DG
30G12	AF500018	881/717	Pioneer (93% identity to 4G05)		–	DG	DG
32E03*	AF500036	701/588	Pioneer		–	DG	DG
33A09	Ay125963	461/270	Pioneer		DG	DG	
33E05	AF502392	684**	Pioneer		DG	DG	
34B08	AF500037	974/735	Pioneer		DG	DG	DG
45D07	AF520565	928/819	Chorismate mutase—*Globodera pallida*	276/2e^{-73}	DG	DG	DG

[a] Clones with an asterisk encode secretory proteins with predicted nuclear localization signals.
[b] Sequences submitted to GenBank with exception of AF273728 and AF006052, which were already in the databases.
[c] Size of the full-length clone with predicted open reading frame (ORF) size; ** indicates not full length.
[d] In situ hybridization of cDNA probes to mRNA specifically within the single dorsal esophageal gland cell (DG) or the two subventral esophageal gland cells (SvG) in preparasitic second-stage juveniles (Pre-J2), parasitic J2 (Par-J2), or parasitic J3, J4, or young adult stages (J3-A) of *Heterodera glycines*.
[e] Novel transcript with no homology to any genes in the public databases.
[f] Not detected.
[g] Percent identity in the amino acid residues of predicted protein.

ISOLATION AND FUNCTIONAL ANALYSIS OF AN ANNEXIN-LIKE PARASITISM GENE OF CYST NEMATODES (*HETERODERA* SPP.)

Abstract

Secretions of proteins encoded by parasitism genes expressed within the esophageal gland cells of cyst nematodes are important in the establishment of specialized feeding cells within the host. Parasitism gene *4f01* originally cloned from *Heterodera glycines* encodes an annexin-like protein (Hg4F01). The *4f01* cDNA homolog was isolated from *Heterodera schachtii* (*Hs4f01*) which is able to infect the model plant *Arabidopsis thaliana*. *Hs4f01* sequence analysis revealed a 97% nucleotide identity with a 3% nucleotide sequence gap and 92% predicted amino acid identity with the Hg4F01 homolog. mRNA *in-situ* hybridization and immunolocalization studies confirm the expression of this gene exclusively within the dorsal esophageal gland and gland extension within *H. shachtii*. Hs4F01 expression within Arabidopsis did not result any obvious morphological differences but the resulting plants were moresusceptible to *H. shachtii* infection. Further, the Hs4F01 was able to complement the plant annexin mutant (*Atann1*) indicating that the nematode annexin-like protein may play a role similar to that of plant annexin 1. Experiments with plant transformed with RNAi vectors indicate that lines expressing dsRNA to *Hs4f01* resulted in a suppression of females three weeks post inoculation.

Introduction

Plant-parasitic cyst nematodes in the genus *Heterodera* are microscopic worms that comprise a major agronomically important group of plant pathogenic nematode species

(Mitchum et al., 2007; Lilley et al., 2005). The infective cyst nematode second-stage juvenile (J2) hatches from its eggshell in soil, penetrates host plant roots near the tip, and migrates intracellularly through the root cortex to the vascular cylinder (Niblack et al., 2006). The nematode will subsequently induce elaborate changes in host plant cells to form a specialized feeding site called a syncytium that will provide the permanent source of nutrition for the nematode as it swells, becomes sedentary, and molts three more times to reproductive maturity. This specialized feeding site has characteristic features such as proliferation of organelles, increased cytoplasmic density, decreased main vacuole replaced by smaller secondary vacuoles and enlargement and increased number of nuclei (Endo, 1964). The syncytium is formed by the incorporation of root vascular cells adjacent to an initial syncytical cell through cell wall dissolution and incorporates new host cells throughout the cyst nematode parasitic cycle (Endo, 1964; Hussey and Grundler, 1998; Davis et al., 2000). The cell walls surrounding the syncytia thicken and develop ingrowths enhancing the surface area through which solute uptake can occur (Lilley et al., 2005; Hussey and Grundler, 1998). Evidence indicates that the parasitic process beginning with host root penetration, migration, and formation of the syncytium within host roots is regulated, directly or indirectly, by a suite of proteins secreted from the nematode stylet (hollow, protrusible oral spear) that are developmentally-regulated to alter host cell physiology to promote parasitism (Baum et al., 2006; Davis et al., 2004 and Mitchum et al., 2007). The protein secretions originate from three elaborate secretory cells within the nematode esophagus and are released through complex valves via exocytosis and secreted through the nematode stylet into host tissues (Hussey 1989; Davis et al., 2004).

Over fifty developmentally expressed parasitism genes encoding predicted secreted proteins have been isolated from the esophageal gland cells of the soybean cyst nematode, *Heterodera glycines* (Gao et al., 2001, 2003; Wang et al., 2001) and only about thirty percent of these *H. glycines* parasitism genes have database homologs (Mitchum et al., 2007). One of the parasitism genes isolated, *Hg4F01* (Gao et al., 2003), is expressed exclusively within the single dorsal esophageal gland cell of parasitic life stages and has sequence similarity to annexins in the nematode *Caenorhabditis elegans* and to annexins in *Arabidopsis thaliana*. Annexins are a diverse family of proteins that are involved in a number of calcium-regulated activities associated with membrane surfaces (Creutz, 1992). They have several conserved domains including a 60-70 amino acid motif that is repeated at least four times within which the calcium and phospholipid binding site are located (Clark et al., 2001).

Limited localization studies of annexins in nematodes including *C. elegans* and the potato cyst nematode, *Globodera pallida* indicate that annexins associate with the reproductive organs (Daigle and Creutz, 1999; Fioretti et al., 2001). In plants, annexins are thought to play diverse roles and several potential functions exist in a number of plant species. For example, annexins in peas and corn concentrate in secretory cell types of root caps and developing xylem and phloem (Clark et al., 1992; Clark et al., 1994). In cotton, annexins are thought to decrease callose synthase enzyme activity (Delmer and Potikha, 1997). In *Arabidopsis thaliana* there are at least eight distinct annexin genes, some of which are thought to be linked to abiotic stress responses (Clark et al, 2001; Cantero et al., 2006). There is strong evidence that Annexin 1 gene (*AnnAt1*) in Arabidopsis can protect cells against oxidative stress and also restore the ability of OxrR mutants of *E. coli* to survive and

grow in hydrogen peroxide (Gorecka et al., 2005). *AnnAt1* was also shown to be involved in osmotic stress response where mutant annAt1 lines showed a significant reduction in germination efficiency, indicating its possible role in salt stress response (Lee et al., 2004). Further, the upregulation of each annexin in Arabidopsis in response to abiotic stresses indicates that annexins play a role in cold stress, hot stress, water stress and osmotic stress (Cantero et al., 2006).

Confirmation of an annexin-like function of the Hg4F01 protein and the potential significance of a secreted annexin-like protein in the cyst nematode-plant parasitic interactions remains unclear. The available functional information and genetic resources for annexins in Arabidopsis (Clark et al, 2001; Cantero et al., 2006; Lee et al., 2004) provide a model to evaluate the potential function of the Hg4F01 parasitism protein in the plant-nematode interaction. Since Arabidopsis is not a host for *H. glycines* but is a host (Sijmons et al., 1991) for the closely-related *Heterodera schachtii* (Subbotin et al., 2001), the isolation of a potential *Hg4f01* homolog in *H. schachtii* would present a tenable model pathosystem.

The potential to induce RNA-mediated interference (RNAi) of target nematode genes (Fire et al., 1998) through introduction of complementary double-stranded RNA (dsRNA) also provides potential for functional analyses through specific silencing of target nematode parasitism genes (Mitchum et al., 2007). Target-specific RNAi of several different plant-parasitic nematode genes has been achieved through *in vitro* soaking in complementary dsRNA (Lilley et al., 2007), and more recently, via plant host-derived RNAi (Gheysen and Vanholme, 2007). The 16D10 parasitism gene of *Meloidogyne incognita* encodes a peptide that stimulates root growth when expressed in *Arabidopsis thaliana* and functions as a ligand

for a putative plant transcription factor (Huang et al., 2006a). The expression of dsRNA to the 16D10 gene in transgenic Arabidopsis resulted in host-derived RNAi producing an almost 90% reduction in nematode reproduction in the transgenic plants (Huang et al., 2006b). This extraordinary result suggests that host-derived RNAi can provide significant biological information when targeting parasitism genes involved in the plant-nematode interaction. Functional analyses of the annexin-like parasitism gene (*4f01*) of cyst nematodes is presented here using plant host-derived RNAi and the Arabidopsis-cyst nematode pathosystem.

Materials and Methods

Nematode culture

Heterodera schachtii were propagated on roots of greenhouse-grown cabbage plants (*Brassica oleracea var. capitata*) and *Heterodera glycines* were propagated on roots of greenhouse-grown soybean plants (*Glycine max*). Eggs were collected from crushed cysts of *H. schachtii* and *H. glycines* as previously described for other cyst nematode species (Goellner et al., 2001). *Meloidogyne incognita* (root-knot nematode) were propagated on roots of greenhouse grown tomato (*Lycopersicon esculentum* cv Rutgers) plants, and *M. incognita* eggs were extracted from host roots with 0.05% sodium hypochlorite as previously described (Hussey and Barker, 1973). Eggs of both nematode species were hatched over water at 28C on a Baermann pan to collect 24h-cohorts of pre-parasitic second-stage juveniles (pre-J2s). Mixed parasitic stages of *H. schachtii* were collected from within cabbage roots by the root blending and sieving method of Ding et al. (1998).

Southern Blot analysis

Frozen *H. shachtii* and *H. glycines* pre-J2 pellets were mixed with lysis solution (100mM NaCl, 100mM Tris-HCl [pH 8.5], 50mM EDTA [pH 7.4], 1% SDS, 1% β-mercaptoenthanol and 100μg/ml proteinase K and incubated at 65°C for 45min. The DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA was resuspended in 10mM Tris-HCl [pH 8] and treated with RNase *If* according to manufacture's instructions (New England Biolabs, Ipswich, MA).

Five micrograms of *H. schachtii* and *H. glycines* genomic DNA was completely digested overnight at 37°C with Bam*HI* and Hind*III* (New England Biolabs, Ipswich, MA), separated by 0.7% agarose gel electrophoresis, transferred onto a Hybond-N membrane through capillarity (Sambrook et al., 1989). A digoxygenin-labeled (DIG) *4f01* probe was generated using the PCR DIG Probe Synthesis kit (Roche Applied Science, Indianapolis, IN) with *Hg4f01* cDNA (GeneBank Accession AF469059) template and primer pair B4F01p 5' AAGCAGGCGTATGAGCAGTT3' and 5'GTCGTGTGCCAATACAATGC3'. Hybridization of the probe was performed at 42°C for 16 hours in DIG Easy Hyb solution (Roche Applied Science, Indianapolis, IN). After the stringency washes the blot was detected using the DIG Wash and Block Buffer Set (Roche Applied Science, Indianapolis, IN). The membrane was exposed to x-ray film for 10min and hybridized bands were observed.

Isolation of *Hs4f01* parasitism cDNA clone.

Frozen pellets of mixed parasitic stages of *H. schachtii,* or frozen plant material, were ruptured with Lysis Matrix D beads (Q-Biogene, Irvine, CA,) and liquid nitrogen in a mini

beadbeater (Biospec Products Inc. Bartlesville, OK). Nematode total RNA was extracted using the Micro-Midi Total RNA purification system (Invitrogen, Carlsbard, CA) following the manufacturer's instructions including digestion with DNAseI.

The original *Hg4f01* cDNA clone (GeneBank Accession AF469059) was isolated 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 homolog of *Hg4f01* in *H. schachtii*, 3' and 5' cDNA ends were amplified from total *H. schachtii* RNA using GeneRacer kit (Invitrogen, Carlsbard, CA) and PCR primers dervived from the Hg4f01 cDNA sequence. 5' RACE was performed using GeneRacer 5'primer and GSP Hg4F01-1 5'GCGAGTGGCCAACACCTGGTTGAACA 3' with RACE-ready first strand *H. schachtii* cDNA template. 3' RACE was performed using Gene Racer 3' primer (oligo dT) and GSP Hg4F01-2 5'TTGCTCAGCTGCTCTCGCGAAGAAAA3'. The RACE product was cloned into pCR4-TOPO vector (Invitrogen, Carlsbard, CA,) for sequencing. Based on the sequencing results of the 3' and 5' RACE products, forward primer 5'ATGCTCCAAA ACGGCCTTACCATT3' and reverse primer 5'TCACTGCTCCGTGTTGCCCTT 3' were used to amplify the full-length *4f01* cDNA clone from template *H. schachtii* RNA. The *Hs4f01* cDNA was subsequently cloned into pCR4-TOPO vector (Invitrogen, Carlsbard, CA,) for DNA sequence confirmation.

Sequence analyses

Comparison of the nucleotide and predicted amino acid sequences of the *H. schachtii 4f01* cDNA homolog to the *H. glycines 4f01* parasitism gene (AF469059) and to the amino acid sequences of *Arabidopsis thaliana* annexin 1 gene (At1g35720), *C. elegans* annexin 1 (NP498109), and *Globodera pallida* annexin 1 (CAC33829) were conducted using the BLAST alignment algorithm at the National Center for Biotechnology Information (NCBI) (Tatusova and Madden, 1999). Sequence similarity searches to genes in NCBI database were conducted using BLASTp program on NCBI. Prediction of a signal peptide for secretion and the cleavage site was performed using the SIGNAL P 3.0 program (Bendtsen et al. 2004). NCBI Conserved Domain search was also used to compare the predicted protein sequences of Hs4F01, Hg4F01 (AF469059), *Globodera pallida* annexin 1 (CAC33829), *C. elegans* annexin 1 (NP498109) and *A. thaliana* annexin 1 (NP174810) (Marchler-Bauer and Bryant, 2004).

mRNA *in situ* hybridization

Localization and developmental expression analyses of *Hs4f01* were performed in fixed mixed parasitic stages of *H. schachtii* prepared as previously described (De Boer et al., 1999). Specific forward and reverse primers for each cDNA clone were used to synthesize DIG-labeled sense (control) and antisense cDNA probe by asymmetric PCR (Gao et al., 2001). The cDNA probes that hybridized within the nematode specimens were detected by the DIG labeling system and observed by light microscopy using the method of De Boer et al. (1998).

Immunolocalization

Polyclonal antibodies to *H. glycines* 4F01 were produced (Eurogentec, Inc., Belgium) by concomitantly immunizing individual rabbits with two specific synthetic peptides (H2N-CEEDIKAKTLPKS-CONH2 and H2N-KGLGTRDSDLIRLVIS-CONH2). The localization of expressed Hs4F01 gene products within nematode specimens with the anti-Hg4F01 sera was detected by immunoflorescence microscopy of fixed mixed parasitic stages of *H. schachtii* according to the method Goellner et al. (2000).

Expression and Complementation of *annAt1* T-DNA insertion mutant with *Hs4f01*

Expression assays of *Hs4f01* in transformed wildtype and annexin-mutant Arabidopsis plants were conducted. The β-glucuronidase gene of the binary vector pB121 (Clonetech, Palo Alto, CA) was replaced at the *Bam HI* and *Sac II* sites with the coding region of *Hs4f01* with and without the predicted signal peptide sequence. The (35S::Hs4F01) constructs were each introduced into wild-type Arabidopsis (ecotype Columbia-0) via *Agrobacterium*-mediated floral dip transformation (Clough and Bent, 1998). The 35S::Hs4F01 construct without the signal peptide was also transformed into an *annAt1* Arabidopsis T-DNA Insertional mutant (SALK_015426) background. Transgenic Arabidopsis lines were selected on 50 μg/mL kanamycin. Transgenic Arabidopsis lines were observed for altered phenotype compared to wildtype and annexin-mutant lines as appropriate. Total RNA from plant material was extracted using RNeasy Plant Mini Kit following manufacture's instructions (Qiagen, Valencia, CA). Expression of the *Hs4f01*

transgene (with or without signal peptide sequence) was confirmed using RT-PCR on total RNA of leaf tissues on T3 lines using the first strand cDNA synthesis using Superscript II RT kit and PCR amplification using 4F01 primers 5'AGTTGGACAGAAGGCATCAGCAC3' and 5'AGAAGAGTTG CGGACATATTTGA3'. Homozygous Arabidopsis lines that expressed *Hs4f01* were grown in Murashige-Skoog (MS) media plates placed vertically for root growth assays. Homozygous *annAt1-1/Hs4F01* Arabidopsis lines were germinated on Murashige-Skoog (MS) media supplemented with 75mM sodium chloride (NaCl) to test the complementation efficiency (Lee et al., 2004) compared to the mutant and germination on standard MS media. Germination rates were scored 7 days post incubation.

