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Nrupali Patel
*North Carolina State University*

Noureddine Hamamouch
*North Carolina State University*

Chunying Li
*North Carolina State University*

Tarek Hewezi
*Iowa State University*

Richard S. Hussey
*University of Georgia*

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RESEARCH PAPER

A nematode effector protein similar to annexins in host plants

Nrupali Patel1,*, Noureddine Hamamouch1, Chunying Li1, Tarek Hewezi2, Richard S. Hussey3, Thomas J. Baum2, Melissa G. Mitchum4 and Eric L. Davis1,†

1 Department of Plant Pathology, North Carolina State University, Raleigh, NC 27607, USA
2 Department of Plant Pathology, Iowa State University, Ames, IA 50011, USA
3 Department of Plant Pathology, University of Georgia, Athens, GA 30602, USA
4 Division of Plant Sciences and Bond Life Sciences Center, University of Missouri, Columbia 65211, USA

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Abstract

Nematode parasitism genes encode secreted effector proteins that play a role in host infection. A homologue of the expressed Hg4F01 gene of the root-parasitic soybean cyst nematode, Heterodera glycines, encoding an annexin-like effector, was isolated in the related Heterodera schachtii to facilitate use of Arabidopsis thaliana as a model host. Hs4F01 and its protein product were exclusively expressed within the dorsal oesophageal gland secretory cell in the parasitic stages of H. schachtii. Hs4F01 had a 41% predicted amino acid sequence identity to the nex-1 annexin of C. elegans and 33% identity to annexin-1 (annAt1) of Arabidopsis, it contained four conserved domains typical of the annexin family of calcium and phospholipid binding proteins, and it had a predicted signal peptide for secretion that was present in nematode annexins of only Heterodera spp. Constitutive expression of Hs4F01 in wild-type Arabidopsis promoted hyper-susceptibility to H. schachtii infection. Complementation of an AnnAt1 mutant by constitutive expression of Hs4F01 reverted mutant sensitivity to 75mM NaCl, suggesting a similar function of the Hs4F01 annexin-like effector in the stress response by plant cells. Yeast two-hybrid assays confirmed a specific interaction between Hs4F01 and an Arabidopsis oxidoreductase member of the 2OG-Fe(II) oxygenase family, a type of plant enzyme demonstrated to promote susceptibility to oomycete pathogens. RNA interference assays that expressed double-stranded RNA complementary to Hs4F01 in transgenic Arabidopsis specifically decreased parasitic nematode Hs4F01 transcript levels and significantly reduced nematode infection levels. The combined data suggest that nematode secretion of an Hs4F01 annexin-like effector into host root cells may mimic plant annexin function during the parasitic interaction.

Key words: Arabidopsis thaliana, cyst nematode, Heterodera, parasitism gene, RNAi, secretions.

Introduction

Plant-parasitic cyst nematodes in the genus Heterodera comprise a major agronomically important group of plant pathogens (Mitchum et al., 2007; Davis et al., 2008). The infective second-stage juveniles (J2) of these microscopic worms hatch from eggs in soil, penetrate host plant roots near the tip, and migrate intracellularly through the root cortex to the vascular cylinder (Niblack et al., 2006). Each nematode will subsequently induce elaborate changes in selected root vascular parenchyma cells to form a specialized feeding site (syncytium) that will provide the permanent source of nutrition for the nematode as it swells, becomes sedentary, and moults three more times to reproductive maturity. The syncytium has characteristic features such as the proliferation of organelles, increased cytoplasmic density, and enlargement and an increased number of nuclei (Endo, 1964). It is formed by the coalescence of root
vascular cells adjacent to an initial syncytial 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 to increase plasmalemma surface area and to enhance solute uptake by the parasite (Hussey and Grundler, 1998; Lilley et al., 2005). The parasitic process from host root penetration through formation and maintenance of the syncytium is regulated, directly or indirectly, by a suite of effector proteins secreted from the nematode stylet (a hollow, protrusible oral spear) that are developmentally-regulated to alter host cell physiology to promote parasitism (Baum et al., 2007; Mitchum et al., 2007; Davis et al., 2008, 2009). The secreted effector proteins originate in secretory granules in three elaborate secretory gland cells within the nematode oesophagus and are released through complex valves via exocytosis for secretion through the nematode stylet into the host tissues (Wyss and Zunke, 1986; Hussey, 1989; Davis et al., 2008).

Over 60 developmentally-expressed parasitism genes encoding predicted secreted effector proteins have been identified from the oesophageal gland cells of the soybean cyst nematode, *Heterodera glycines* (Gao et al., 2001, 2003; Wang et al., 2001) and only about 30% of the *H. glycines* parasitism genes have database orthologues (Mitchum et al., 2007). One of the parasitism genes isolated, *Hg4F01* (Gao et al., 2003), is expressed exclusively within the single cyst nematode dorsal oesophageal gland secretory cell during syncytium formation 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 sites are located (Clark et al., 2001).

