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RESEARCH PAPER

The interaction of the novel 30C02 cyst nematode effector protein with a plant β-1,3-endoglucanase may suppress host defence to promote parasitism

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Abstract

Phytoparasitic nematodes secrete an array of effector proteins to modify selected recipient plant cells into elaborate and essential feeding sites. The biological function of the novel 30C02 effector protein of the soybean cyst nematode, *Heterodera glycines,* was studied using *Arabidopsis thaliana* as host and the beet cyst nematode, *Heterodera schachtii*, which contains a homologue of the *30C02* gene. Expression of *Hg30C02* in *Arabidopsis* did not affect plant growth and development but increased plant susceptibility to infection by *H. schachtii*. The 30C02 protein interacted with a specific (AT4G16260) host plant β-1,3-endoglucanase in both yeast and plant cells, possibly to interfere with its role as a plant pathogenesis-related protein. Interestingly, the peak expression of *30C02* in the nematode and peak expression of *At4g16260* in plant roots coincided at around 3–5 d after root infection by the nematode, after which the relative expression of *At4g16260* declined significantly. An *Arabidopsis At4g16260* T-DNA mutant showed increased susceptibility to cyst nematode infection, and plants that overexpressed *At4g16260* were reduced in nematode susceptibility, suggesting a potential role of host β-1,3-endoglucanase in the defence response against *H. schachtii* infection. *Arabidopsis* plants that expressed dsRNA and its processed small interfering RNA complementary to the *Hg30C02* sequence were not phenotypically different from non-transformed plants, but they exhibited a strong RNA interference-mediated resistance to infection by *H. schachtii*. The collective results suggest that, as with other pathogens, active suppression of host defence is a critical component for successful parasitism by nematodes and a vulnerable target to disrupt the parasitic cycle.

Key words: *Arabidopsis thaliana*, *At4g16260*, *Heterodera schachtii*, nematode secretions, PR protein, RNAi.

Introduction

The soybean cyst nematode, *Heterodera glycines*, is a microscopic worm and an obligate endoparasite of host plant roots, and is the most damaging pathogen of soybeans grown in the USA (Niblack *et al.*, 2006; Wrather and Koenning, 2006). Cyst nematode second-stage juveniles (J2) hatch from eggs, penetrate host plant roots behind the root tip, and migrate intracellularly through the root cortex to the vascular cylinder (Wyss and Zunke, 1986). When the nematodes reach the vascular tissue, they transform selected root cells into a specialized feeding site called the syncytium, which becomes

Abbreviations: DIG, digoxygenin; GFP, green fluorescent protein; J2, second-stage juveniles; p.i., post-infection; PR, pathogenesis-related; RNAi, RNA interference; SE, standard error; siRNA, small interfering RNA; WT, wild type; YFP, yellow fluorescent protein.

the permanent food source for the nematodes as they develop through subsequent sedentary life stages. Syncytium formation includes profound changes in host root cells including DNA endoreduplication, disappearance of large vacuoles, proliferation of organelles, peripheral cell-wall thickening and ingrowths, and targeted cell-wall dissolution between adjacent plant cells to allow syncytium expansion via cellular fusion (Jones, 1981; Endo, 1991; Grundler *et al.*, 1997).

Cyst nematodes secrete effector proteins through their stylet (a hollow, protrusible oral spear) to induce and maintain the syncytium (Hussey, 1989; Williamson and Hussey, 1996; Davis *et al.*, 2000, 2008; Gheysen and Mitchum, 2011). The effector proteins originate in three elaborate secretory gland cells within the nematode oesophagus, and effector synthesis and secretion are developmentally regulated throughout the parasitic cycle (Wyss and Zunke, 1986; Hussey, 1989; Davis *et al.*, 2008). Functional genomics approaches have identified multiple putative effector-encoding 'parasitism genes' that are expressed within the oesophageal gland cells of *H. glycines* (Gao *et al.*, 2001, 2003; Wang *et al.*, 2001). While some cyst nematode parasitism genes encode proteins with database matches such as those encoding cell-wall-modifying enzymes, the majority of these identified parasitism genes have no database orthologues (Davis *et al.*, 2008; Gheysen and Mitchum, 2011) and may encode effector proteins that are unique to cyst nematodes.