Western Blot

The transgenic Arabidopsis lines above confirmed to express *Hs4f01* by RT-PCR were grown for 10 days on MS media after which the seedlings were harvested, frozen immediately and ground in liquid nitrogen. Total protein was extracted by incubating the ground tissue in extraction buffer [50mM Tris, pH 8.0, 2mM EDTA, 2mM DTT, 0.25M sucrose and protease inhibitor cocktail] and centrifuging at 8000g for 15min at 4°C. The supernatant was collected and protein concentration was estimated by Bradford assay using BSA as a standard (Bradford, 1976). Proteins were separated on 4-20% polyacrylamide gel (Biorad, Hercules, CA) and transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, Billerica, MS). The protein blot was incubated for 2.5hrs at room temperature in anti-Hg4F01 primary sera (1:3,000) and potential transgene

products were detected by enhanced chemiluminescence after 1.5 incubation in (1:2000) anti rabbit-alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO).

Host-derived RNAi – vector construction and analyses of transgenic lines

Full-length *Hs4f01* cDNA sequence was cloned in the sense and antisense orientation at the *XhoI – KpnI and BamHI – HindIII* restriction sites, respectively, of the pHANNIBAL RNAi vector containing the CaMV 35S promoter (Wesley et al., 2001). A control host-derived RNAi was also constructed in pHANNIBAL that contained the cDNA sequence (U87974) of the green fluorescent protein (GFP). The *Ntcel7*- RNAi vector was constructed by replacing the CaMV 35S promoter in pHANNIBAL with the promoter of the *Nicotiana tabacum* cellulase 7 gene that has demonstrated upregulation within nematode feeding cells (Goellner et al., 2001; Wang et al., 2007). All pHANNIBAL clones containing the sense and antisense gene were subcloned as *NotI* fragments into the binary vector pART27 (Gleave, 1992). The constructs were transformed into wildtype Arabidopsis (ecotype Columbia-0) via agrobacterium-mediated floral dip transformation (Clough and Bent, 1998). Transformant (T1) lines expressing dsRNA via the *Ntcel 7* promoter were selected on kanamycin for 10 days after which some seedlings were transferred to soil for T2 seed collection. PCR analysis was used to confirm the presence of the transgene within the genome of each plant line by amplifying the full length *Hs4f01* sense gene from DNA template extracted from transformed plant samples. Subsamples of DNA extracted from whole 10-day old *Ntcel7*-driven seedlings were used to analyze transgene expression of the PDK intron (Wesley et al., 2001) using RT-PCR. Constitutive (CaMV 35S) transgene expression of the Arabidopsis PDK intron of the

hairpin dsRNA (Wesley et al., 2001) in each confirmed transgenic line was analyzed using RT-PCR from leaf samples of 3 week-old plants. Total RNA from plant material was extracted using RNeasy Plant Mini Kit following manufacture's instructions (Qiagen, Valencia, CA). RT-PCR analysis for the PDK intron expression was conducted using the First-Strand cDNA Synthesis kit using Supercript II RT (Invitrogen, Carlsbard, CA). Gene specific primer PDK-RT-R: 5'ATCAATGATAACACAATGACATGATCT3' was used to make first strand cDNA which was used as a template for amplification of a 300bp amplicon using primers pIntronF: 5'GACGAAGAAGATAAAAGTTGAGAG3' and pIntronR: 5'TTGATAAATTACAAGCAGATTGGA3'. Products of RT-PCR were separated on an agarose gel to assess expression of the RNAi construct among transgenic Arabidopsis lines.

Nematode infection assays

Twenty-four seedlings (5 days post germination) from each Arabidopsis line that were selected on MS media supplemented with 50 μg/ml kanamycin were aseptically transferred one seedling per well in six-well culture plates (Falcon, Lincoln Park, NJ) containing 6mls 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 growth chamber with 16 hour light/8hour dark cycle for 7 days before nematode inoculation. Hatched *H. schachtii* preparasitic J2 or *M. incognita* preparasitic J2 were collected as described above and surface-sterilized by incubating for 10min in 0.004% Mercuric chloride, 0.004% sodium azide, and 0.002% Triton X-100 and washed 3 times with sterile distilled water. The sterilized nematodes were

suspended at a concentration of 10 J2/10 µl in 35°C 1.5% low melting point agarose (LMA) to allow even distribution of nematodes to each plant and to facilitate the movement of the J2 into the solid plant growth medium. Each plant was inoculated with approximately 50-60 J2 after which the plates were re-sealed with parafilm and placed in the growth chamber as described above. The infection rates and growth of *H. schachtii* on each Arabidopsis line were assessed over the same time course for comparative results. Statistical differences were determined by the paired *t*-test with an alpha level of 0.05 using SAS software (Cary,NC).

Quantitative RT-PCR (qRT-PCR) of parasitism genes in *H schachtii* infecting dsRNA-expressing Arabidopsis lines

RNAi Arabidopsis plants confirmed to express hairpin dsRNA were grown on modified knops media as described above (Sijmons, et al., 1991) with 0.8% Daishin agar (Brunschwig Chemie BV, Amsterdam, Netherlands) and the plates were grown in a growth chamber at 24°C with 16 hour light/8hour dark cycle. Each plate was grown vertically for vertical root growth for 10 days before being inoculated with sterilized pre-parasitic *H schachtii* J2. Plates were viewed under a dissecting microscope and parasitic J3 nematodes were hand-dissected out of the roots using sterilized forceps.

The parasitic J3 *H. schachtii* excised from dsRNA-expressing *Arabidopsis* lines were pooled together and mRNA was isolated after mechanical disruption using the Dynabeads mRNA DIRECT micro kit (Invitrogen, Carlsbard, CA) and DNase treated with Turbo DNA-free kit (Ambion, Austin, TX) according to the manufacturers instructions. First strand cDNA synthesis was made from 10ng of mRNA using SuperScript II RT (Invitrogen,

Carlsbard, CA). The cDNA was used as a template for real-time quantitative PCR (qRT-PCR) which was performed in a DNA Engine Opticon2 (Biorad, Hercules, CA). qRT-PCR primers used to quantify expressed *Hs4f01* transcript were 5'AGATTGAGAAGGGCATTGAG3' and 5'CGTTGCG GACATATTTGAT3' and the primers used to quantify expression of a non-RNAi target parasitism gene Hgsyv46 (Wang et al., 2001) were 5'ACCGCTGGGCTGCTCTTCAC3' and 5'CGGCATTTCCTCCC TGAGCA3'. Primers used to quantify *H. schachtii* actin for normalization were 5'CGTGACCTCACTGACTACCT3' and 5'CGTAGCACAACTTCTCCTTG3'. A single 20µl PCR reaction included 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5µl cDNA template and 5µM each forward and reverse primers. The PCR cycling parameters were set at 95°C for 10min followed by 40 cycles of 95°C for 15s and 60°C. After the completion of the cycling parameters, dissociation melt curve analyses (60-90°C every 0.5°C for 1sec) was conducted to discount the effects of primer-dimer formation and contamination. 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. The fold change relative to the nematodes infecting the control lines was calculated according to the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001). The paired *t*-test with an alpha level of 0.05 was used to compare relative transcript level means using the statistical software package of SAS (Cary, NC).

Results

Isolation and analyses of *Hs4f01*.

Isolation of the *H. schachtii 4f01* (*Hs4f01*) cDNA resulted in a 1023bp nucleotide sequence that showed 97% nucleotide identity and 3% gaps within the sequence when compared with the 1026 bp *H. glycines 4f01* (AF469059) cDNA homolog (*Hg4F01*) (Fig. 1). Southern blot analysis was used to determine the gene copy number of *4f01* in *H. schachtii* and *H. glycines* genome (Fig. 2). The Hg*4f01*-DIG labeled probe hybridized to DNA within both nematode genomes and indicated that there were at least 2 copies of the *4f01* gene present in each cyst nematode genome.

The open reading frame of *Hs4f01* encoded a predicted protein of 340 amino acids with a calculated protein mass of 36kDa and showed a 92% amino acid identity to the Hg4F01 protein sequence. The amino acid sequence of Hs4F01 was analyzed for the presence of a signal peptide using the program Signal P 3.0 (Bendtsen et al. 2004) and predicted a hydrophobic signal peptide set for eukaryotic cleavage between position 21 (Alanine) and 22 (Asparagine) indicating that the protein is potentially secreted by the cell, a characteristic of *H. glycines* parasitism gene products (Davis et al., 2004). NCBI conserved domain search (Marchler-Bauer and Bryant, 2004) indicated that the protein encodes the four conserved annexin domains (Clark et al., 2001) typical of the annexin family of calcium and phospholipids binding proteins (Fig. 3). A BLASTp search of the database showed highest similarity to a hypothetical protein from *C. elegans* with E-value of $4e^{-72}$ and showed a 41 % identity to the *nex-1* gene (NP_498109) protein (Daigle and Creutz, 1999). The protein sequence comparison of the *Globodera pallida* annexin-2 (CAC33829) also showed a

sequence identity of 45% to Hs4F01. To observe sequence similarities to annexins in the plant kingdom, protein alignment of the plant annexin (annexin-1) from Arabidopsis (NP_174810) revealed a 33% identity to the Hs4F01 annexin. Sequence similarities of Hs4F01 to annexins in other species were aligned to the conserved domain regions (Fig. 3).

Expression of 4F01 within *H. schachtii*

The tissue localization *4f01* transcripts within the different parasitic stages of *H. schachtii* were analyzed by *in situ* mRNA hybridization. The digoxigenin-(DIG) labeled antisense *Hs4f01* cDNA probe specifically hybridized exclusively within the dorsal esophageal gland cell of parasitic stages J2, J3 (Fig. 4), and J4 of *H. schachtii*, but not within any tissues of hatched, preparasitic J2 nor males. No hybridization signals were detected with the control sense *Hs4f01* cDNA probe in any developmental stage of *H. schachtii*. Polyclonal sera raised to synthetic peptides predicted in Hg4F01 bound exclusively to the dorsal esophageal gland cell lobe, extension, and ampullae (Fig. 5) within *H. schachtii* specimens excised from host plant roots. No specific binding with rabbit pre-immune serum was detected in the nematode specimens.

Expression of Hs4F01 in wildtype Arabidopsis

Since it is hypothesized that the parasitism gene products 4F01 are secreted into the host to initiate host parasite interactions, the first assay to assess the role of 4F01 was to constitutively (CaMV 35S promoter) overexpress *Hs4f01* cDNA in transformed Arabidopsis lines with and without the predicted signal peptide sequence. The exclusion of the signal

peptide should target the protein within the cytoplasm of the transformed plant cells while the presence of the signal peptide target the protein to the secretory pathway for export from the plant cell. Four independent transgenic lines were generated from individual transformations. After selection of transformed Arabidopsis lines with antibiotic resistance, the expression of *Hs4f01* was confirmed by RT-PCR (Fig. 6A). The presence of Hs4F01 protein within the plants was confirmed by western blot analysis with approximate protein size of about 36 kDa, the theoretical molecular weight value (Fig. 6B). Root phenotype was preferentially assessed as it is the area of nematode-host interaction and formation of the specialized feeding cells. No significant difference in root growth was observed in Arabidopsis that overexpressed Hs4F01 with or without the signal peptide (Fig. 6C) as compared to the transformed Arabidopsis controls that expressed β-glucuronidase (GUS). In addition, no significant differences were observed in the shoots of the transgenic Hs4F01 Arabidopsis lines and the control lines.

Infection assays with *H. schachtii* were conducted on Arabidopsis lines confirmed to express *Hs4f01* without signal peptide sequence (predicted overexpressed 4F01 localization within plant cell cytoplasm). An agar-based infection assays using six-well culture plates were used to grow the Arabidopsis lines and inoculated with approx 50-60 *H. schachtii* preparasitic J2s. Interestingly, a significantly (paired *t*-test, $p \leq 0.05$) greater number of *H. schachtii* females developed on plant lines that overexpressed Hs4f01 as compared to the control transgenic GUS line (Fig. 7A). Further, to test whether this increased susceptibility is a phenomenon that occurs with the infection of other plant parasitic nematodes, the Hs4F01 overexpressing Arabidopsis lines were infected with another sedentary nematode,

Meloidogyne incognita (root knot nematode) which share similar life cycles to cyst nematode but have unique methods of root infection (Hussey and Grundler, 1998). No significant difference in infection by root-knot nematodes (Fig. 7B) was observed between transgenic Arabidopsis that overexpressed Hs4F01 and the control transgenic GUS lines.

Complementation of an Arabidopsis annexin mutant with Hs4F01

Similar to the expression of Hs4F01 in Arabidopsis, the Arabidopsis annexin 1 mutant (*annAt1*) shows no observable morphological phenotype (Lee et al., 2004). No significant effect in *H. schachtii* infection or development in roots of *annAt1* plants was observed (data not shown). Germination of the *annAt1* mutant is reduced under high salt stress (Lee et al., 2004), so this phenotype was used to assess whether the nematode Hs4F01 was functionally similar to the Arabidopsis annexin 1 gene. The vector construct 35S::Hs4F01 without the signal peptide was transformed into the T-DNA insertional mutant Arabidopsis lines (SALK_015426). The absence of the Arabidopsis annexin 1 gene trancripts (*AnnAt1*) in the mutant lines and the expression Hs4F01 trangenes were confirmed with RT-PCR (Fig. 8A). Total protein from Hs4F01-expressing lines was isolated from 14 day old seedlings using mainly root tissue since the roots are almost deficient of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisco), the most abundant protein in leaves (Lee et al., 2004), allowing the visualization of low abundance proteins (Lee et al., 2004). Protein extracts were analyzed in a western blot probed with the anti-Hg4F01 poloyclonal sera (Fig. 8B). Four independent homozygous annAt1 mutant lines confirmed to express Hs4F01, the control *annAt1-1* mutant line, and wildtype (Columbia) Arabidopsis were then grown on

75mM NaCl to determine germination efficiency under osmotic stress. Under osmotic stress *annAt1* mutants displays only 16 % germination efficiency while the wildtype seedlings show 93% germination efficiency. Mutant *annAt1* lines expressing *Hs4F01* were germinated in MS media supplemented with 75mM NaCl and displayed 89 – 93 % germination rate (similar to wildtype Arabidopsis (93%), indicative of functional complementation of *annAt1* by Hs4F01 (Fig 8C and 8D).

Host-derived RNA-interference (RNAi) of *Hs4f01*

Plant host-derived RNAi (Gheysen and Vanholme review 2007) was used as a method to potentially silence the expression of the *Hs4f01* parasitism gene within the nematode and to observe the consequential effects on parasitism. Two pHANNIBAL (Wesley et al, 2001) constructs (Fig 9A) were generated with different promoters to express the *Hs4f01* dsRNA in transformed Arabidopsis. The two promoters included the CaMV 35S (constitutive) promoter and the tobacco *Cel7* gene promoter (*Ntcel7*) that has limited tissue-specific expression among plant tissues and was demonstrated to be upregulated in cyst nematode feeding sites in heterologous plants species (Wang et al., 2007) .

After kanamycin selection of transgenic Arabidopsis lines possessing the sense and antisense *Hs4f01*, the expression of the dsRNA was confirmed with RT-PCR of the PDK intron (Fig. 9A) which forms a single-stranded loop of the hairpin dsRNA structure and is spliced out during RNA processing (Wesley et al., 2001). Thus, the amplification of the spliced intron in Arabidopsis is a good indicator that the RNAi vector construct is expressing the dsRNA via each promoter. Since the *Ntcel7* promoter is not constitutive and has limited

expression within specific organs at different life stages of the plant (Wang et al., 2007), whole 10 day-old seedlings were harvested to yield the maximum amount of tissue expressing the dsRNA. RT-PCR confirmed the expression of the PDK intron (dsRNA) within Arabidopsis lines transformed with *Hs4f01*-RNAi driven by the *Ntcel7* promoter (Fig. 9B) and 35S promoter (Fig 9C). No significant plant morphological differences were observed between the control empty vector line and any transgenic *Hs4f01*-RNAi Arabidopsis lines. Four independently transformed homozygous lines that express *Hs4f01* dsRNA behind *Ntcel7* promoter and a single transformed line expressing the original empty vector as a control were used to conduct agar-based *H. schachtii* infection assays. Three weeks post inoculation, a significant (*t*-test, $P \leq 0.05$) decrease (45%) in the number of females was observed in *Ntcel7-Hs4F01* dsRNA expressing lines compared to the empty vector control lines (Fig. 9B). A control 35S-RNAi line expressing dsRNA of the green fluorescent protein (GFP) (non-nematode gene) was generated as an additional control in *35-Hs4f01* RNAi infection assays. No significant plant morphological differences were observed between the *35S-GFP* RNAi lines, wild-type, and the transgenic *35S-Hs4f01* RNAi Arabidopsis lines. Three weeks post inoculation, a significant (*t*-test, $P \leq 0.05$) decrease (36%) in number of developed *H. schachtii* females was observed in *35S-Hs4f01* RNAi plants as compared to the *35S-GFP* RNAi plant lines (Fig. 9C).