Previous localizations of annexins within nematodes including *C. elegans* and the potato cyst nematode, *Globodera pallida* were within the reproductive organs (Duigle and Creutz, 1999; Fioretti et al., 2001). In plants, annexins play diverse roles and several potential functions exist in a number of plant species. For example, annexins in peas and corn concentrate in the secretory cell types of root caps in developing xylem and phloem (Clark et al., 1992, 1994). In cotton, annexins appear to decrease callose synthase enzyme activity (Andrawis et al., 1993; Delmer and Potikha, 1997). In *Arabidopsis thaliana* there are at least eight annexin genes, including annexins associated with abiotic stress responses (Clark et al., 2001; Cantero et al., 2006). There is strong evidence that the Annexin 1 gene (*annAt1*) in *Arabidopsis* can protect cells against drought stress (Konopka-Postupolska et al., 2009), oxidative stress (Gorecka et al., 2005), and also play a role in the osmotic stress response (Lee et al., 2004).

Confirmation of an annexin-like function of the *Hg4F01* effector and the potential significance of a secreted annexin-like protein in the cyst nematode–plant parasitic interactions remain unclear. The available functional information and genetic resources for annexins in *Arabidopsis* (Clark et al., 2001; Lee et al., 2004; Cantero et al., 2006) provide a model to evaluate the potential function of the *Hg4F01* effector 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* homologue in *H. schachtii* would present a tenable model pathosystem.

A lack of non-lethal mutants of the obligate-parasitic cyst nematodes and current inability to transform them with desired gene constructs also confound analyses of phytoparasitic nematode gene function. The potential to induce RNA-mediated interference (RNAi) of target nematode genes (Fire et al., 1998) through the introduction of complementary double-stranded RNA (dsRNA), however, provides prospective functional analyses through specific silencing of targeted nematode genes (Davis et al., 2009; Gheyse and Vanholme, 2007; Mitchum et al., 2007). Target-specific RNAi of several different plant-parasitic nematode genes has been achieved through in vitro soaking in solutions of complementary dsRNA (Lilley et al., 2007) and, alternatively, via nematode ingestion from transgenic plant host-derived RNAi of targeted nematode parasitism genes (Huang et al., 2006a; Patel et al., 2008; Sindhu et al., 2009). Functional analyses of the annexin-like effector 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* cv. Lee 74). Eggs were collected from crushed cysts 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 plants (*Lycopersicon esculentum* cv. Rutgers), and eggs were extracted from roots with 0.05% sodium hypochlorite as previously described (Hussey and Barker, 1973). Nematode eggs were hatched over water at 28°C on a Baermann pan to collect 24 h-cohorts of preparasitic 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).

**DNA gel blot analysis**

*H. schachtii* and *H. glycines* pre-J2s were mixed with lysis solution [100 mM NaCl, 100 mM TRIS-HCl (pH 8.5), 50 mM EDTA (pH 7.4), 1% SDS, 1% β-mercaptoethanol, and 100 μg ml⁻¹ proteinase K] and incubated at 65°C for 45 min. The DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA was resuspended in 10 mM TRIS-HCL (pH 8) and treated with RNase according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA).
Five micrograms of \textit{H. schachtii} and \textit{H. glycines} genomic DNA was digested overnight at 37 °C with \textit{Bam}HI and \textit{Hind}III (New England Biolabs, Ipswich, MA), separated by 0.7% agarose gel electrophoresis and transferred onto a Hybond-N membrane by capillarity (Sambrook et al., 1989). A digoxigenin-labelled (DIG) 4F01 probe was generated using the PCR DIG Probe Synthesis kit (Roche Applied Science, Indianapolis, IN) with a Hg401 cDNA (AF469059) template and the primer pair B4F01-p 5'-AAG-CAGGCCGTATGACGTGTT-3' and 5'-GTCGGTGCCATA-CAATGC-3'. Hybridization of the probe was performed at 42 °C for 16 h in DIG Easy Hyb solution (Roche Applied Science, Indianapolis, IN). After the stringency washes the blot was detected using the DIG Wash, Block Buffer Set, and DIG chemiluminescence detection reagent (Roche Applied Science, Indianapolis, IN). The membrane was exposed to X-ray film for 10 min and hybridized bands were observed.

**Isolation of the Hs4F01 parasitem cDNA clone**

Mixed parasitic stages of \textit{H. schachtii}, were ruptured with Lysis Matrix D beads (Q-Biogene, Irvine, CA) and liquid nitrogen in a mini bead beater (Biospec Products Inc. Bartlesville, OK). Nematode total RNA was extracted using the Micro-Midi Total RNA purification system (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions including digestion with DNaseI.