As cyst nematodes are obligate biotrophs embedded within host roots and cannot currently be genetically engineered or routinely used in forward genetic assays, analyses of the functions of their novel parasitism genes remains a challenge. The use of *Arabidopsis thaliana* as the test plant species for investigations of nematode parasitism gene function, however, has provided a wealth of genetic resources on the host side of the interaction that has enabled considerable progress in such functional studies (Wang *et al.*, 2005; Huang *et al.*, 2006*b*; Hewezi *et al.*, 2008, 2010; Patel *et al.*, 2010; Wang *et al.*, 2010; Lee *et al.*, 2011; Replogle *et al.*, 2011). As *A. thaliana* is not a host for *H. glycines*, the closely related beet cyst nematode, *Heterodera schachtii* (Subbotin *et al.*, 2001), provides an excellent surrogate, as it can infect the roots of *Arabidopsis* (Sijmons *et al.*, 1991). Several studies have demonstrated that *H. schachtii* possesses many of the same parasitism genes as *H. glycines* with an almost identical nucleotide and predicted amino acid sequence (Patel *et al.*, 2008; Sindhu *et al.*, 2009; Lee *et al.*, 2011; Wang *et al.*, 2011). Coupled with functional analyses in *Arabidopsis*, the RNA interference (RNAi) technology developed in the nematode *Caenorhabditis elegans* (Fire *et al.*, 1998) has been adapted to provide host plant-derived silencing of target phytonematode parasitism gene transcripts via ingested dsRNA from transformed host plants (Huang *et al.*, 2006*a*; Patel *et al.*, 2008, 2010; Sindhu *et al.*, 2009). Some target plant proteins that interact with secreted nematode effector proteins have also been identified through yeast two-hybrid analyses and have led to further functional characterization in plants for their roles in plant–nematode interactions (Huang *et al.*, 2006*b*; Hewezi *et al.*, 2008, 2010; Rehman *et al.*, 2009; Patel *et al.*, 2010; Lee *et al.*, 2011). In the present study, the function of a novel cyst nematode parasitism gene initially identified as *30C02* (Gao *et al.*, 2003) was investigated using *A. thaliana* as a model plant host and *H. schachtii* where appropriate. The results suggested that the 30C02 effector protein is essential for successful plant parasitism by cyst nematodes and interacts with a plant β-1,3-endoglucanase to potentially suppress plant defence.

Materials and methods

Nematode culture and infection assays

Cyst nematodes of *H. schachtii* and *H. glycines* were propagated on the roots of cabbage plants (*Brassica oleracea* var. *capitata*) and soybean plants (*Glycine max* cv. Lee 74) grown in soil, respectively. Eggs were collected from crushed cysts as described previously for other cyst nematode species (Goellner *et al.*, 2001). *Meloidogyne incognita* (root-knot nematodes) were propagated in soil-grown tomato plants (*Solanum lycopersicum* cv. Rutgers) and eggs were extracted as described previously (Hussey and Barker, 1973). All nematode eggs were hatched over water at 28 °C on Baermann pans for 48 h, after which the hatched pre-parasitic second-stage juveniles (pre-J2s) were collected and surface sterilized for 10 min in sterilization solution (0.004% mercuric chloride, 0.004% sodium azide, 0.002% Triton X-100) followed by three washes with sterile distilled water. Mixed parasitic stages of *H. schachtii* and *H. glycines* were collected from within the roots of cabbage and soybean plants, respectively, by root blending and sieving as described by Ding *et al.* (1998). Nematode infection assays and data collection were performed as described previously (Hamamouch *et al.*, 2011). For analysis of nematode infection rate, cysts (for beet cyst nematode) and galls (for root-knot nematodes) developed in wild-type (WT) and transgenic plants were counted 3–4 weeks post-infection (p.i.), respectively, using a dissecting microscope, and the mean and standard error (SE) of 20 replicates per treatment were calculated. Statistical differences in the mean (n=20) were determined by paired t-test with an alpha level of 0.05 using SAS software (Cary, NC).