A reduction in expression of the endogenous *Hs4f01* gene in the nematodes was detected by quantitative real-time PCR of J3 *H. schachtii* that were excised from the roots of *Hs4f01*-RNAi Arabidopsis lines (Table 1). Expression levels of the nematode actin gene, which was used to normalize the qRT-PCR data, were not significantly different in the

nematodes infecting the RNAi expressing lines and the controls plants. No reduction in endogenous *Hs4f01* expression was observed in nematodes recovered from control plant lines, nor was any reduction in expression of the non-complementary *Hssyv46* nematode parasitism gene (Wang et al., 2006) observed in roots of any *Hs4f01*-RNAi plants. The RNAi silencing effect using the nematode-inducible *Ntcel7* promoter showed a 2.5-3.5 significant ($p \leq 0.05$) fold reduction in *Hs4F01* transcript level, while the RNAi silencing effect using the 35S promoter resulted in a 0.7-2.5 fold reduction in *Hs4f01* transcript levels (Table 1).

Discussion

Functional analyses of the products of *Heterodera glycines* parasitism genes in host plants can be a challenging task due to the technical demands of soybean molecular genetic analyses. The relatively close phylogeny of *H. schachtii* to *H. glycines* (Subbotin et al., 2001) and the ability of *H. schachtii* to infect the model host plant *Arabidopsis thaliana* (Sijmons et al., 1991), however, suggest that this model pathosystem may assist in gene function assessment of *H. glycines*. The close identity in nucleotide and predicted amino acid sequence between the *4f01* annexin-like cyst nematode parasitism genes of both species, and the confirmation of expression of *Hs4f01* and its translated product exclusively within the dorsal esophageal gland secretory cell of parasitic stages of both nematode species, suggests that *H. schachtii* may use similar parasitism genes products to infect its host and therefore can be used as a model organism to understand *H. glycines* infecting soybean.

The *Hs4f01* parasitism gene encodes a protein structurally homologous to the annexin family of calcium-dependant phospholipids binding proteins. These proteins generally have a

variable N-terminal region and a conserved core region composed of at least four repeats (Reynal and Pollard, 1994). The variable N-terminal region is thought to confer the gene function to each annexin member (Reynal and Pollard, 1994). In this study, the *Heterodera* annexins are of similar amino acid length and contain the conserved annexin domains as the annexins from plants and other nematode species.

Annexins have been studied in animal cells including the free living nematode, *C. elegans* where annexins were shown to localize within the reproductive organs of the spermathecal valve and the vulva as well as the gland cell bodies within the terminal bulb of the pharynx of the intestinal cells (Daigle and Creutz, 1999). Furthermore, annexins have been studied in the plant parasitic nematode, *Globodera pallida* (potato cyst nematode), where they were immunolocalized within the amphids, genetial primodium and in the constraining muscles above and below the metacorpus pump chamber of second stage *G. pallida* (Fioretti et al., 2001). Interestingly, the annexin-like genes identified in *C. elegans* and *G. pallida* do not contain the predicted signal peptide for secretion present in *Hg4F01* and Hs4f01 which are characteristic of parasitism genes isolated from the esophageal gland cells of plant-parasitic nematodes (Davis et al., 2004). This observation combined with the developmental expression data of *Hg4f01* and *Hs4f01* suggest the potential adaptation of nematode annexin-like proteins specifically for secretion to modulate the parasitic process in host tissues.

The expression of nematode parasitism genes within a transformed host to assess potential affects in plant phenotype and nematode susceptibility gives initial insight into the function of the nematode parasitism proteins when secreted during infection (Wang et al.,

2005; Huang et al., 2006). Expression of *Hs4f01* in Arabidopsis did not produce any observable morphological phenotype in contrast to phenotypes observed by expression of some nematode parasitism genes (Wang et al., 2005; Huang et al., 2006), but is consistent with the lack of observed phenotype in the *annAt1* mutant (Lee et al., 2004). Hence, the lack of obvious phenotypic change in annexin-augmented plants does not exclude the possibility of Hs4F01 playing a role at a molecular and/or biochemical level in the host. Further, the potential role of annexins in stress related environments may indicate their visible phenotypes only under suitable stress environments, such as the abiotic stress or nematode infection. Plants that expressed Hs4F01 were more susceptible to infection by *H. schachtii* suggesting that an increase in accumulation of annexin-like protein by the nematode (eg. by secretion) may promote the parasitic interaction. The biological basis of the increased susceptibility to *H. schachtii* in Hs4F01 expressing lines remains unclear, but the ability for Hs4F01 to complement the *Arabidopsis* annexin I mutants in restoring its ability to germinate under high salt stress (Lee et al., 2004) suggests functional similarity between nematode secreted annexins and plants annexins. Understanding the effect of endogenous plant annexins during nematode parasitism could suggest potential functional roles of secreted nematode annexins. In *Arabidopsis*, annexins play a role in ameliorating various abiotic stress responses and annexin 1 levels in particular were shown to be elevated during treatment with mannitol and polyethylene glycol indicating that the protein is sensitive general osmotic stress (Lee et al., 2004 Cantero et al., 2006). Microarray studies assessing plant gene expression in response to cyst nematode infection has indicated that plant annexins homologs are induced. Arabidopsis roots infected with *H. schachtii* show a 3-fold

increase in Annexin 4 (*AnnAt4*) levels at 3 days post inoculation (Puthoff et al., 2003). In more recent studies, the analysis of soybean transcript levels within nematode syncytia cells isolated through laser capture microdissection method revealed an Arabidopsis annexin 7-like homolog was upregulated in initial syncytial cells but was down regulated up to 10 days post infection (Ithal et al., 2007a). Similarly, the expression levels of *Hg4f01* in *H. glycines* show initial upregulation but a significant decrease over the course of soybean infection from 2 dpi to 10 dpi (Ithal et al., 2007b) suggesting that both nematode secreted annexin and selected plant annexins play a significant role in initial plant infection and formation of the initial syncytial cells. The roles of the each Arabidopsis annexin member during *H. schachtii* infection, however still need to be assessed and could potentially be involved in later stages of nematode infection as the secreted Hs4F01 levels decrease.

The recent discovery that cyst nematodes secrete homologs of plant peptides is providing evidence that plant parasitic nematodes may mimic plant physiological processes to aid in the successful parasitism of its host. One such example is the functional complementation of the plant CLAVATA 3(CLV3)/CLE polypeptide with the nematode parasitism protein SYV46 (Wang et al., 2005). In Arabidopsis, the CLV3 ligand binds to CLV1/CLV2 receptor kinase complex in order to maintain shoot meristem cell proliferation and differentiation (Wang et al., 2005). Further, it is thought that other CLE proteins which are expressed within the roots of Arabidopsis may be involved in the SYV46 peptide interactions. Although the exact role of SYV46 in relation to the CLAVATA 3 protein family is still unclear, it is evident that cyst nematodes exploit certain plant signal transduction pathways for parasitism. Similarly, perhaps secreted Hs4F01 may interact with the plasma

membrane of the syncytia to influence multiple aspects of cell regulation to promote parasitism. For the successful formation and maintenance of the syncytia the nematode secretions should promote the increased metabolic and transfer capacity of the syncytia to meet the nematodes' nutritional demands throughout feeding. In addition, the potential that nematode secretions suppress potential defense and wound responses of the plant (Hussey and Grundler, 1998) may suggest a role for stress remediation by secreted annexins.

One important aspect of annexin function is that they bind to membranes in response to elevated levels of calcium (Clark et al., 2001). The gross modifications of syncytia formation and maintenance could potentially involve elevated levels of cystolic calcium. One characteristic that is observed during initial syncytia formation is the decrease in the central vacuole followed by the formation of many new small secondary vacuoles (Hussey and Grundler, 1998). As observed in tobacco and Arabidopsis plant cells, annexins associate with vacuolar vesicles and are potentially involved in vacuole biogenesis (Seals and Randall, 1997). Perhaps, Hs4F01 plays a role in the formation of the secondary small vacuoles within the syncytia.

The evidence that Hs4F01 complements plant osmotic stress responses could suggest that Hs4F01 plays a role in ameliorating initial osmotic stress created within the feeding sites which are known to have high turgor pressure and low water potential due to the high transfer of solutes into the feeding site (Hussey and Grundler, 1998). A plant promoter study of Arabidopsis *At17.1* on cyst nematode response showed that this promoter is induced in nematode feeding sites but is also responsive to hormones and osmotics stress indicating that perhaps nematodes share plant responses with abiotic stresses and hormone effects (Mazarei

et al., 2004). Annexins could also be involved in maintaining the nutritional demands of the syncytium through solute transport. Specific membrane bound sugar transport proteins are shown to be activated within the nematode induced syncytia (Juergensen et al., 2003). Although no studies have been shown to demonstrate the role of annexins with sugar transport proteins, there is some evidence that sucrose synthase may be associated with callose and cellulose synthase where annexins may anchor accessory proteins such as sucrose synthase to the plasma membrane (Delmer and Potikha, 1997). Perhaps, nematode secreted annexins aid in the membrane associated sugar transport into the syncytium. Further, callose synthase which is an enzyme that is localized within the plasma membrane is inhibited by the production of annexins (Andrawis et al., 1993). Callose production is observed as a plant wound response during nematode stylet penetration of the cell wall (Hussey and Grundler, 1998). Although callose deposition around the stylet in infecting cells is a common wound response, this response does not appear to inhibit the feeding by nematodes (Hussey et al., 1992). It is tempting to speculate that the nematode may use Hs4F01 to limit callose production and minimize or suppress basal defense responses during compatible interactions.

To further investigate whether nematode secreted Hs4F01 is essential in the compatible interaction of the host, silencing of the gene encoding this annexin-like protein was performed using host-derived RNAi. Since plants display post-transcriptional gene silencing mechanism similar to RNAi in nematodes, constructs that express dsRNA can be cleaved using the dicer-mediated digestion resulting in small interfering RNAs (siRNAs) in the plant (Baulcombe, 2004). The siRNA can potentially be ingested by established cyst nematodes through the feeding tube which has a purported size exclusion limit of 28 kDa

(Bockenhoff and Grundler, 1994) to potentially silence expression of the endogenous nematode gene. The effects of host-derived RNAi against *Hs4f01* during nematode feeding site formation were assessed using two different plant promoters. The 35S CaMV promoter is a constitutive promoter but in response to cyst nematode infection the promoter appears to be repressed particularly as the feeding site matures (Bertiolli et al., 1999). Even though this promoter is thought to be repressed during later stages of the feeding site, Urwin et al. (1995) provided evidence that certain proteinase inhibitors driven by the 35S promoter in transgenic roots resulted in enhanced levels of resistance to *Globodera pallida*, indicating that the use of this promoter with cyst nematode infection can provide effective transgene expression within the feeding site. The expression of the dsRNA constitutively throughout the plant can potentially result in silencing off-target genes although no difference in phenotype was observed in 35S-*Hs4f01*RNAi plants as compared to wildtype and plants that expressed dsRNA of GFP. Off target silencing of Arabidopsis annexins seems unlikely since there are no significant similarities between *Hs4f01* and Annat1 nucleotide sequences. The *Nicotiana tabacum* cellulase 7 (*Ntcel7*) promoter was recently shown to be limited in its expression throughout Arabidopsis and is specifically induced within the nematode feeding sites in heterologous plant species (Wang et al., 2007). High activity of this promoter within cyst and root-knot nematode feeding cells was reported at least 7 days post inoculation. The use of this promoter to target dsRNA expression specifically within feeding sites was shown to be effective, achieving a higher level of downregulation of the target nematode Hs4F01 gene than the 35S promoter, so it may provide an increased level of dsRNA for ingestion from the feeding site. However, introduction of the dsRNA transgene construct using either promoter

was not sufficient to induce complete silencing (knock-out) of the *Hs4f01* gene. Fairbairn et al., 2007 also observe similar results in using the *TobRB7* tobacco promoter (Opperman et al., 1994), which is a root specific promoter induced within feeding cells of plant-parasitic root-knot nematodes, to silence the transcription factor MjTIS11 via plant host-derived dsRNA. The siRNA amplification process is present in plants and nematodes where the siRNA can serve as primers for the synthesis of dsRNA by RNA-dependant RNA polymerases resulting in further production of siRNAs (Baulcombe, 2004). The amplification of siRNA or production of secondary siRNA is not possible within the host plant since the target gene is not available within the plant genome. This was also observed by Fairbairn et al., 2007 where the detection of nematode siRNA appeared to be less that the GUS siRNA produced in GUS expressing lines. However, if the amplification process is truly effective, even a small amount of initial siRNA uptake by the nematode should result in the amplification of target siRNA within the nematode since the target gene is present in the nematode genome.

The effect of the dsRNA/siRNA to *Hs4F01* in infecting nematodes caused a significant reduction (36% – 45%) in established nematode females. The only other study of host derived-RNAi to *H. glycines* showed significant reduction in the number of cysts and eggs in lines expressing dsRNA to the major sperm protein (MSP) (Steeves et al., 2006). Similar to this study, the effect of the siRNA to the MSP resulted in a reduction of infection and not the total control of the nematodes as seen with RNAi to root knot nematode genes (Yadav et al., 2005; Huang et al., 2006). However, since the results of the effects of host-derived RNAi driven by the CaMV 35S promoter vary, and that feeding cell-enhanced

expression can increase the gene silencing effect, the combined data suggest that the nematode gene targeted is critical for success of host-derived RNAi. This observation may underscore the essential nature of the target gene, such as the high transcript abundance of *Hs4f01* at early stages of the infection process within the nematode requiring large amounts of siRNAs through nematode feeding or the presence of functionally redundant genes that could assist the nematodes in completing the lifecycle without the presence of sufficient amounts of Hs4F01.

The quantitative levels of endogenous *Hs4f01* transcripts within the nematodes that are infecting the RNAi lines, could give an indication to the level of silencing within a particular infecting stage. The overall quantitative data presented here indicates that the decrease in transcript levels of *Hs4f01* was specific to this gene and shows no effect on non-target genes analyzed in the same sample. The level of *Hs4f01* on J3 stage nematode, however only showed an approximate 2-3 fold reduction in trancript, which may be associated with the partial resistance observed in the RNAi lines. The low levels of reduction in these genes could be attributed to the fact that the nematodes at this stage perhaps escape the siRNA effect and these nematodes are those that are counted as established nematodes. The samples used for these analyses were, of necessity, biased for viable nematodes and may simply exclude nonviable specimens where the RNAi effect was large enough to completely inhibit nematode development. Perhaps the effect of RNAi to the *Hs4f01* may have occurred much earlier at the initial feeding cell development at around 36-48 hours post infection (Endo 1964) where the target *4f01* transcript levels is the highest (Ithal et al., 2007b). At this stage it was very difficult to dissect the nematodes out of the roots for real-time PCR. In

another study, the effect of the siRNA by stimulated *in vitro* ingestion of the *H. glycines β-1,4-endoglucanse* dsRNA resulted in an initial fold reduction in transcript level followed by a 5-10 day recovery period where high message levels were detected after 10 days post treatment (Bhakhetia et al., 2007). Perhaps, the level of *Hs4F01* transcripts detected at the J3 stage are at the recovery stages and hence allowing the nematode to establish itself on the host RNAi lines. The ingested dsRNA "dose" vs. endogenous expression of the target gene in the nematode is stoichiometrically related, and a sufficient feeding cell promoter may be needed for relatively high abundance nematode gene transcripts. The Ntcel7 promoter is induced during early stages of syncytium stages up to 14 days post infection (Wang et al., 2007) and RNAi effect observed with this promoter was more efficient than the constitutive 35S promoter which is thought to be repressed at least 10 days post infection (Bertioli et al., 1999).