To obtain the cDNA homologue of Hs4F01 in \textit{H. schachtii}, 3' and 5' cDNA ends were amplified from total RNA using the GeneRacer kit (Invitrogen, Carlsbad, CA) and PCR primers derived from the Hg4F01 cDNA sequence. 5' - RACE was performed using the GeneRacer 5' primer and GST Hg4F01-1 5'-GCGAGTGTCACACCTGTTGGA-3' with the RACE-ready first-strand \textit{H. schachtii} cDNA template. 3' - RACE was performed using the GeneRacer 3' primer (oligo dT) and GST Hg4F01-2 5'-TTGCTCAGTGCTCTTGCGAAGA-3'. The RACE product was cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) for sequencing. Based on the sequencing results of the 3' and 5' RACE products, forward primer 5'-ATGCTCAGACAGCCTACATT-3' and reverse primer 5'-TCACTGTCCTGTTGCCCC-3' were used to amplify the full-length 4F01 cDNA clone from template \textit{H. schachtii} RNA. The Hs4F01 cDNA was subsequently cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and the cDNA inserts of ten clones were sequenced.

**Sequence analyses**

Comparison of the nucleotide and predicted amino acid sequences of the \textit{H. schachtii} 4F01 cDNA homologue to the \textit{H. glycines} 4F01 parasitism gene, including the amino acid sequences of \textit{Arabidopsis thaliana} annexin 1 gene (At1g35720), \textit{C. elegans} annexin 1 gene (NP174810), and \textit{G. pallida} annexin 1 gene (CAC33829), \textit{C. elegans} annexin 1 (NP498109), and \textit{A. thaliana} annexin 1 (NP174810) (Marchler-Bauer and Bryant, 2004).

**mRNA in situ hybridization**

Localization and developmental expression analyses of \textit{Hs4F01} were performed in fixed mixed parasitic stages of \textit{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-labelled sense (control) and antisense cDNA probes by asymmetric PCR (Gao et al., 2001). The cDNA probes that hybridized within the nematode specimens were detected by the DIG labelling system and observed by light microscopy using the method of De Boer et al. (1998).

**Immunolocalization**

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

**Expression and complementation of the annAt1 T-DNA insertion mutant with \textit{Hs4F01}**

The \(\beta\)-glucuronidase gene of the binary vector pBI21 (Clontech, Palo Alto, CA) was replaced at the \textit{Bam}HI and \textit{Sac}I sites with the coding region of \textit{Hs4F01} with and without the predicted signal peptide sequence. The 35S::Hs4F01 constructs were each introduced into wild-type \textit{Arabidopsis} (ecotype Columbia-0) via Agrobacterium-mediated floral dip transformation (Clough and Bent, 1998). The 35S::Hs4F01 construct with and without the signal peptide was transformed into an \textit{annAt1 Arabidopsis} T-DNA inserional mutant (SALK_015426) background. Expression of the \textit{annAt1} gene in both the mutant and wild-type plants was monitored by RT-PCR using primers specifically to amplify a 150 bp product of the 3'-UTR of the \textit{annAt1} transcript (Lee et al., 2004), 5'-GCTTAATACTCAACTCCTCC-3' and 5'-CTCAAAACACACACAGAGA-3'. An internal control for plant gene expression used primers 5'-GGCGATGAAGCCT-CAATCCAAAG-3' and 5'-GGTCGACGGACGAGAT-CAGAGC-3' specifically to amplify (Lee et al., 2004) a 400 bp product of an \textit{Arabidopsis actin} gene (AT2G37620) by RT-PCR. Transformants were selected on Murashige-Skoog (MS) media supplemented with 50 μg ml\(^{-1}\) kanamycin. Transgenic lines were observed for altered phenotype compared to the wild type and annexin-mutant lines as appropriate. Total RNA from plant material was extracted using the RNaseasy Plant Mini Kit following the manufacturer’s instructions (Qiagen, Valencia, CA). Expression of the \textit{Hs4F01} transgene (with or without a signal peptide sequence) was confirmed using RT-PCR on the total RNA of leaf tissues using first-strand cDNA synthesis (Invitrogen, Carlsbad, CA) and PCR amplification using the \textit{Hs4F01} primers 5'-AGTTT-GGACAGAGGCATCGACACGAC-3' and 5'-AGAAGAGGTTCGG-GACATATTGGA-3'. Homozygous lines expressing \textit{Hs4F01} were grown in MS media plates placed vertically for root growth assays. One hundred seeds of each homozygous \textit{annAt1-11/Hs4F01 Arabidopsis} line were germinated on Murashige-Skoog (MS) media supplemented with 75 mM sodium chloride (NaCl) to test complementation efficiency (Lee et al., 2004) compared to mutant and wild-type seeds treated under identical storage, stratification, and assay conditions. Seed germination rates were scored at 7 d post-incubation.