DNA gel blot analysis

Extraction of *H. schachtii* and *H. glycines* genomic DNA was performed as described by Patel *et al.* (2010). Genomic DNA (5 μg) was digested overnight at 37 °C with *Eco*RI, separated by 0.7% agarose gel electrophoresis, and transferred by capillarity (Sambrook *et al.*, 1989) on to a positively charged nylon membrane (GE Healthcare Biosciences, NJ). Genomic DNA isolated from *M. incognita* and WT *A. thaliana* (Col-0) plants was used as negative controls. A digoxygenin (DIG)-labelled probe was synthesized using a PCR DIG Probe Synthesis kit (Roche Applied Science) and *Hg30C02* cDNA (GenBank accession no. JF896103) as template. Hybridization of the probe to the target sequence(s) was performed at 42 °C, and subsequent washes and detection were carried out following the manufacturer's instructions (Roche Applied Science; Indianapolis, IN). To visualize hybridization signals, the membrane was exposed to Lumi-Film chemiluminescent detection film (Roche Diagnostics, Indianapolis, IN) for 1–2 min (Fig. 1).

Isolation of expressed 30C02 *and sequence analysis*

H. schachtii and *H. glycines* mRNAs were extracted from mixed parasitic stages of each nematode species using a Dynabeads mRNA DIRECT kit (Invitrogen; Carlsbad, CA) and treated with DNase using a Turbo DNA-free kit (Ambion; Austin, TX) according to the manufacturer's instructions. cDNA synthesis of *30C02* was conducted using SuperScript II reverse transcriptase (Invitrogen), amplified using a *30C02*-specific (Gao *et al.*, 2003) primer pair (5′-ATGAGGAAACTTCCATTCTTGC-3′ and

5′-GTGTTCTGCTGGTGAAATGC-3′), and cloned into pGEM-T vector for sequencing. Alignments of *Hs30C02* (GenBank accession no. JF896102) and *Hg30C02* (JF896103) cDNA and predicted amino acid sequences (Fig. 2), and predictions of a signal peptide for secretion, were performed using the Clustal W (Thompson *et al.*, 1994) and SIGNAL P 3.0 (Bendtsen *et al.*, 2004) programs, respectively. Specimens of each individual parasitic stage of *H. schachtii* were identified under a microscope and collected for RNA extraction and quantitative RT-PCR (qRT-PCR) assays as described below.

30C02 *mRNA* in situ *hybridization in nematode specimens*

In situ hybridization within nematode specimens was performed as described previously (de Boer *et al.*, 1999) using mixed parasitic stages of *H. schachtii*. *Hg30C02*-specific primers were used to synthesize DIG-labelled sense (negative control) and antisense cDNA probes. Hybridization signals within the nematodes were detected with alkaline phosphatase-conjugated anti-DIG antibody, and the treated specimens were observed by light microscopy.

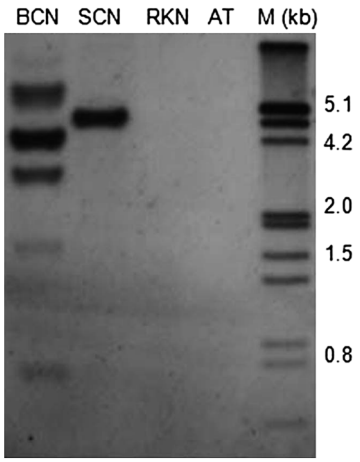

Fig. 1. Genomic DNA digested with *EcoRI*, separated by agarose gel electrophoresis and hybridized on gels blots with a full-length *Hg30C02* (JF896103) DIG-labelled cDNA probe. BCN, beet cyst nematode (*H. schachtii*); SCN, soybean cyst nematode (*H. glycines*); RKN, root-knot nematode (*M. incognita*); AT, *A. thaliana*. At least five copies of *30C02* are present in *H. schachtii* and a single copy is present in *H. glycines*. No hybridization signal was detected in *M. incognita* or *A. thaliana* genomic DNA. M, DIG-labelled DNA marker.