Dissection of the potential pathways of activity of nematode annexins secreted into plant cells as suggested above, perhaps through relative profiles of plant gene expression in annexin lines or RNAi plants, may shed further light on their biological functions. Analyses of potential protein-protein interactions between nematode annexin and plant proteins could suggest potential modes of action as was observed in yeast two-hybrid analyses of a root-knot nematode parasitism protein with Arabidopsis proteins (Huang et al., 2006a). The significant reduction in nematode infection of plants observed from host-derived RNAi of *Hs4f01* combined with the functional complementation of annexins in *Arabidopsis thaliana*, suggest a functional role for secreted nematode annexins in the cyst-nematode host-parasite interaction.

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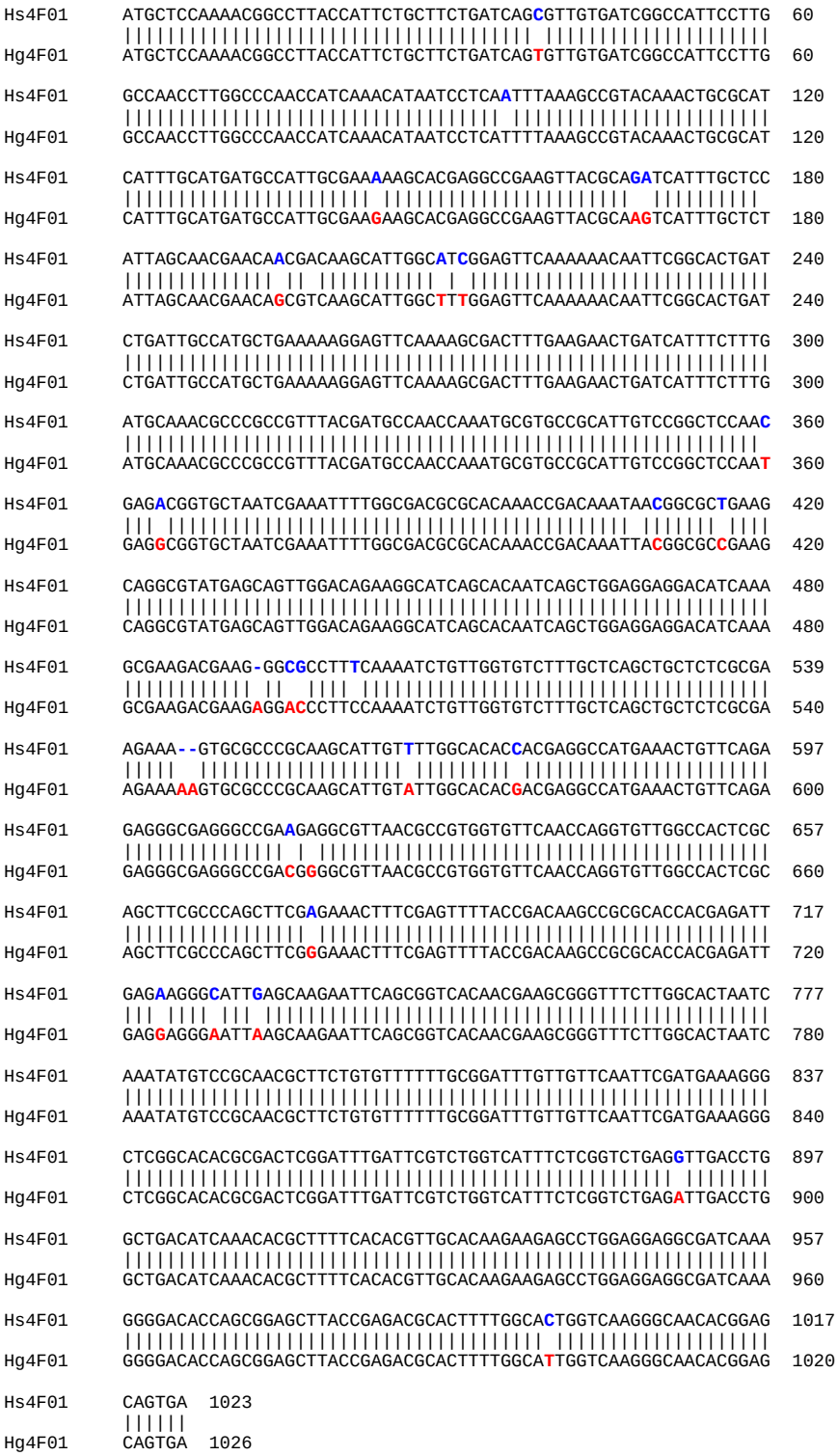

Figure 1. Nucleotide sequence alignment of *4f01* parasitism gene from *H. schachtii* (*Hs4f01*) and *H. glycines* (*Hg4f01*) (AF469059). Alignment conducted using the NCBI sequence local alignment program (Tatusova and Madden (1999)) showing 97% nucleotide identities.

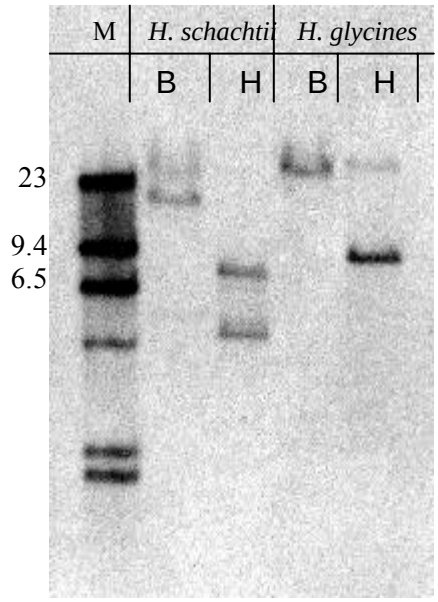

Figure 2. Southern blot analyses of nematode 4F01 annexin-like gene from *H. schachtii* and *H. glycines*. Genomic DNA from *H. schachtii* and *H. glycines* was digested with *BamH I* (B) and *Hind III* (H) with a 162bp Hg4F01 DIG-labeled cDNA probe. M: molecular weight marker shown in Kb.

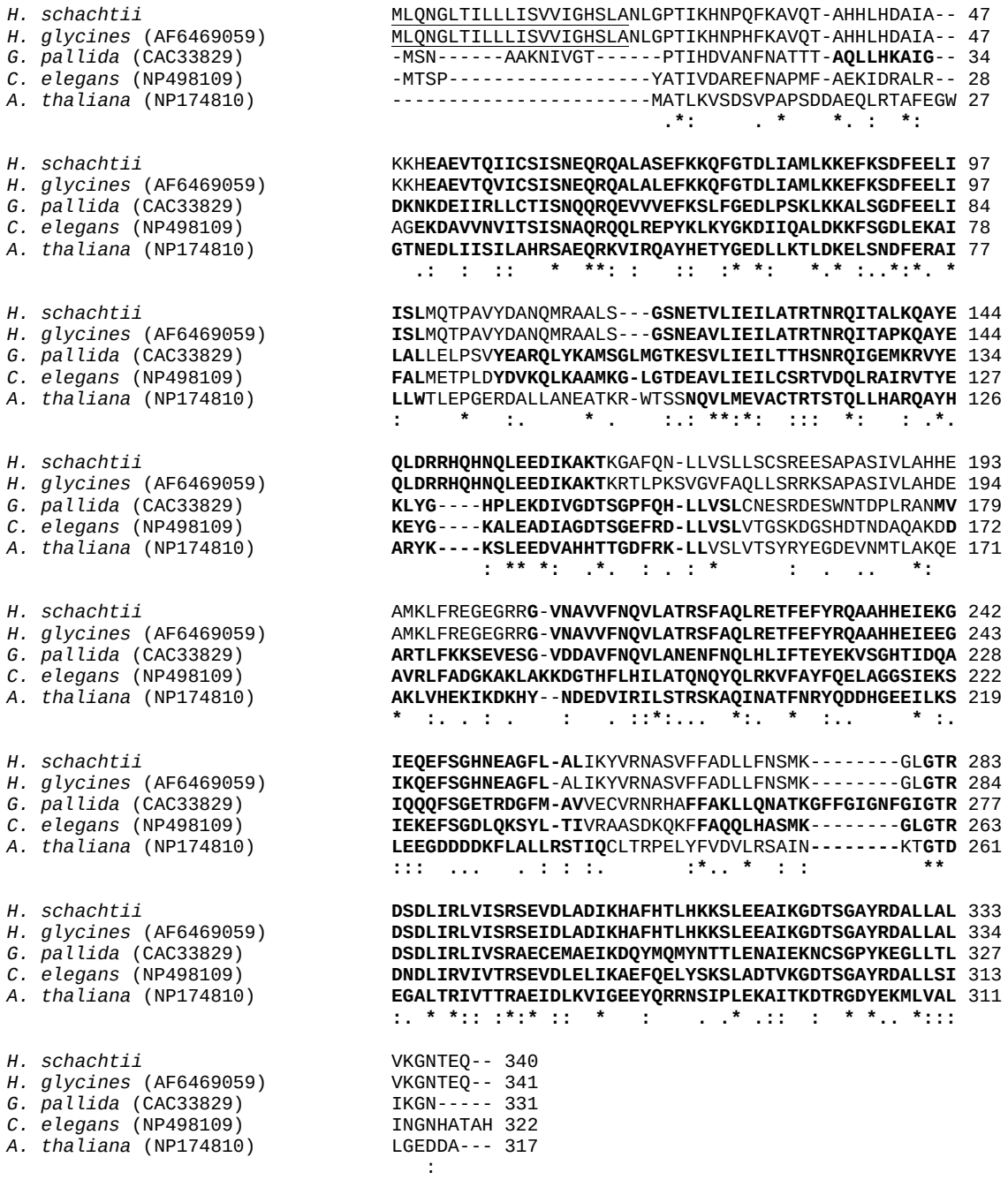

Figure 3. Alignment of *H. schachtii* predicted annexin protein with annexin protein from plant parasitic nematode *H. glycines* and *Globodera pallida* and predicted annexin from *C. elegans* and *A. thaliana*. (*): Absolutely conserved residues, (:) residues conserved in at least four sequences, (.): residues conserved in at least 2 sequences. The underlined sequences indicate predicted signal peptides present in the N-termini of the sequences. Bold sequences represent the conserved annexin repeats as predicted by the NCBI Conserved Domain search.

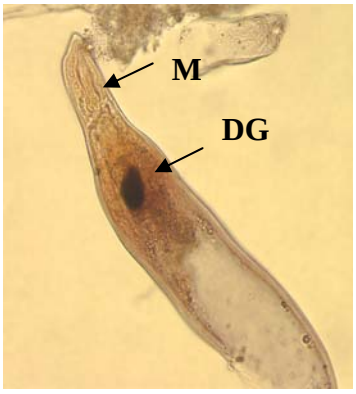

Figure 4. Hybridization of digoxigenin-labeled antisense cDNA probe (dark staining) of the *Hs4f01* annexin-like parasitism gene to transcripts specifically expressed within the dorsal esophageal (DG) gland cell of a third-stage juvenile of *Heterodera schachtii* that was excised from host plant roots. M: nematode metacorpus.

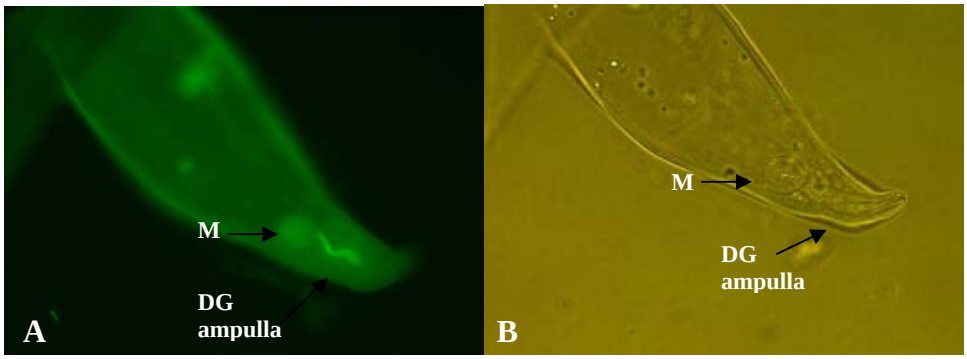

Figure 5. Indirect immunofluorescence (FITC) microscopy demonstrating the binding of polyclonal antibodies generated to synthetic peptides of *H. glycines* 4F01 parasitism gene products. **A:** Binding specifically within the dorsal esophageal gland cell ampulla of a parasitic stage of *H. schachtii*. **B:** Brightfield image showing the nematode gland cell ampulla. M: nematode metacarpus; DG: Dorsal gland.

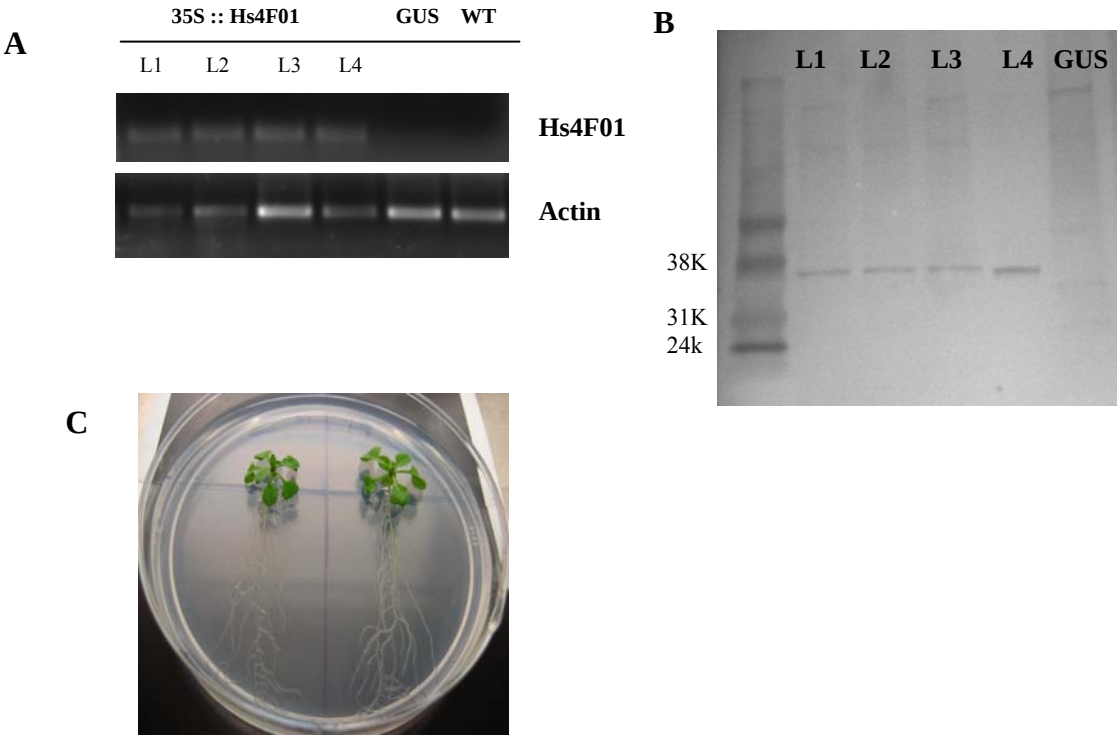

Figure 6. Expression of Hs4F01 in independent transgenic lines of *Arabidopsis thaliana*. **A** and **B**: Relative reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analyses for the expression of *Hs4f01* in four transgenic homozygous lines (L2, L3, L4 and L5). **C**: Root phenotype analysis of *Hs4f01* expressing line (left) and GUS expressing line (right) showing no difference in root and shoot phenotype 14 days post germination. No phenotypic differences were observed with the presence and absence of the signal peptide.