**Western blot**

The transgenic \textit{Arabidopsis} lines confirmed to express \textit{Hs4F01} by RT-PCR were grown in liquid nitrogen. Total protein was extracted by incubating the ground tissue in extraction buffer (50 mM TRIS, pH 8.0, 2 mM EDTA, 2 mM DTT, 0.25 M sucrose, and protease inhibitor cocktail) and centrifuging at 8000 g for 15 min at 4 °C. The supernatant was collected and protein concentration was estimated by the Bradford assay using BSA as
a standard (Bradford, 1976). Proteins (10 μg lane−1) were separated on 4–20% polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, Billerica, MS). The protein blot was incubated for 2.5 h at room temperature in anti-Hs4F01 primary sera (1:3000) and potential transgene products were detected by enhanced chemiluminescence after 1.5 h incubation in (1:2000) anti rabbit-alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich, St Louis, MO).

Yeast two-hybrid assay
A yeast two-hybrid screening was carried out as described in the BD Matchmaker™ Library Construction and Screening Kits User Manual (Clontech, Mountain View, CA). The coding sequence of 4F01 without its putative signal peptide was fused to the GAL4 DNA-binding domain (BD) of pGBKKT7 vector and then introduced into Saccharomyces cerevisiae strain Y187 to generate the bait strain. cDNA libraries from A. thaliana roots of ecotype C24 at 7 d after H. schachtii infection were generated in S. cerevisiae strain AH109, as fusion to the GAL4 activation domain (AD) of pGADT7 vector (Hewezi et al., 2008). Screening for interacting proteins and subsequent analyses were performed as described in Clontech protocols. The putative positive interactions were tested against an unrelated protein (laminin C) fused to GAL4 DNA BD and empty plasmid pGBKTK7 to discard false positives.

Host-derived RNAi vector construction and analyses of transgenic lines
The 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) to express hairpin dsRNA of Hs4F01 in transformed Arabidopsis plants. A negative 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 up-regulation within nematode feeding cells (Goelner et al., 2001; Wang et al., 2007). All subsequent pHANNIBAL vectors were subcloned as NosI fragments into the binary vector pART27 (Gleave, 1992). The constructs were transformed into wild-type Arabidopsis (ecotype Columbia-0) via Agrobacterium-mediated floral dip transformation (Clough and Bent, 1998). 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. Total RNA extracted using the RNAeasy Plant Mini Kit (Qiagen, Valencia, CA) from whole 10-d-old Ntcel7-driven seedlings and leaf samples of 3-week-old CaMV 35S-driven plants was used to analyse transgene expression of the PDK intron of hairpin dsRNA (Wesley et al., 2001) using RT-PCR with the First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA). Gene-specific primer PDK-RT-R: 5′-ATCAATGATAA CACAATGACATGATC-3′ was used to make first-strand cDNA which was used as a template for amplification of a 300 bp amplicon using primers pIntronF: 5′-GAGCAAGAA GATAAAAGTGAGAG-3′ and pIntronR: 5′-TTGATAAAT TACAAAGCAGATTGGA-3′.

Nematode infection assays
Twenty-four seedlings (5 d post-germination) from each Arabidopsis line that were selected on MS media supplemented with 50 μg ml−1 kanamycin were asexually transferred one seedling per well in six-well culture plates (Falcon, Lincoln Park, NJ) containing 6 ml of sterile modified Knops medium (Sijmons et al., 1991) solidified with 0.8% Daishin agar (Bruschwieg Chemie BV, Amsterdam, Netherlands). The plates were placed in a 24 °C growth chamber with 16/8 h light/dark cycle for 7 d before nematode inoculation. Hatched H. schachtii preparasitic J2 or M. incognita preparasitic J2 were collected as described above and surface-sterilized by incubating for 10 min in 0.004% mercuric chloride, 0.004% sodium azide, and 0.002% Triton X-100 and washed three times with sterile distilled water. The surface-sterilized nematodes were suspended at a concentration of ten J2/10 μl in 35 °C 1.5% low melting point agarose to allow an 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 J2s 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 using an inverted microscope over the same time-course for comparative results. The mean and standard error of 24 replicates of each treatment (line) were calculated in each experiment, and each experiment was repeated three times. 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
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 (Bruschwieg Chemie BV, Amsterdam, Netherlands) and the plates were placed in a growth chamber at 24 °C with a 16/8 h light/dark cycle. Each plate was placed vertically for vertical root growth for 10 d before being inoculated with sterilized 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, Carlsbad, CA) and DNase treated with the Turbo DNA-free kit (Ambion, Austin, TX) according to the manufacturer’s instructions. First-strand cDNA synthesis was made from 10 ng of mRNA using SuperScriptII RT (Invitrogen, Carlsbad, CA). qRT-PCR was performed in a DNA Engine Opticon2 (Bio-Rad, Hercules, CA) using primers 5′-AGATTGA GAAAGGGCATTGAG-3′ and 5′-CGTTGGC GACATTTTT- GAT-3′ to quantify Hs4F01 transcript and primers 5′- ACCGCTGGGCTGTCCTAC-3′ and 5′-CGGGATTTCT CCCTTGAGAC-3′ to quantify expression of a non-RNAi target parasite gene HgSYV46 (Wang et al., 2001). The primers used to quantify the H. schachtii actin gene for normalization were 5′- CGTGAACCTCAGCTACACT-3′ and 5′-CGTACGACAAC TCTTCCTTG-3′. A single 20 μl PCR reaction included 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5μl cDNA template, and 5 μM each of the forward and reverse primers. The PCR cycling parameters were set at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After the completion of the cycling parameters, dissociation melt curve analyses (60–90 °C every 0.5 °C for 1 s) 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 RNA extracted from the nematodes and water to check for DNA contamination in the samples. 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−ΔΔCt 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 of an expressed H. schachtii 4F01 annexin-like parasitism gene