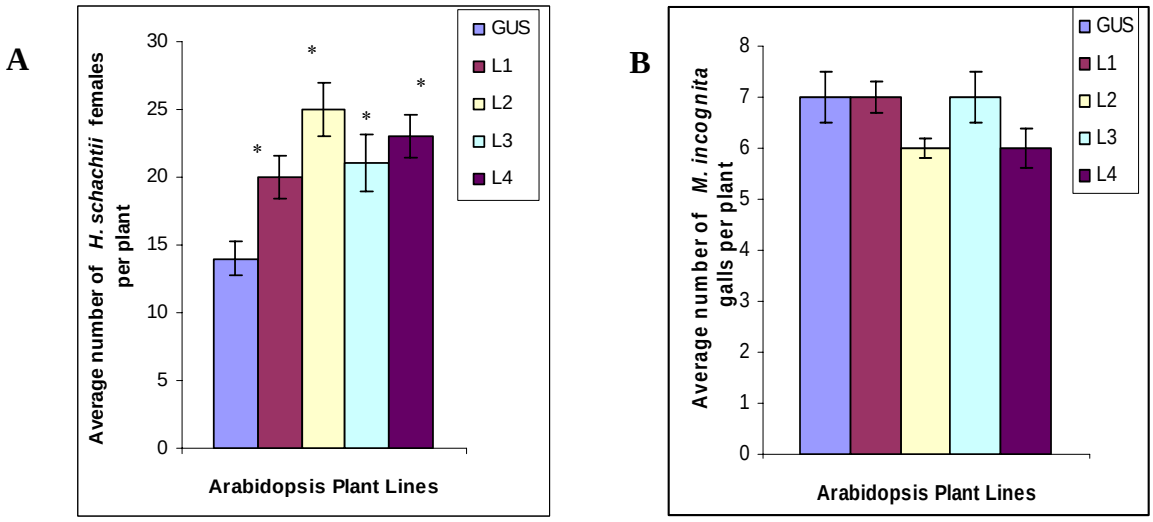

Figure 7. Nematode development in roots of the independent transgenic lines (L1-L4) of *Arabidopsis thaliana* shown in Figure 6 that express Hs4F01. A significant (*t*-test, P≤0.05) increase (*) in average number of *H. schachtii* females per plant (Panel A), and no significant effects on development of galls by the root-knot nematode, *Meloidogyne incognita* (Panel B), were observed at 21 days post inoculation on Hs4F01 overexpressing Arabidopsis lines as compared to nematode development on control lines that expressed beta-glucuronidase (GUS).

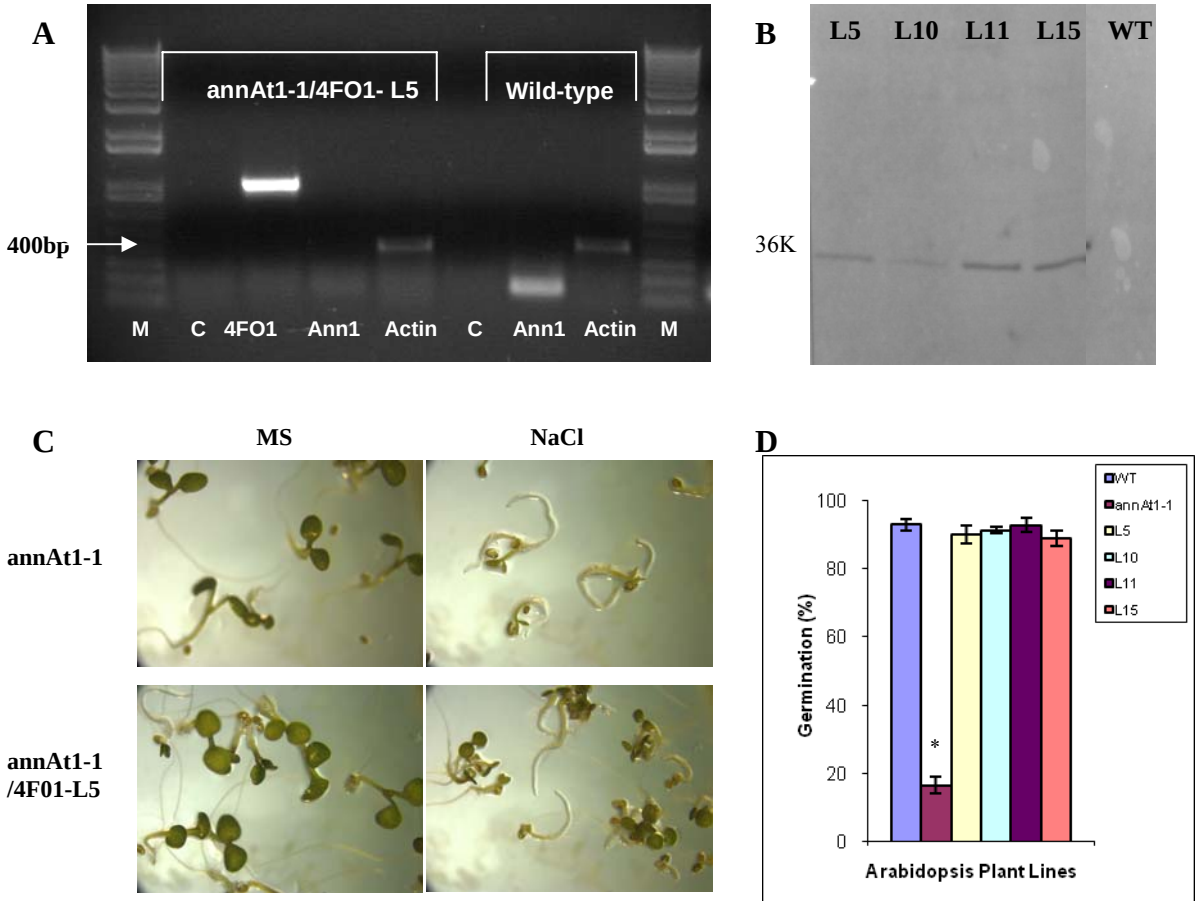

Figure 8. Complementation of the Arabidopsis annexin 1 mutants (*annAt1-1*; Lee et al., 2004) by the cyst nematode annexin-like parasitism gene *Hs4f01*. **A:** RT-PCR confirmation of expression of *Hs4f01* in the *annAt1-1* background. No endogenous *annAt1* transcripts were detected in the mutant compared to the wildtype. **B:** Western blot confirming Hs4F01 protein in the transgenic *annAt1-1/Hs4f01* lines. **C:** Differential germination and growth of the *annAt1* mutant and transgenic (L5) line of *annAt1-1/Hs4f01* L5 grown on MS medium with and without supplemental 75mM NaCl at seven days after germination. **D:** Percent germination of wildtype, *annAt1-1* and transgenic *annAt1/Hs4f01* Arabidopsis lines L5, 10, 11, 15 on MS media supplemented with 75mM NaCl. Bars indicate ± standard error; Asterix (*) represents a significant difference (*t*- test, $P \leq 0.05$ versus control ($n \geq 100$).

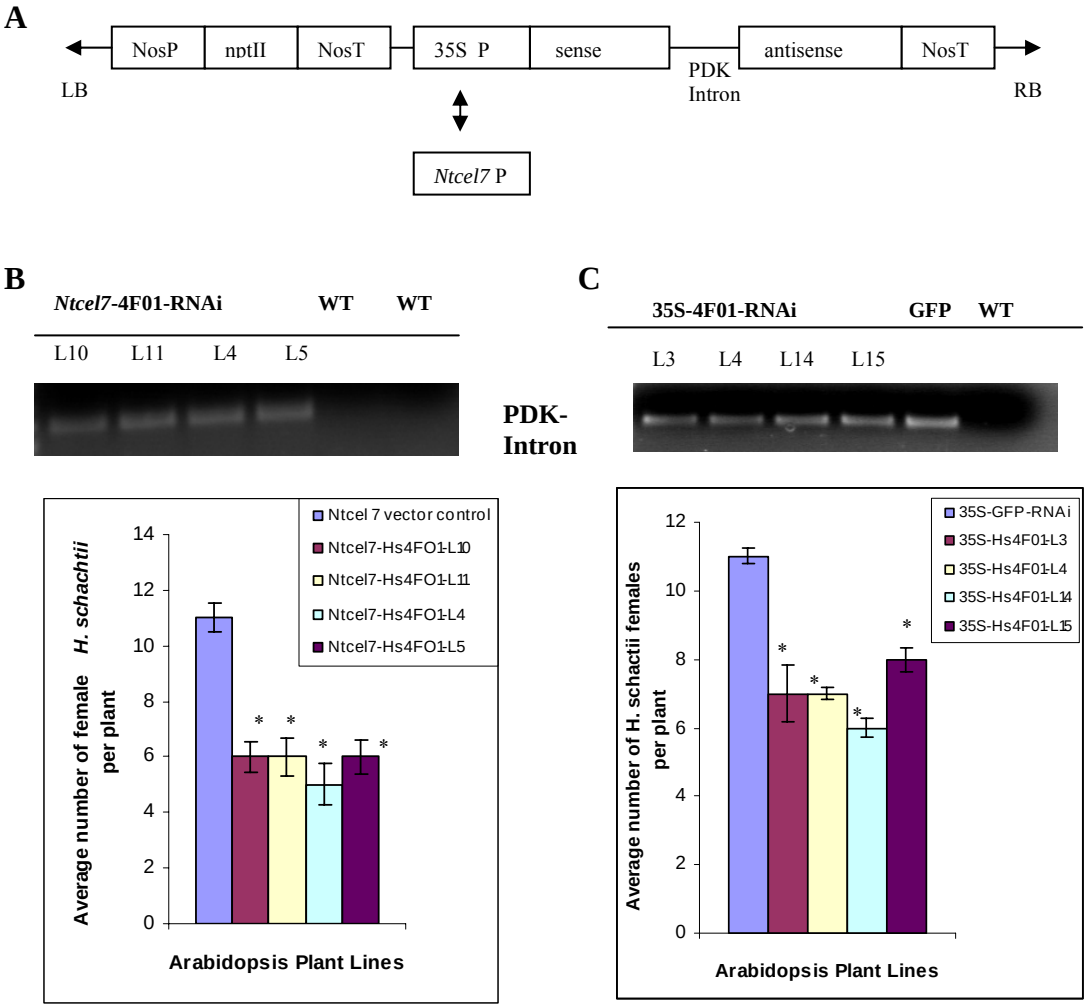

Figure 9. Host-derived RNA-interference of *Hs4f01*. **A:** Illustration of the T-DNA region of the binary vector pArt27 containing the sense and antisense of *Hs4f01*. 35S CaMV promoter (35S P) was originally in the vector and was replaced with the *Nicotiana tabacum* cellulase 7 promoter (*Ntcel7* P). **B:** Expression of the intron region of the dsRNA product at 10 days post germination. *H. schachtii* infection data on RNAi lines expressing the *Hs4f01* dsRNA via nematode inducible *Ntcel7* promoter showing a reduction in the number of females per plant compared to the control **C:** Expression of the intron region on the 35S CaMV-*Hs4f01* Arabidopsis lines. *H. schachtii* infection on RNAi lines constitutively expressing *Hs4f01* dsRNA showing a reduction in females three weeks post infection. Bars indicate ± standard error; n=30. Asterisk (*) represents a significant difference (*t*- test, P≤0.05 versus control).

Table 1. Relative endogenous expression levels of *Hs4f01*and *Hssyv46* (non-target gene) within J3 stage *H. schachtii* that were excised from transgenic Arabidopsis plant lines expressing dsRNA to *Hs4f01* compared to the *Hs4f01* or *Hssyv46* levels in nematodes infecting the control 35S-GFP-RNAi plant lines. Significant ($P \leq 0.05$) differences in parasitism gene expression in nematodes from RNAi plants as compared to nematodes excised from control plants are noted by an asterisk.

Nematode infecting Arabidopsis RNAi Plant Lines	**Parasitism gene tested**	**Relative fold Difference**
Ntcel7-4F01-L4	4F01	-2.5 ± 0.2*
Ntcel7-4F01-L5	4F01SYV46	-3.5 ± 0.1*
Ntcel7-4F01-L4	SYV46 (non-target gene)	0.52 ± 0.4
35S-4F01-L14	4F01	-1.5 ± 0.5
35S-4F01-L15	4F01	2.5 ± 0.34*
35S-4F01-L15	SYV46 (non-target gene)	0.54 ± 0.8

FUNCTIONAL ANALYSES OF SELECTED CANDIDATE CYST NEMATODE PARASITISM GENES.

Abstract

The secreted proteins encoded by "parasitism genes" expressed exclusively within the esophageal glands cells of cyst nematodes play important roles in nematode infection and parasitism of the plant host. The *syv46*, *4e02* and *5d08* parasitism gene cDNA clones originally isolated from *H. glycines* were also present in *H. shachtii* and were confirmed to be expressed within the esophageal glands cells. Expression of Hs4E02 and Hs5D08 in *Arabidopsis thaliana* did not result in any visible phenotypic changes in the roots and shoots during early plant development. Infection of the lines expressing Hs4E02 with *H. schachtii* did not affect the number of females established within the host as compared to the control. Further, host-derived RNAi of *Hssyv46* was conducted using two vectors containing the nematode-inducible promoter (*Ntcel7*) and the 35S CaMV promoter. RNAi lines expressing *Hssyv46* dsRNA via *Ntcel7* and 35S promoters resulted in a reduced number of established females three weeks post infection. The transcript level of endogenous *Hssyv46* in nematodes excised from the RNAi lines was reduced approximately two-fold. No difference in the female counts was observed for host-derived RNAi of *Hs4e02* and *Hs5d08* using both promoters. Quantitative mRNA levels of *Hs4e02* in nematodes infecting RNAi lines showed a two-fold reduction but no significant difference in transcript level of *Hs5d08* in nematodes was observed.

Introduction

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. Infective second-stage juveniles (J2) penetrate host plant roots and migrate intracellulary releasing cell wall-degrading enzymes and piercing the cell wall with their 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 (Niblack et al., 2006). Cyst nematodes use their stylet to penetrate the cell wall of an initial syncytial cell and secrete proteins to induce syncytium formation by 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). Secretions from the nematode stylet originate within the three enlarged esophageal gland secretory cells, two located subventral and one cell located dorsal (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). A battery of nematode parasitism genes encoding cell wall-modifying proteins have been isolated (summarized in Mitchum et al., 2007) including the first secreted endoglucanases of animal origin (Smant et al., 1998; Wang et al., 1999) that were hypothesized to be the product of ancient horizontal gene transfer (Smant et al., 1998). Parasitism genes encoding secreted chorismate mutase have also been isolated from both

root-knot and cyst nematodes (Mitchum et al., 2007), and nematode chorismate mutase was shown to alter root development when expressed within host roots (Doyle and Lambert; 2003). The homology of these nematode parasitism genes with genes of known function within public databases provided a basis to investigate their biological role.

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 *syv46* (*Hgsyv46*) (Wang et al., 2001) encodes a novel protein whose C-terminus was predicted by domain analyses (Olsen & Skriver, 2003) to be similar to a plant peptide in the CLAVATA3/Endosperm surrounding region (CLE/ESR) family. CLAVATA3 functions as a signaling peptide involved in plant meristem differentiation and stem cell maintenance (Clark, 2001; Mitchum et al., 2008). Amazingly, expression of *Hgsyv46* in a transgenic *Arabidopsis thaliana clv3*-1 mutant background complemented the phenotype and negatively regulated expression of the Arabidopsis *wuschel* gene, similar to the effects of overexpression of *clv3* itself in plants (Wang et al., 2005; 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 *clv3* 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). Recent *in planta* localization studies of these candidate parasitism genes indicated that 4E02 (without the presence of the signal peptide) showed nuclear localization in both onion cells and Arabidopsis protoplasts (Elling et al., 2007). In contrast, 5D08 (without the presence of the signal peptide) accumulated only in the cytoplasm when expressed in onion and Arabidopsis cells (Elling et al., 2007). The presence of a functional NLS in 4E02 suggests that this protein may play a direct regulatory role within the plant cell nucleus to promote parasitism of the host. Even though 5D08 did not show active nuclear uptake, this parasitism protein could play important roles as a cytoplasmic effector.

In this study, we make use of the plant model *Arabidopsis thaliana* as a host to infer function of the *H. glycines* candidate parasitism genes *syv46*, *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 *syv46, 4e02* and *5d08* 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 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 second stage juveniles (pre-J2s). 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 homologs

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, Carlsbard, CA) following the manufacturer's instructions including extract digestion with DNAse I to remove potential reaction contaminants.

The original *syv46, 4e02 and 5d08* cDNA clones were isolated 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, Carlsbard, 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, Carlsbard, CA) for sequencing. Based on the sequencing results of the 3' and 5' RACE products for each gene, forward and reverse gene specific primers of *syv46, 4e02 and 5d08* (Table 1) were used to amplify the full-length *H. schachtii* parasitism gene cDNA clones. The cDNA clones were subsequently cloned was into pCR4-TOPO vector (Invitrogen, Carlsbard, CA) for DNA sequence confirmation.

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 BLAST Alignment algorithim at National Center for Biotechnology Information (NCBI) (Tatusova and Madden, 1999). Sequence similarity searches to genes in NCBI database were performed using 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 the PSORT II (Nakai and Horton, 1999).

mRNA *in situ* hybridization

Localization and developmental expression analyses of *Hssyv46, Hs4e02* and *Hs5d08* were performed on fixed mixed parasitic stages of *H. shachtii* (De Boer et al., 1998). Specific forward and reverse primers for each cDNA clone were used to synthesize digoxigenin (DIG)-labeled sense (control) and antisense cDNA probe by asymmetric PCR (Gao et al., 2001). The cDNA probes that hybridized to transcripts within the nematode specimens were detected colorimetrically using the method of De Boer et al., (1998).

Immunolocalization of nematode parasitism proteins

Polyclonal antibodies to *H.glycines* SYV46, 4E02 and 5D08 were produced by immunizing individual rabbits with two synthetic peptides designed to specific predicted

amino acid sequence of each gene product including; SYV46 – NH2-STGDKKTANDGSGNN-COOH; NH2- PVNESKRLSPSG PDPH-COOH; 5D08 – NH2-SKPNPGQKPSGERRK-COOH; NH2-VNRNGWENTGTPTGGR-COOH; and 4E02 NH2-RGGWPWDWAGK-COOH; NH2-KQLCKTSANCKCKD-COOH (Eurogentec, Inc., Belgium). The localization of expressed gene products with each specific antiserum within nematode specimens was detected by immunoflorescence microscopy of fixed mixed parasitic stages of *H. schachtii* according to Goellner 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 *Bam HI* and *Sac II* 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). Transgene expression of *Hs4e02* and *Hs5d08* were confirmed by RT-PCR on total RNA of leaf tissues on homozygous lines by first strand cDNA synthesis using Superscript II RT kit and PCR amplification using 4E02 primers 5'TTCTGGCCGCTCTTTGCCTGTCCT3' and 5'TTAATGTTTGGGCTTCT TCCCGC3' and 5D08 primers 5' TGCCGTGTTTATAATTGGCATTAA3' and

5'TCATCGTCCGCCGGTTGGAGCGC3'. Four homozygous Hs4E02- and Hs5D08-expressing *Arabidopsis* lines were grown in Murashige-Skoog (MS) media plates placed vertically for root growth assays.

Host derived-RNAi – vector construction and analyses of transgenic lines

Full-length *Hs4e02*, *Hs5d08* and partial *Hssyv46* cDNA sequences (70bp-240bp) were cloned in the sense and antisense orientation at the *XhoI – 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 (*Ntcel7*) gene that has demonstrated upregulation within cyst and root-knot nematode feeding cells (Goellner 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 *Ntcel 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 gene-specific primes in PCR. Leaf samples 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 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 Supercript II RT (Invitrogen, Carlsbard, CA). Gene-specific primer PDK-RT-R: 5'ATCAATGAT AACACAATGACATGATCT3' was used to make first strand cDNA which was used to a template for amplification of a 300bp amplicon using primers pIntronF: 5'GACGAAGAAGATAAAAGTTGAGAG3' and pIntronR: 5'TTGATAAATTACAAG CAGATTGG A3'. Products of RT-PCR were separated on an agarose gel to assess expression of the RNAi construct among 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 6mls 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 growth chamber with 16 hour light/8hour dark cycle for 7 days before nematode inoculation. Hatched *H. schachtii* preparasitic 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 subsequent three rinses with sterile distilled water. The surface-sterilized pre-J2 were suspended at a concentration of 10J2/10 μl in 35°C 1.5% low melting point agarose (LMA)

to allow even distribution of nematodes to each plant and to facilitate the penetration of the J2 into the solid growth medium. Each plant was inoculated with approximately 60 J2 on *Ntcel 7*-RNAi plants lines and 100 J2 on the 35S –RNAi plants lines after which the plates were re-sealed with parafilm and place in the plant growth chamber. Statistical differences were determined by the paired *t*-test with an alpha level of 0.05 using the statistical software package SAS (Cary, NC).

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 media (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/8hour dark cycle. Each plate was grown vertically for vertical root growth 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, Carlsbard, 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, Carlsbard,

CA). The cDNA was used as a template for qRT-PCR which was performed in the DNA Engine Opticon2 (Biorad, Hercules, CA). A single 20µl PCR reaction included 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 3µl cDNA template and 5µM each forward and reverse primers (Table 2). The PCR cycling parameters were set at 95°C for 10min followed by 40 cycles of 95°C for 15s and 60°C. After the completion of the cycling parameters, dissociation melt curve analyses (60-90°C every 0.5°C for 1sec) 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 $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). qRT-PCR of a non-target parasitism gene in each reaction was also included to very 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 statistical software package SAS (Cary, NC).

Results

Isolation of selected parasitism gene homologs in *H. schachtii*

A cDNA (*Hs4e02*) of 279bp encoding a predicted 93 amino acid protein was amplified from mRNA of *H. schachtii* using PCR primers designed from the *H. glycines 4e02* (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 database

('pioneer' protein) other than Hg4E02. Alignment of the *Hs4e02* homolog with *Hg4e02* revealed a 99% nucleotide identity and a predicted 100% amino acid identity, indicating that the protein is conserved within the two species (Fig.1; Table 3). Similar to the *H .glycines* cDNA, *Hs4e02* also possessd a secretion signal peptide predicted by SignalP that is cleaved between position 27 (Serine) and 28 (Glutamic acid) of the protein sequence.

The gene *Hg5D08* (AF473828) is also a candidate parasitism gene (Gao et al., 2003) that does not have significant homology to any known sequence in the NCBI database (a 'pioneer' protein). The *Hs5d08* cDNA (411bp) was amplified from *H. schachtii* mRNA using *Hg5d08* PCR primers and showed a 98% nucleotide identity and a 96% predicted amino acid identity to the Hg5D08 sequence (Fig.2; Table 3). 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) which suggest that the secreted proteins are potentially targeted for import into the host cell nucleus. The *Hgsyv46* parasitism gene product is unique (Mitchum et al., 2008; Wang et al., 2001, 2005) in that the C-terminal domain contains a motif similar to that of the CLAVATA3/ESR-related (CLE) family in *Arabidopsis thaliana* (Table 3). An *Hssyv46* cDNA was amplified from *H. schachtii* mRNA using *Hgsyv46* PCR primers that had a 93% nucleotide identity and a 92 % predicted amino acid identity to *Hgsyv46* (AF473827) (Fig.3; Table 3). HsSYV46 possessed a predicted secretion signal peptide that is cleaved between position 24 (Threonine) 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 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 preparasitic J2 and parasitic J2, J3 and J4 of *H. schachtii* (Fig. 4A and B). The Hs*syv46* and Hs*5d08* DIG-probes hybridized to transcripts expressed within the single dorsal esophageal gland cell of parasitic J2, J3 and J4 (Fig 4A and 4B) 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 to translated HsSYV46 within the dorsal gland cell of *H. schachti* 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 overexpressed (with and without the signal peptide) constitutively in transformed Arabidopsis. The exclusion of the signal peptide should target the protein within the cytoplasm of the transformed plant cells and the presence of the signal peptide should target the protein to the secretory pathway. The expression of *Hs4e02* in three independent transgenic Arabidopsis lines was confirmed by RT-PCR (Fig. 6C) of the full-length transcript. No visible differences in root or shoot growth were observed in plants that overexpressed Hs4E02 with or without the signal peptide compared to the control transgenic Arabidopsis plant that expressed GUS (Fig. 6A and 6B). 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 (Fig. 6D).

The Hs5D08 parasitism gene encodes a novel protein with a NLS sequence predicted by PSORT II analysis. Similar to Hs4E02, although Hs5D08 expression was confirmed in transgenic Arabidopsis lines (Fig. 7B), there were no visible effects of Hs5D08 expression on plant phenotype (Fig. 7A) compared to control plants.

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 8A) were generated with different promoters to express the dsRNA in

transformed Arabidopsis. The two promoters included the CaMV 35S (constitutive) promoter and the tobacco *Cel7* gene promoter (*Ntcel7*) 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 independently-transformed homozygous Arabidopsis lines with either the *Ntcel 7* promoter or the 35S promoter to drive hairpin dsRNA expression of *Hssyv46*, *Hs4e02*, and *Hs5d08* were assessed for dsRNA expression by RT-PCR of the single-stranded hairpin loop (Wesley et al., 2001) of the PDK intron (Fig. 8A). 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 three weeks post inoculation.

Homozygous Arabidopsis L8 and L10 transgenic lines that expressed *Ntcel7* promoter-driven dsRNA complementary to *Hssyv46* showed a significant ($P \leq 0.05$) 36% reduction in the number of females compared to the control *Ntcel7* empty vector RNAi plant lines (Fig 8B). Two T2 lines expressing CaMV 35S-driven dsRNA of *Hssyv46* showed a significant ($P \leq 0.05$) 32% reduction in the number of established females when compared to the control lines that expressed 35S-driven dsRNA of the non-target green fluorescent protein (GFP) gene (Fig. 8C). Quantitative real-time PCR of nematodes excised from the Arabidopsis *Hssyv46*-RNAi lines indicated that transcript levels of endogenous *Hssyv46*

were significantly ($P \leq 0.05$) reduced compared to nematodes infecting control plants expressing dsGFP (Table 4). 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 plantss, indicating that the observed 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 *Ntcel7* or CaMV 35S promoter (Figs. 9 and 10). Although significant silencing of endogenous nematode *Hs4E02* was observed compared to controls in *Hs4e02*-RNAi plants (Table 4), a significant fold increase the non-target *Hssyv46* gene was also observed in those nematode infecting those plants. No significant silencing of endogenous nematode *Hs5d08* was observed compared to controls in *Hs5d08*-RNAi plants (Table 4), however, silencing of endogenous *Hs5d08* was approximately one and a half-fold greater than endogenous non-target *Hssyv46*.

Discussion

The striking inter-species sequence identity between the *H. schachtii* homologs of the *H. glycines syv46*, *5d08* and *4e02* parasitism genes suggests that the two nematode species share common parasitic mechanisms even though their host plant species may vary. This comparison was further supported by similar developmental expression of the *H. shachtii*

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 *Arabidopsis thaliana* and *H. schachtii* as a model for functional analyses of *H. glycines* parasitism genes in soybean.

The *syv46* parasitism gene (Wang et al., 2001) is unique in that it contains a C-terminal domain of the CLAVATA/ESR (CLE) family in *Arabidopsis thaliana* (Mitchum et al., 2008; Olsen and Shriver, 2003; Wang et al., 2005). In Arabidopsis shoots and floral meristems, CLV3 purportedly binds with the CLV1/CLV2 receptor complex and negatively regulates WUSHEL expression to maintain the size of the stem cell population (Mitchum et al., 2008). Interestingly, the expression of HgSYV46 in wild-type Arabidopsis resulted in premature termination of the shoot apical meristem and short root phenotype characteristic of *wushel*-like phenotype (Wang et al., 2005). Such a strong and discrete phenotype within the host indicates that this parasitism protein is specifically active in plant cells and may function in a similar manner to affect the formation and/or maintenance of cyst nematode feeding cells. This hypothesis is further supported by the remarkable ability of expressed HG-SYV46 to complement the shoot phenotype of an Arabidopsis *clv3-1* mutant (Wang et al., 205). The exclusion of the nucleotide sequence encoding the 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 pant phenotype were observed. 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 J2 of *H. glycines* induced to ingest *syv46* 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 shift to male development that is a characteristic of nematode stress environment (Lilley et al, 2005). The partial resistance observed on the *Hssyv46*-RNAi plants and non-lethal nematode phenotype observed in RNAi-soaking assays could be the result of a very high level of *Hssyv46* transcript abundance (Bakhetia et al., 2007) that makes it difficult to silence the gene to a level that completely disrupts parasitism. DNA gel blots indicate the presence of at least five copies of the *syv46* gene within the nematode genome (Mitchum M.G., unpublished data) suggesting the further potential for functional redundancy to compensate for RNAi silencing of some members of the *syv46* gene family. The use of the entire coding region of *Hssyv46* (minus CLE domain) for RNAi constructs here would suggest the likelihood of sufficient nucleotide identity for siRNA effects among gene family members, however, but evidence that the region of the transcript targeted can affect RNAi efficacy has been reported for cyst nematodes (Sukno et al., 2007).

The transcript levels of endogenous nematode *Hssyv46* recovered from nematodes infecting *Hssyv46*-RNAi transgenic Arabidopsis plants showed only a 2.2 fold reduction compared to the control at the J3 stage of infection (Table 4). Even though a quantitative measurement of transcript abundance was determined for *syv46* in *H. glycines* throughout the life cycle, Bhaketia et al. (2007) could not provide transcript levels after the dsRNA soaking

treatment and attributed this to the very low levels of *Hgsyv46* before root invasion making it difficult to detect any further reduction following the RNAi effect. Assessing the *Hgsyv46* transcript levels throughout the *H. glycines* lifecycle (up to 21 day post invasion) show a significant increase in mRNA abundance by 2 day post invasion however later stages in the timecourse *Hgsyv46* remained stably high (Bhaketia 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 *Hssyv46*-RNAi plants. The seemingly minimal reduction in *Hssyv46* expression determined from viable nematodes excised from RNAi plants could also likely be an artificat 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 represent an escape from a threshold dose of ingested dsRNA due to differential localized or temporal activity of the transgene promoter. Both CaMV 35S and *Ntcel7* promoters have been reported to descrease in activity within syncytia at the later 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 regulatory 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 or 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 *Hs4e02*-RNAi plants. 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.

For similar reasons as with *Hssyv46*-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 of 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 nematode parasitism genes observed here substantiates a diversity of the roles that each gene could play in parasitism of a plant host.

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Table 1. Primers used to isolate full length *H. shachtii* parasitism genes using the 5' Gene racer system.

Primer	**Sequence 5' to 3'**
5'4E02-Gene Racer-Reverse:	CCTTGCAACTTGCAATTTGCCGATGTT
Nested 5'4E02-Gene Racer-Reverse:	CCTCCCTCTTCCGACGAAGTGACAA
Hs-4E02-ORF-Forward:	ATGTCCCTTCTCCGTCCTCAATCG
Hs-4E02-ORF-Reverse:	TTAATGTTTGGGCTTCTTCCCGC
5'SYV46-Gene Racer-Reverse:	CGGCATTTCCTCCCTGAGCATTGCTTC
Nested 5'SYV46-Gene Racer-Reverse:	CGTACCAATCCCAGCTGATGAGTTGT
Hs-SYV46-ORF-Forward:	ATGTCAAACATTTTCAAAATC
Hs-SYV46-ORF-Reverse:	CTAATGATGACGTGGGTCGGGTC
5'5D08-Gene Racer-Reverse:	TTGGCCGCCGATTGTTTGCCATTCAATAA
Nested 5'5D08-Gene Racer-Reverse:	GCGTTTGGAGGGGCGGCGCCT
Hs-5D08-ORF-Forward:	ATGTTCAGCTCTTCCAATTTGTCTG
Hs-5D08-ORF-Reverse:	TCATCGTCCGCCGGTTGGAGC

Table 2. The forward and reverse PCR primers used for q-RTPCR to evaluate the differential expression of selected parasitism genes from *H. schachtii* infecting RNAi lines.

Primers	**Forward**	**Reverse**
HsActin	CGTGACCTCACTGACTACCT	CGTAGCACAACTTCTCCTTG
Hs4E02	ATTGGGCCGGCAAACAACTG	GCCGCGTAGCCTTCCGACTT
Hs5D08	TGATTGGGCAAAGAGTCAGTGGA	GTTGAAGCGCCGGTGTTTTC
HsSYV46	ACCGCTGGGCTGCTCTTCA	CGGCATTCCTCCCTGAGCA

Table 3. Comparison of selected *H. schachtii* parasitism gene homologs showing high nucleotide and amino acid identities with the *H. glycines* parasitism genes.