A 1023 bp full-length 4F01 cDNA clone was generated from RNA of H. schachtii using PCR primers designed from the Hg4F01 cDNA (GenBank Accession AF469059) and 5’ and 3’ amplification of cDNA ends. The Hs4F01 cDNA (GenBank Accession FJ768021) had 97% nucleotide identity and 3% gaps when aligned with the 1026 bp Hg4F01 cDNA. A DIG-labelled Hg4F01 probe hybridized to at least two DNA fragments of both H. schachtii and H. glycines on DNA gel blots (Fig. 1) which may represent two members of an annexin gene family or restriction sites for both enzymes within the same species. Although two copies of 4F01 may exist within the H. schachtii genome, only the 1023 bp Hs4F01 cDNA sequence was generated from all expression analyses of H. schachtii life stages and, thus, this sequence was used for all subsequent studies.

The open reading frame of Hs4F01 encoded a predicted protein of 340 amino acids with a calculated protein mass of 36 kDa and 92% predicted amino acid identity to Hg4F01 (Fig. 2). Signal P 3.0 (Bendtsen et al., 2004) predicted a hydrophobic 5’ signal peptide in the Hs4F01 set for cleavage between position 21 (alanine) and 22 (asparagine) indicating that the protein can be secreted by the cell, a characteristic of H. glycines parasitism gene products (Davis et al., 2004, 2008). BLASTp of Hs4F01 determined a 41% sequence identity to nex-1 (NP_498109) of C. elegans, a 45% identity to annexin-2 (CAC33829) of G. pallida, and 33% identity to AnnAt1 (NP_174810) of Arabidopsis thaliana. Four conserved domains typical of the annexin family of calcium and phospholipids binding proteins (Clark et al., 2001) were present in an alignment of the Hs4F01 sequence with the above annexins.

Expression of 4F01 within H. schachtii

The tissue localization 4F01 within the different parasitic stages of H. schachtii was analysed by in situ mRNA hybridization. The digoxigenin-(DIG) labelled antisense Hs4F01 cDNA probe specifically hybridized to transcripts expressed exclusively within the dorsal oesophageal gland cell of parasitic stage J2, J3 (Fig. 3), and J4 females of H. schachtii, but not within any tissues of hatched, preparasitic J2s or males. No hybridization signals were detected with the control sense Hs4F01 cDNA probe in any developmental stage of H. schachtii. Antisera raised to synthetic peptides were designed from predicted Hg4F01 solvent-exposed domains bound exclusively to Hs4F01 within secretory granules of the dorsal oesophageal gland cell lobe, cell extension, and ampulla (Fig. 3) within H. schachtii specimens that were excised from host plant roots. The movement of Hs4F01 to the secretory gland cell ampulla is consistent with the observed activity of gland cells immediately prior to secretion from the stylet (Hussey, 1989; Wang et al., 2005; Wyss and Zunke, 1986). No binding with rabbit pre-immune serum was detected in the nematode specimens (data not shown).

Expression of Hs4F01 in wild-type Arabidopsis and interaction of Hs4F01 with Arabidopsis proteins

Hs4F01 was constitutively expressed in transformed Arabidopsis lines with and without the predicted nematode signal peptide sequence to monitor potential effects of the nematode effector proteins on plant phenotype. 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, if functional in host plant cells, should target the protein to the secretory pathway for export from the plant cell. The expression of Hs4F01 (Fig. 4A) and the presence of Hs4F01 protein within four independent transformed Arabidopsis lines were confirmed by RT-PCR and Western blot analysis, respectively, with a protein size of the theoretical molecular weight value of 36 kDa (Fig. 4B). No visible phenotypic differences were observed in shoots and roots of plants expressing Hs4F01 compared with the control lines (data not shown).

Infection assays with H. schachtii were conducted on homozygous Arabidopsis lines confirmed to express Hs4F01 without a signal peptide sequence to the mimic cytoplasmic accumulation of the effector protein. Agar-based infection assays were used to grow the Arabidopsis lines and were inoculated with approximately 60 H. schachtii infective juveniles. Interestingly, a significantly (P < 0.05) greater number of H. schachtii females developed on plant lines that expressed Hs4F01 compared to the control line (Fig. 4C). Further, the increased susceptibility appeared to be unique to H. schachtii infection because the
Hs4F01-expressing Arabidopsis lines infected with another sedentary phytoparasitic nematode, Meloidogyne incognita (root knot nematode), showed no significant difference in infection rates (Fig. 4D).