SCN Clones	NCBI Accession Number	Full-length cDNA *H. schachtii* homolog nucleotide identity (%)	Full-length *H. schachtii* homolog amino acid identity (%)	BLASTP of BCN homolog/Domain Search (NCBI)
4EO2	AF473826	99%	100%	Pioneer
SYV46	AF473827	93%	92%	CLAVATA, *Arabidopsis thaliana*
5DO8	AF473828	98%	96%	Pioneer

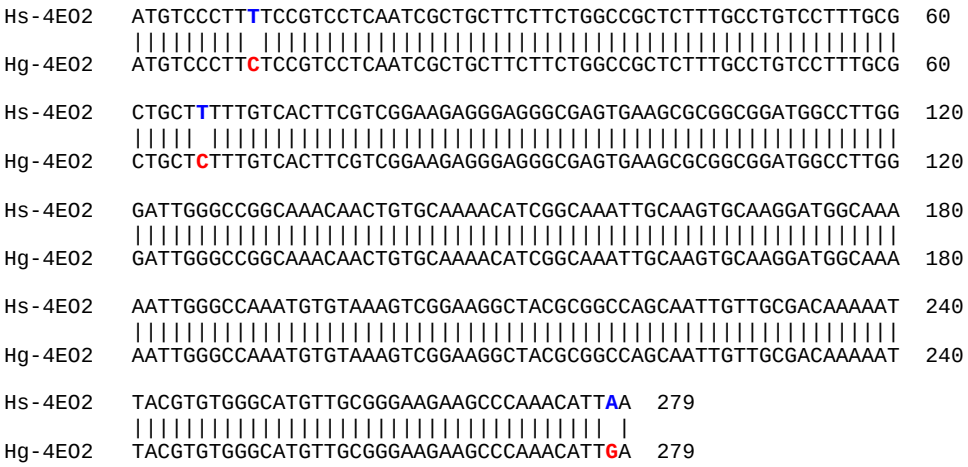

Figure 1. The nucleotide alignment of full length cDNA of parasitism gene *4e02* of *H. schachtii* (*Hs4e02*) and *H. glycines* (*Hg4e02*) (AF473826) showing 99% nucleotide sequence identity.

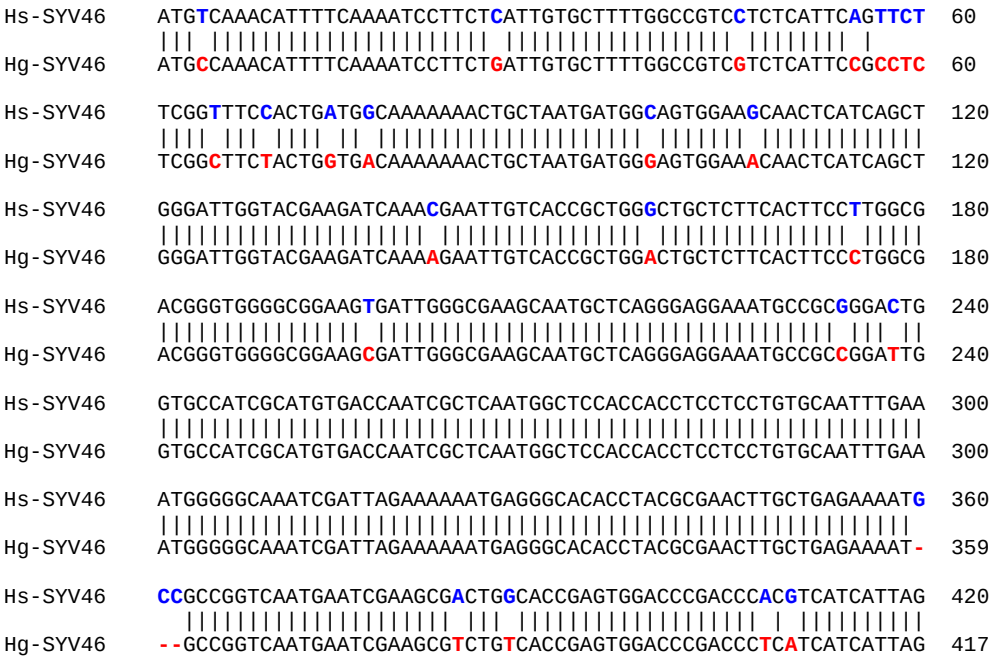

Figure 2. The nucleotide alignment of full length cDNA of the parasitism gene *syv46* of *H. schachtii* (*Hssyv46*) and *H. glycines* (*Hgsyv46*) (AF473827) showing 93% nucleotide sequence identity.

```
Hs-5D08   ATGTTCAGCTCTTCCAATTTGTCTGCTCTCTTTTTGGCCTCCTCCGTTTTTGCCGTGTTT  60
          |||||||||||||||||||||||||||||||||||||||||||||||||| |||||| ||
Hg-5D08   ATGTTCAGCTCTTCCAATTTGTCTGCTCTCTTTTTGGCCTCCTCCGTTTTGGCCGTGCTT  60

Hs-5D08   ATAATTGGCATTAAAATGGACGGACCGACGGAGGCAAAAGGCGCCGCCCCTCCAAACGCC  120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Hg-5D08   ATAATTGGCATTAAAATGGACGGACCGACGGAGGCAAAAGGCGCCGCCCCTCCAAACGCC  120

Hs-5D08   GCGGGGCCAATGGGACTTTTGCTTTTATTGAATGGCAAACAATCGGCGGCCAATGAAAAG  180
          |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Hg-5D08   GCGGGGCCAATGGGACTTTTGCTTTTATTGAATGGCAAACAATCGGCGGCTAATGAAAAG  180

Hs-5D08   GGAAAAGCGCCCTCTGGCGAAAGTAAGCCAAATCCGGGGCAGAAGCCGAACGGAGAACGG  240
          |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Hg-5D08   GGAAAAGCGCCCTCTGGCGAAAGTAAGCCAAATCCGGGGCAGAAGCCGAGCGGAGAACGG  240

Hs-5D08   CAAAAGAGGGACGTTTTGGGGCACGCCGGCGGATACGTCGGAGGATGGGACCATCCCATT  300
          | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Hg-5D08   CGAAAGAGGGACGTTTTGGGGCACGCCGGCGGATACGTCGGAGGATGGGACCATCCCATT  300

Hs-5D08   GACTCGACAGTTGATTGGGCAAAGAGTCAGTGGAATGATGCCAATTGGCTCGCCGATGTT  360
          |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Hg-5D08   GACTCGACACTTGATTGGGCAAAGAGTCAGTGGAATGATGCCAATTGGCTCGCCGATGTT  360

Hs-5D08   GTCAACAGAAACGGATGGGAAAACACCGGCGCTCCAACCGGCGGACGATGA  411
          |||||||||||||||||||||||||||||| ||||||||||||||||||||
Hg-5D08   GTCAACAGAAACGGATGGGAAAACACCGGCACTCCAACCGGCGGACGATGA  411
```

Figure 3. Nucleotide alignment of cDNA sequences of *5d08* parasitism gene between *H. schachtii* (*Hs5d08*) and *H. glycines* (*Hs5d08*) (AF473828) showing 98% nucleotide identity.

Figure 4. Hybridization of digoxigenin-labeled antisense cDNA probes (dark staining) of selected parasitism genes clones to transcripts specifically expressed within the subventral or dorsal esophageal gland cells of *Heterodera schachtii*. **A**: cDNA probe: 4E02 binding to the subventral gland (SvG) of preparasitic J2 *H. shachtii.* **B**: cDNA probe: 4EO2 binding to the subventral gland (SvG) cells of parasitic J3 *H. schachtii*. **C:** cDNA probe: SYV46 binding to the dorsal gland (DG) cells of parasitic J3 *H. schachtii*. **D**: cDNA probe: 5DO8 binding to the dorsal gland cells of parasitic J4 *H. schachtii*.

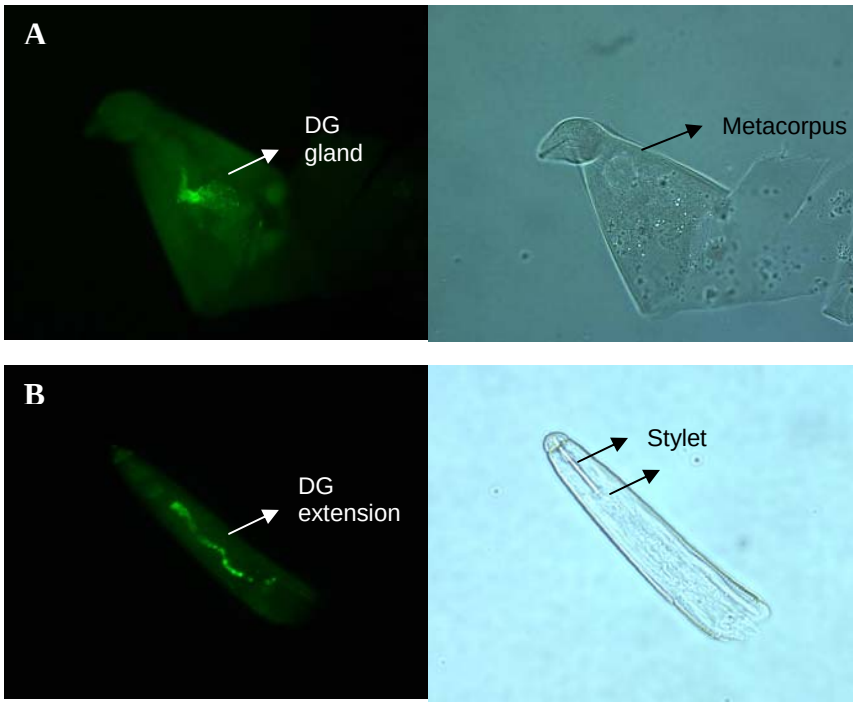

Figure 5. Binding of polyclonal antibodies specific to *H. glycines* parasitism gene products (peptides) SYV46 (**A**), and 5D08 (**B**) specifically within the dorsal esophageal gland cell (DG) and dorsal esophageal gland cell ampulla in parasitic stages of *H. schachtii* that were dissected from host plant roots.

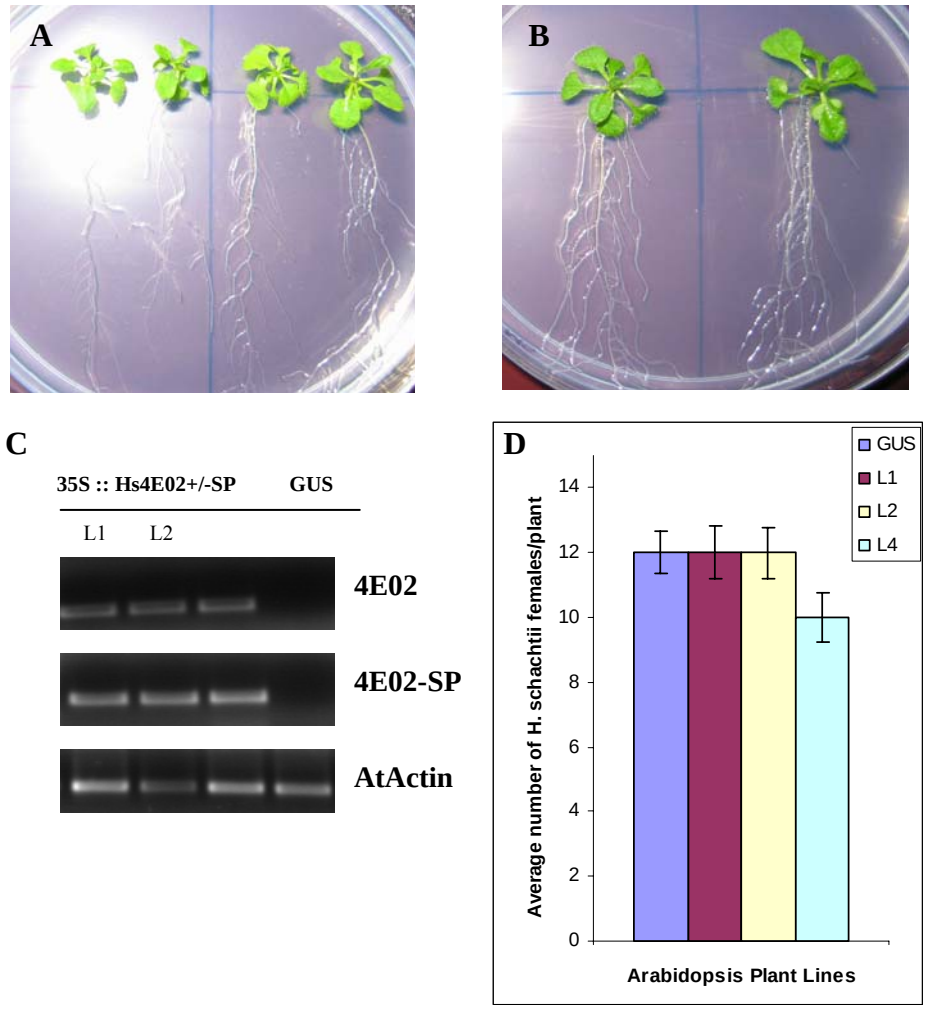

Figure 6. Expression of Hs4E02 in *Arabidopsis thaliana*. **A**: Root length phenotype analysis of Hs4E02+signal peptide (SP) expressing line (left) and GUS expressing control line (right) showing no difference in root and shoot phenotype 14 days post germination. **B**: Root length phenotype in Hs4E02-SP overexpressing line (left) and GUS expressing control line (right) showing no difference in root and shoot phenotype 14 days post germination. **C**: Reverse transcriptase PCR confirming the expression of *Hs4e02* and *Hs4e02*-SP in three independent Arabidopsis homozygous lines. **D**: Average number of *H. schachtii* females per plant 21 days post inoculation on Hs4E02 (without the signal peptide) overexpressing lines and control GUS Arabidopsis lines. Bars indicate ± standard error; n=27. (*t*- test, $P<0.05$ versus control).

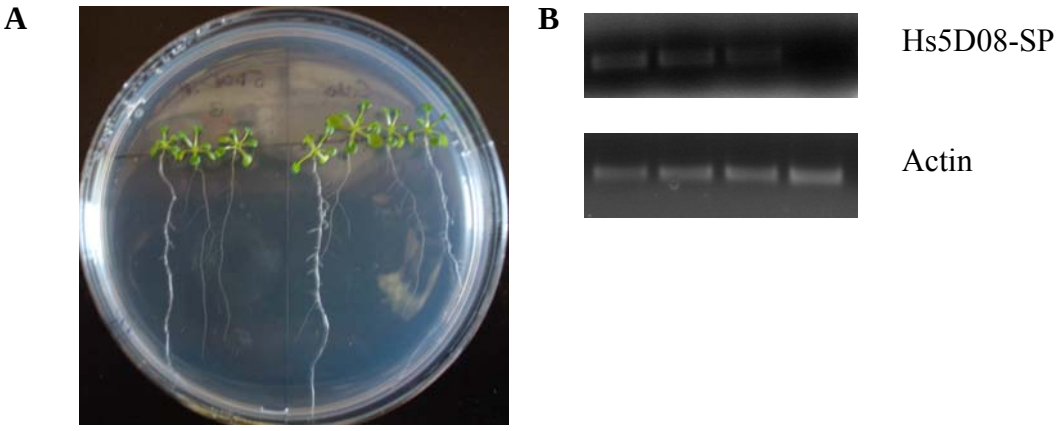

Figure 7. Expression of Hs5D08 in *Arabidopsis thaliana*. **A**: Root length phenotype analysis of Hs5d08-SP (without signal peptide) expressing line (left) and GUS expressing control line (right) showing no difference in root and shoot phenotype 8 days post germination. **B**: Reverse transcriptase PCR confirming the expression of *Hs5d08*-SP in three independent Arabidopsis homozygous lines.

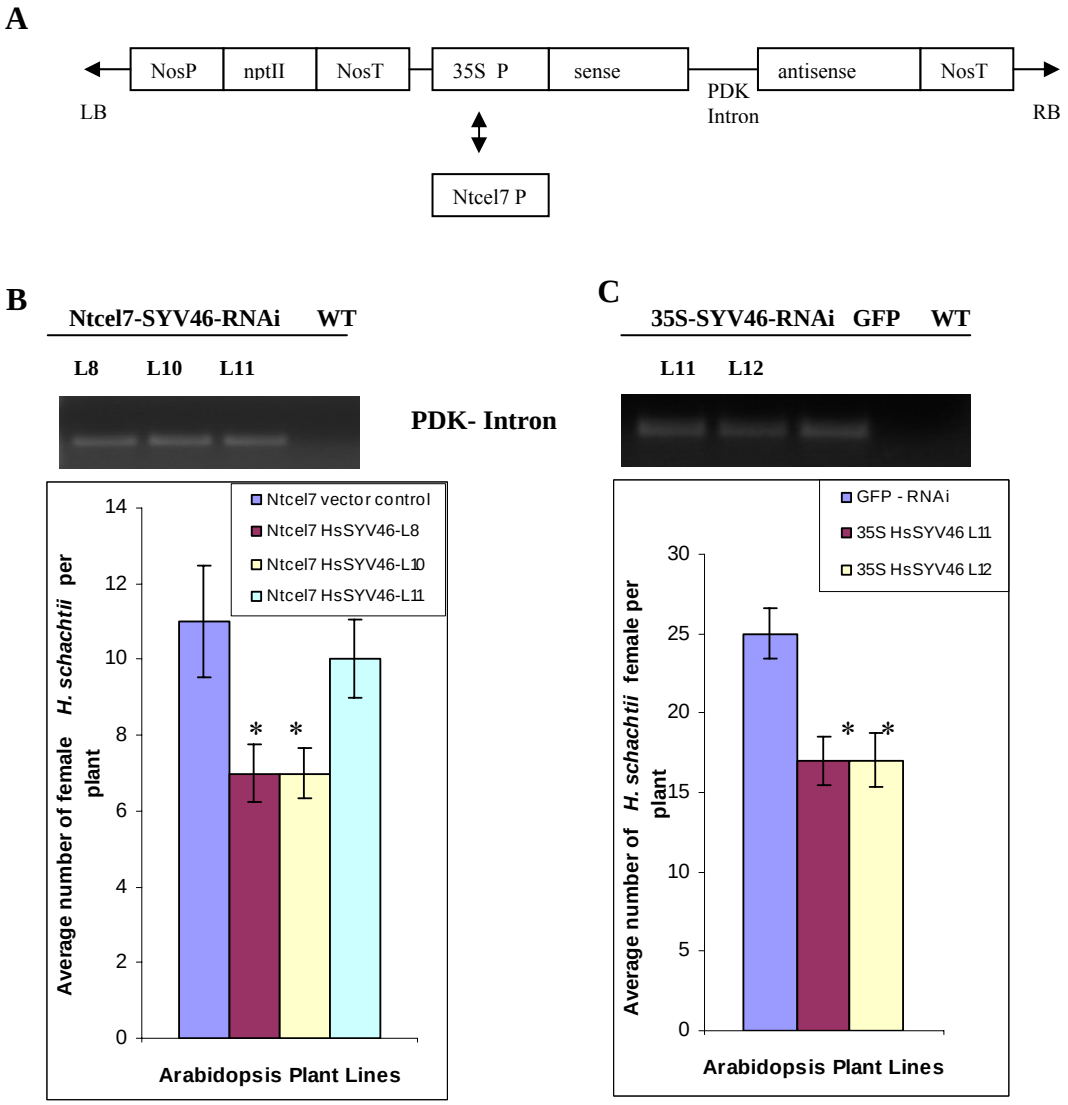

Figure 8. Significant (*t*-test, P≤0.05) reductions (*) in number of *Heterodera schachtii* females that developed on roots of transgenic Arabidopsis lines that expressed RNAi to the nematode parasitism gene *Hssyv46*. **A:** Illustration of the T-DNA region of the binary vector pART27 containing the sense and antisense gene of interest. 35S CaMV promoter (35S P) was originally in the vector and was replaced with the tobacco *Cel 7* promoter (*Ntcel7* P). **B:** Expression of the intron region of the dsRNA in *Ntcel7* RNAi lines that were inoculated with 60 *H. schachtii* J2s. Infection on L8 and L10 show significantly reduced (40%) females compared to the control empty vector infection. Although the intron is expressing in L11 there was no significant difference in the number of females. **C:** Expression of the intron region on the 35S CaMV*Hssyv46* Arabidopsis lines that were inoculated with 100 *H. schachtii* J2s. Infection on RNAi lines constitutively expressing *Hssyv6* dsRNA showed a significant reduction (32%) in established females when compared to the control. Bars indicate ± standard error; n=24.

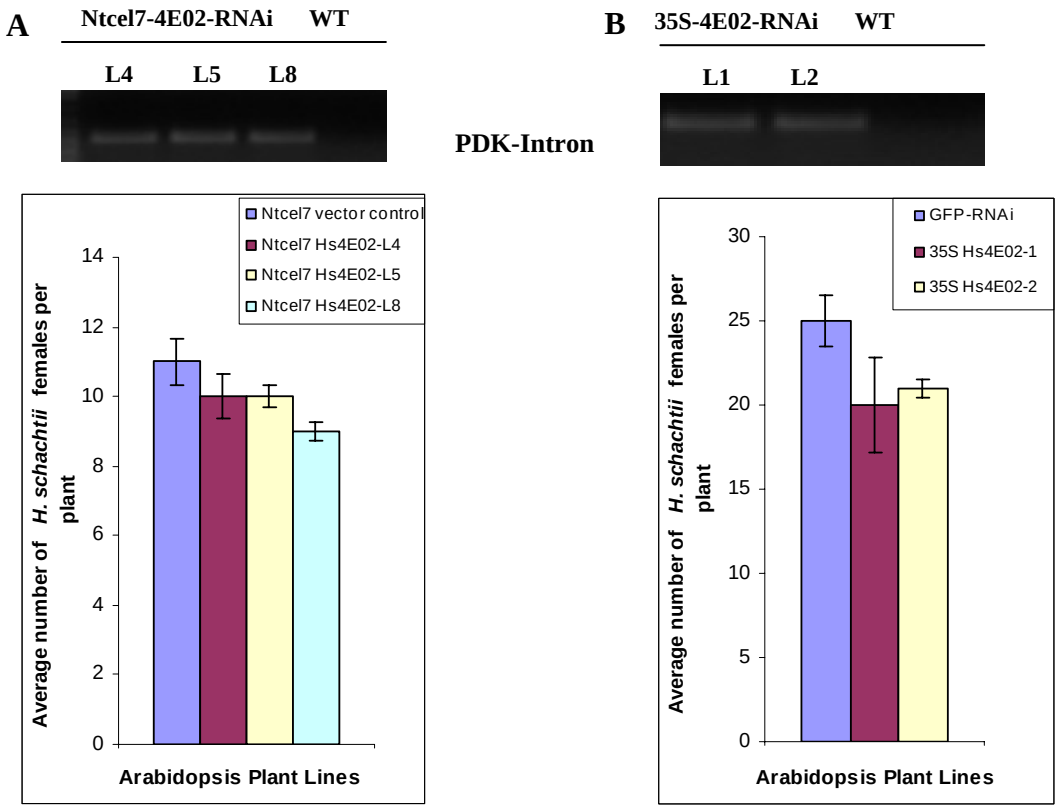

Figure 9: Reductions in number of *Heterodera schachtii* females that developed on roots of transgenic Arabidopsis lines that expressed RNAi to the nematode parasitism gene *Hs4e02* were not significant. **A:** Expression of the intron region of the dsRNA In *Ntcel7* RNAi lines that were inoculated with 60J2. *H. schahctii* infection data on RNAi lines expressing the *Hs4e02* dsRNA via nematode inducible *Ntcel7* promoter. Although the intron is expressing in L4, L5, L8 there was no significant difference in the number of females compared to the control. **B:** Expression of the intron region on the 35S *Hs4e02* Arabidopsis lines. 35S-RNAi lines were inoculated with 100 J2. *H. schachtii* infection on RNAi lines constitutively expressing *Hs4e02* dsRNA showing no significant reduction in established females when compared to the control. Bars indicate ± standard error; n=24. (*t*- test, P≤0.05 versus control).

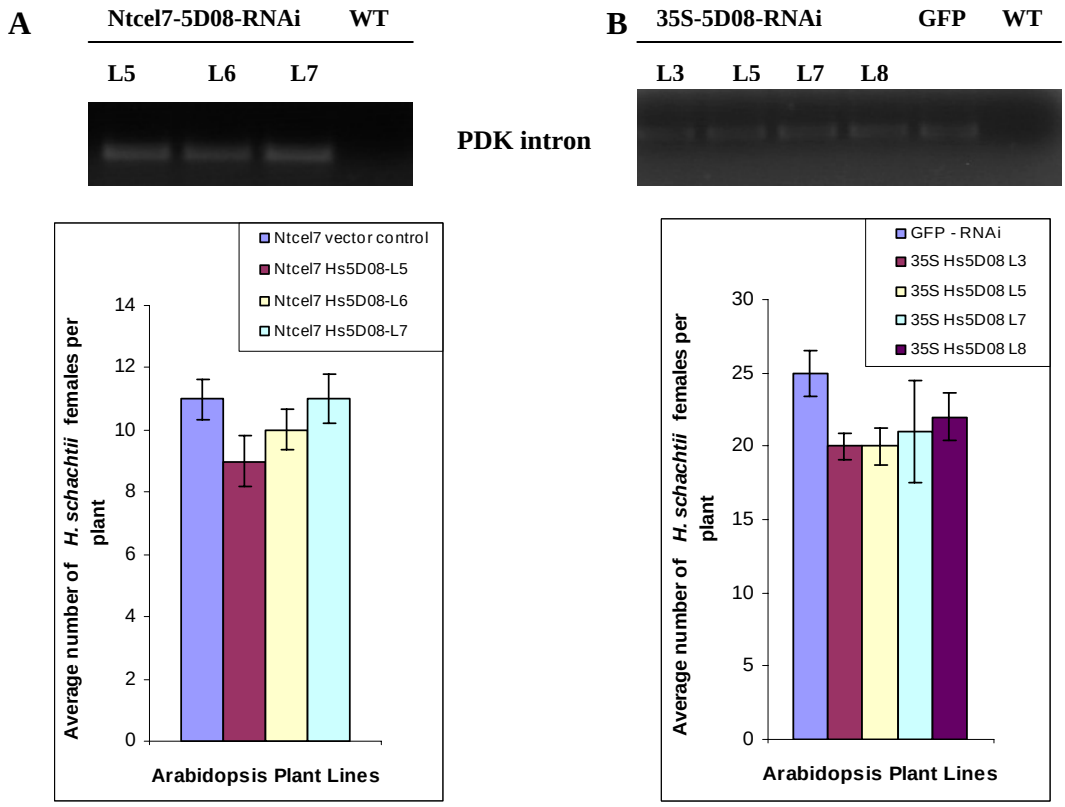

Figure 10: Host derived RNA-interference of *Hs5d08*. **A:** Expression of the intron region of the dsRNA product in *Ntcel7* RNAi lines that were inoculated with 60J2. *H. schachtii* infection data on RNAi lines expressing the *Hs5d08* dsRNA via nematode inducible *Ntcel7* promoter. Although the intron is expressing in L5, L6, L7 there was no significant difference in the number of females compared to the control. **B:** Expression of the intron region on the 35S CaMV-*Hs5d08* Arabidopsis lines. 35S-RNAi lines were inoculated with 100 J2 showing no significant reduction in established females when compared to the control. Bars indicate ± standard error; n=24. (*t*- test, P≤0.05 versus control).

Table 4. Relative endogenous expression levels of parasitism genes within *H. schachtii* specimens that were excised from transgenic Arabidopsis plant lines that expressed RNAi targeted to individual parasitism genes. A significant (*) ($P \leq 0.05$) fold difference in parasitism gene expression when compared to nematodes excised from control 35S-GFP-RNAi plants is noted by an asterisk.

Arabidopsis RNAi Plant Lines	**Parasitism gene tested**	**Relative fold Difference**
35S-4E02-L1	4E02	-2.18 ± 0.22*
35S-4E02-L1	SYV46 (non-target gene)	2.8 ± 0.76 *
35S-SYV36-L11	SYV46	-2.2 ± 0.04*
35S-SYV46-L11	4E02 (non-target gene)	-0.5 ± 0.4
35S-5D08-L7	5D08	-1.2 ± 0.4
35S-5D08-L7	SYV46 (non-target gene)	0.5 ± 0.3

Appendix

Southern Blot analyses

Southern blot analysis was conducted on the Arabidopsis transgenic lines to assess the copy number of the Hs-4f01 T-DNA insertion within the plant genome.

Leaf tissue from transgenic lines was frozen in liquid nitrogen DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacture's instructions. The eluted DNA was precipitated with ethanol. DNA was resuspended in 10mM Tris-HCl [pH 8] and treated with RNase *If* according to manufacture's instructions (New England Biolabs, Ipswich, MA). Three micrograms of plant genomic DNA was completely digested overnight at 37°C with Bam*HI* and Hind*III* (New England Biolabs, Ipswich, MA), separated by 0.7% agarose gel electrophoresis, transferred onto a Hybond-N membrane through capillarity (Sambrook et al., 1989). A digoxygenin-labeled (DIG) *4f01* probe was generated using the PCR DIG Probe Synthesis kit (Roche Applied Science, Indianapolis, IN) with *Hg4f01* cDNA (GeneBank Accession AF469059) template and primer pair B4F01p 5' AAGCAGGCGTATGAGCAGTT3' and 5'GTCGTGTGCCAATACAATGC3'. Hybridization of the probe was performed at 42°C for 16 hours in DIG Easy Hyb solution (Roche Applied Science, Indianapolis, IN). After the stringency washes the blot was detected using the DIG Wash and Block Buffer Set (Roche Applied Science, Indianapolis, IN). The membrane was exposed to x-ray film for 10min and hybridized bands were observed.

Southern blot analyses were conducted on wild-type Arabidopsis lines expressing *Hs4f01* (Fig. 1). According to the blot, Arabidopsis lines 1 and line 2 contained at least 2 or 3 copies of the *Hs4f01* insert (Fig.1). Arabidopsis *annAt1* mutants expressing the *Hs4f01* used for the complementation studies in Chapter 2 had multiple copies of the insert (Fig. 2).

Arabidopsis lines 1 and 10 had multiple copies (~5) and lines 11 and 15 had a single copy of the T-DNA insert (Fig. 2).

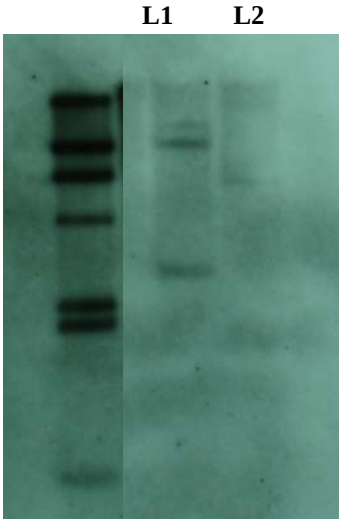

Figure 1. Southern blot analyses of homozygous Arabidopsis plant lines expressing Hs4F01 (35S::4F01). Genomic DNA was digested with *Bam*H I (B) and *Hin*d III (H) with a 162bp 4F01-DIG labeled probe. L1 shows 2 copies of the 4F01 gene and L2 has at least one copy of the 4F01 gene. M: 50 ng DIG-labeled molecular weight marker II in Kb.

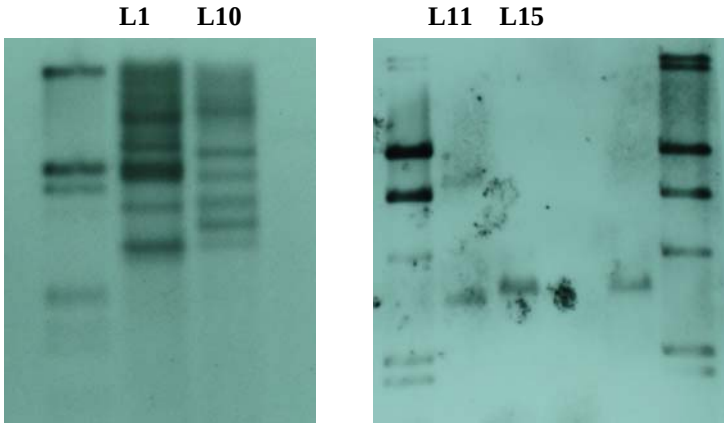

Figure 2. Southern blot analyses of Atann1 mutant plant lines expressing Hs4F01. Genomic DNA was digested with *Bam*H I (B) and *Hin*d III (H) with a 162bp 4F01-DIG labeled probe. L1 and L10 have multiple copies of the 4F01 gene while L11 and L15 have at least one copy. M: 50 ng DIG-labeled molecular weight marker II in Kb.