The increased susceptibility of Hs4F01-expressing lines may be due to Hs4F01 interaction with plant proteins to promote successful nematode parasitism. A yeast two-hybrid screen of a cDNA library derived from roots of cyst nematode-infected Arabidopsis (Hewezi et al., 2008) as prey and Hs4F01 as bait produced a positive interaction with a specific Arabidopsis oxidoreductase (AT5G05600) of the 2OG-Fe(II) oxygenase family (Fig. 5) that was confirmed by yeast co-transformation analysis. This type of enzyme has been associated with plant defence and stress response and...
Fig. 3. Tissue localization of the expressed Hs4F01 annexin-like effector protein and transcripts within specimens of the beet cyst nematode, *Heterodera schachtii*. (A) Specific binding of anti-Hg4F01 polyclonal antibodies to Hs4F01 within the *H. schachtii* J3 dorsal gland secretory cell ampulla, a collecting reservoir for secretion of contents into the plant via the nematode stylet (Wyss and Zunke, 1986). (B) *In situ* hybridization of a digoxigenin-labelled antisense Hs4F01 cDNA probe to transcripts specifically expressed within the dorsal oesophageal (DG) gland secretory cell of a third-stage juvenile (J3) of *Heterodera schachtii* that was excised from host plant roots. M, nematode metacarpus; DG, dorsal gland; bar = 10 μm in both panels.

is required for the susceptibility to the downy mildew pathogen in *Arabidopsis* (van Damme et al., 2008).

**Complementation of an Arabidopsis annexin mutant with Hs4F01**

Similar to the expression of Hs4F01 in *Arabidopsis*, the *Arabidopsis* annexin 1 mutant (*annAt1*) showed no observable morphological phenotype (Lee et al., 2004). Furthermore, *H. schachtii* infection or development in roots of *annAt1* plants was not affected (data not shown). Germination of the *annAt1* mutant is reduced under high salt conditions (Lee et al., 2004), therefore this phenotype was used to assess whether the nematode Hs4F01 was functionally similar to *annAt1*. Expression of Hs4F01 with the signal peptide (extracellular) in the *annAt1* mutant produced only partial and inconsistent complementation, however, the vector construct 35S::Hs4F01 without the signal peptide (cytoplasmic) that was transformed into the T-DNA insertional mutant *Arabidopsis* lines (SALK_015426) provided consistent complementation of the mutant. The absence of the *annAt1* transcripts in the mutant lines and the expression Hs4F01 transgenes (Fig. 6A) were confirmed, as was the presence of the expressed 36 kDa Hs4F01 protein (Fig. 6B) in four independent transgenic lines. Homozygous *annAt1* mutant lines only had 16% germination efficiency while the wild-type seedlings show 93% germination efficiency in growth medium supplemented with 75 mM NaCl. Mutant *annAt1* lines that expressed Hs4F01 had an 89–93% germination rate (similar to wild-type *Arabidopsis*) on 75 mM NaCl media, indicative of functional complementation of *annAt1* by Hs4F01 (Fig. 6C, D).

**Host-derived RNA-interference (RNAi) of Hs4F01**

Plant host-derived RNAi (reviewed in Gheysen and Vanholme, 2007) was used potentially to 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) vector constructs (Fig. 7A) containing either the CaMV 35S (constitutive) promoter or the *Nicotiana tabacum* Cc7 (*Ntcel7*) endoglucanase gene promoter that has limited plant tissue-specific expression and is up-regulated in cyst nematode feeding sites (Wang et al., 2007) were generated to drive expression of hairpin dsRNA complementary to Hs4F01 within transgenic *Arabidopsis*.

*Arabidopsis* lines possessing the RNAi constructs were analysed for the expression of the dsRNA by RT-PCR of the PDK intron (Fig. 7A, B, C) that forms a single-stranded loop of the hairpin dsRNA structure and is spliced out during hairpin dsRNA processing (Wesley et al., 2001). No significant plant morphological differences were observed between the control empty vector line and transgenic Hs4F01-RNAi *Arabidopsis* lines. Homozygous Hs4F01-RNAi lines were inoculated with *H. schachtii*, and at 3 weeks post-inoculation, a significant (*t* test, *P* < 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. 7B). A control 35S-RNAi line expressing dsRNA of the green fluorescent protein (GFP) (non-nematode gene) was generated as an additional control in 35S-Hs4F01-RNAi infection assays. No observable 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* < 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. 7C).

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 *actin* gene (which was used to normalize qRT-PCR data) in nematodes excised from roots of Hs4F01-RNAi plants were not significantly different than actin expression levels in nematodes excised from the roots of control 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., 2001), analysed as a non-target control, observed in roots of any Hs4F01-RNAi plants. The RNAi silencing effect using the nematode-inducible Ntcel7 promoter showed a significant (P =0.05) 2.5–3.5-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 putative effector proteins encoded by *Heterodera glycines* parasitism genes in host plants can be a challenging task due to the technical demands of this obligate parasite combined with current difficulties in 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 gene function analyses 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 oesophageal gland secretory cell of parasitic stages of both nematode species, suggests that *H. schachtii* may use similar effector proteins to infect its host and therefore can be used as a model parasite of *Arabidopsis* to understand *H. glycines* infection of soybean.

**Hs4F01** encodes a protein structurally homologous to the annexin family of calcium-dependent phospholipid binding proteins. These proteins generally have a variable N-terminal region and a conserved core region composed of at least four repeats (Raynal and Pollard, 1994). The
variable N-terminal region is thought to confer the gene function to each annexin member (Raynal and Pollard, 1994). The Heterodera annexins were of similar peptide length and contain the conserved annexin domains as the annexins from plants and other nematode species, suggesting a similar function.

Annexins in the microbivorous nematode, C. elegans were shown to localize within the reproductive organs as well as the gland cell bodies within the terminal bulb of the pharynx (Daigle and Creutz, 1999). Furthermore, in the plant-parasitic nematode G. pallida (pale potato cyst nematode), annexins were immunolocalized within the amphids, genital primodium and in the constraining muscles above and below the metacorpal pump chamber of preparasitic 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 oesophageal gland cells of plant-parasitic nematodes (Davis et al., 2004, 2008). This adaptation combined with the secretory cell-specific developmental expression of Hg4F01 and Hs4F01 and movement of the 4F01 protein to the secretory cell ampulla in parasitic life stages are consistent with the secretion of nematode oesophageal gland proteins observed in planta using video-enhanced microscopy and immunolocalization (Wyss and Zunke, 1986; Hussey 1989; Davis et al., 2008).

The expression of a nematode parasitism gene within a transformed host to assess potential effects in plant phenotype and nematode susceptibility gives initial insight into the function of the nematode effector proteins when secreted during infection (Wang et al., 2005; Huang et al., 2006b). 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., 2006b), 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 suggests that their phenotypes may primarily be observed under abiotic or biotic stress. The biological basis of the increased susceptibility to H. schachtii in Hs4F01-expressing lines remains unclear, however, the identification of a specific oxidoreductase of the 2OG-Fe(II) oxygenase family as an interacting protein with Hs4F01 suggests that this interaction could enhance plant susceptibility, similar to the requirement of another type of functional oxidoreductase of the 2OG-Fe(II) oxygenase (DMR6) for plant susceptibility to the downy mildew pathogen (van Damme et al., 2008). Confirmation and the exact role of the nematode annexin-plant oxidoreductase interaction during host infection remains a subject of further study with complementary Arabidopsis mutants and in planta protein-protein interaction analyses.

The ability of Hs4F01 to complement the Arabidopsis annexin I mutant by restoring its ability to germinate under high salt stress (Lee et al., 2004) suggests a functional similarity between nematode and plant annexins. In Arabidopsis, annexins are involved in ameliorating various abiotic stress responses and annexin 1 levels, in particular, were shown to be elevated during 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 annexin homologues are induced. Arabidopsis roots infected with H. schachtii induce annexin 4 (ann4At) levels at 3 d post-inoculation (Puthoff et al., 2003). In more recent studies, the analysis of soybean transcript levels within nematode syncytia cells isolated through the laser capture microdissection method revealed an Arabidopsis annexin 7-like homologue was up-regulated in initial syncytial cells but was down-regulated by 10 d post-infection (dpi) (Ithal et al., 2007a). Similarly, the expression levels of Hg4F01 in H. glycines show initial up-regulation 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 early plant infection and the formation of initial syncytial cells. The roles of 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. Full

**Figure 5.** A specific interaction between the Hs4F01 annexin-like effector protein and a member of the Arabidopsis oxidoreductase (AT5G05600) in the 2OG-Fe(II) oxygenase family was confirmed in yeast two-hybrid analyses. (a, b) Positive control interaction (blue β-gal product) of SV40 and P53, and no interaction of the SV40 and LamC negative controls, respectively, included in the BD Matchmaker™ yeast two-hybrid system (Clontech, Mountain View, CA). (c, d) No interaction of the Arabidopsis oxidoreductase with the empty vector control or LamC, respectively. (e) A specific positive interaction of Arabidopsis oxidoreductase and Hs4F01.
complementation of *annAt1* was only achieved when *Hs4F01* was expressed in transgenic *Arabidopsis* without the signal peptide for secretion, suggesting that secretion of the nematode annexin into host cell cytosol through the nematode stylet is the probable site of delivery and activity to promote host compatibility.

Evidence that cyst nematodes secrete mimics of endogenous plant peptides suggests that nematodes may directly hijack natural plant physiological processes to aid in the successful parasitism of its host (Davis *et al.*, 2008). One such example is the functional complementation of an *Arabidopsis CLAVATA3* mutant (*clv3-1*) with the 4G12 member of the *H. glycines* SYV46 parasitism gene family (Wang *et al.*, 2005; Davis, 2009). Although the exact role of secreted nematode CLAVATA3/ESR (CLE) peptides in host plant roots remains unclear, it is evident that cyst nematodes probably exploit certain plant signal transduction pathways to augment selected host plant cells for parasitism (Mitchum *et al.*, 2008). Similarly, secreted *Hs4F01* may interact with the plasma membrane of the syncytia to influence multiple aspects of cell regulation to promote parasitism. Recently, it was reported that oxidoreductase is induced in response to elevated levels of exogenous calcium levels (Chan *et al.*, 2008). 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, thereby increasing the levels of oxidoreductase. The observed interaction of *Hs4F01* with host plant oxidoreductase could maintain the level of this protein to limit defence gene expression and to augment the plant stress response to nematode infection.

The significant reduction in parasitic success of cyst nematodes observed in roots of plants that expressed host-derived RNAi targeted to *Hs4F01* suggests an important extension...
role for this nematode annexin gene product in a successful plant–nematode interaction. Constitutive expression of Hs4F01 dsRNA in transgenic Arabidopsis did not alter visible plant phenotype (response of RNAi plants to salt stress was not assessed), suggesting that codon usage between nematode and plant annexins may have differed to a degree that did not induce apparent off-target silencing of endogenous plant annexin genes. Follow-up experiments to monitor expression levels of members of the Arabidopsis annexin family (Cantero et al., 2006) in Hs4F01-RNAi and Hs4F01-overexpression plants combined with nematode infection data in Arabidopsis mutants of different annexin family members will help dissect the contributions of different plant annexins to nematode parasitism. Limiting

Fig. 7. Effect of plant host-derived RNA-interference of Hs4F01 nematode annexin-like parasitism gene on infection of Arabidopsis roots by the beet cyst nematode, Heterodera schachtii. (A) Schematic of the pHANNIBAL (Wesley et al., 2001) hairpin double-stranded RNA (dsRNA) construct containing the sense and antisense coding regions of Hs4F01. The CaMV 35S promoter (35S P) originally in pHANNIBAL was replaced with the nematode-inducible (Wang et al., 2007) Nicotiana tabacum cellulase 7 promoter (Ntcel7 P) in some transgenic Arabidopsis Hs4F01-RNAi lines. (B) RT-PCR of the single-stranded PDK intron of the hairpin dsRNA was used to confirm the expression of Hs4F01-dsRNA in roots of independent transgenic Arabidopsis lines at 10 d post-germination. Heterodera schachtii infection of Arabidopsis lines that expressed Hs4F01 dsRNA via the nematode-inducible Ntcel7 promoter showed a significant reduction in the number of females per plant at 3 weeks post-inoculation compared to the empty vector control line. (C) RT-PCR of the single-stranded PDK intron of the hairpin dsRNA was used to confirm the expression of Hs4F01-dsRNA and non-specific control GFP-dsRNA (RNAi) in roots of independent transgenic Arabidopsis lines at 10 d post-germination. Heterodera schachtii infection of Arabidopsis lines that expressed Hs4F01 dsRNA via the constitutive CaMV 35S promoter showed a significant reduction in the number of females per plant at 3 weeks post-inoculation compared to the GFP-RNAi control line. Bars indicate ± standard error; n=24. An asterisk represents a significant difference (t-test, P <0.05) versus control.
expression of *Hs4F01* dsRNA within transgenic plant tissues using the *Ntcel7* promoter (reported to be up-regulated in nematode feeding sites by Wang et al., 2007) also produced no off-target plant effects while producing a slightly greater reduction in nematode parasitic success as compared to constitutive *Hs4F01* dsRNA expression. With neither *Hs4F01* RNAi construct, however, was strong reduction in nematode parasitic success achieved, as has been observed for some nematode genes targeted by host-derived RNAi (Huang et al., 2006a; Yadav et al., 2006; Klink et al., 2009). Strong reduction in parasitic success using host-derived RNAi, however, has not been achieved for a number of phytoparasitic nematode genes targeted to date, including constructs driven by constitutive (Steeves et al., 2006; Sindhu et al., 2009) and nematode-inducible promoters (Fairbairn et al., 2007; Patel et al., 2008). While the reasons for these latter results are currently unknown, it should be considered that quantitative detection of nematode gene-specific silencing is likely to be biased toward any parasitic nematodes that can be recovered from plant tissues for analyses and may not reflect the potentially high levels of silencing experienced in nematodes that did not survive treatment. Only the single expressed member of the *Hs4F01* family described has been recovered from cyst nematodes to date and its expression in nematode parasitic stages occurs (Ithal et al., 2007b) during the time of reported CaMV35S (Bertioli et al., 1999) and *Ntcel7* (Wang et al., 2007) promoter activity in nematode feeding cells. The significant and specific reductions in *Hs4F01* transcript levels in the host-derived RNAi lines assayed, however, combined with a significant reduction in nematode parasitic success in the RNAi lines, suggests that annexin production and secretion by the nematode plays a substantial role in promoting successful plant infection.

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