Molecular cloning and vector construction

Given the nearly identical predicted amino acid sequences between Hs30C02 and Hg30C02 expressed in the parasitic stages of both nematode species (Fig. 2), the cDNA of *Hg30C02* (JF896103) was used for all subsequent expression vector constructions. The 492 bp cDNA of *Hg30C02* was excised from the pGEM-T Easy vector by digestion with *Sac*II and *Sac*I, and subcloned into pBC plasmid digested with *Sac*II and *Sac*I. The 35S promoter was excised from pBI121 using *Hin*dIII and *Bam*HI, and then subcloned into pBC plasmid upstream of the *30C02* coding sequence. The identity, orientation, and junctions of the resulting *35S::30C02* construct were confirmed by PCR and sequencing. The *35S::GUS* gene of pBI121 plasmid (Chen *et al.*, 2003) was digested with *Hin*dIII and *Sac*I, and replaced with the *35S::30C02* construct resulting in the pBI-30C02 vector.

The full-length cDNA of the *Arabidopsis* β-1,3-endoglucanase (*At4g16260*) encoding a protein that interacted with Hg30C02 was amplified using the forward 5′-TAGGATCCATGACCACG-TTATTCCTCC-3′ and reverse 5′-TAGAGCTCTCACTCAACC-GCCGTA-3′ primer containing *Bam*HI and *Sac*I sites, respectively. The amplified β-1,3-endoglucanase cDNA was cloned in the pBI121 plasmid between the *Bam*HI and *Sac*I sites, resulting in the pBI-β-1,3-endoglucanase vector.

For RNAi vectors, full-length *Hg30C02* was isolated from pGEM-T Easy by *Eco*RI restriction digestion and subcloned in the antisense orientation in pHANNIBAL vector (Wesley *et al.*, 2001) digested with *Eco*RI enzyme. The sense strand of *30C02* was amplified using the primers 5′-ATAAGCTTTGAGGAAACTTCC-ATTCTTG-3′ and 5′-ATTCTAGAGGTGAAATGCGTTTTTCC-3′, which introduced *Hin*dIII and *Xba*I restriction sites (underlined), and cloned into pHANNIBAL. Both sense and antisense strands of *30C02* were under the control of a single 35S promoter. An RNAi vector containing the sense and antisense strands of green fluorescent protein (GFP) was used as a control. *30C02*-RNAi and *GFP* RNAi constructs produced in pHANNIBAL were isolated by restriction digestion with *NotI* and cloned into the pART27 binary vector (Gleave, 1992) for subsequent *Agrobacterium* and plant transformation, resulting in $pART_{27}$-30C02 and $pART_{27}$-GFP, respectively.

Generation of transgenic Arabidopsis *plants*

The binary vectors $pART_{27}$-30C02, $pART_{27}$-GFP, pBI-30C02, pBI-β-1,3-endoglucanase were introduced into *Agrobacterium tumefaciens* strain GV3101 via electroporation and verified by PCR. *A. thaliana* plants (ecotype Columbia) were transformed with *A. tumefaciens* containing the gene construct using the floral dipping method (Clough and Bent, 1998) and seeds were selected on MS medium (Murashige and Skoog, 1962), supplemented with 50 mg l^{-1} of kanamycin. Segregation analyses identified homozygous lines and PCR analysis confirmed the presence of the gene constructs in the genome of the transformed plants. The *Arabidopsis* β-1, 3-endoglucanse T-DNA mutant line (Salk_031479) was obtained

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H.shachtii para-30C02          MRKLPFLLLFSACCYLQRKEVYFAEKNRKKNSSSSEQKTQRRNRGYGRSR
H.glycines para-30C02          MRKLPFLLLFSACCYLQRKEVYFAEKNRKKNSSSSEQKTQRRNRGYGRSR
                               **************************************************

H.shachtii para-30C02          TFSNGNGMYGQSNGFSNGGFGGSSGYSNSGRSNSFPNGGFGGSSGFSSMG
H.glycines para-30C02          TFSNGNGMYGQSNGFSNGRFGGSSVYSNSGRSNSFPNGGFGGSSGFSSMG
                               ****************** ***** ************************

H.shachtii para-30C02          HNSGGLHGFGSGPPAGGYGYGGGFGGRK
H.glycines para-30C02          HNSGGLHGFGSGPPSGGYGYGSGFGGRK
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Fig. 2. A 98% predicted amino acid sequence identity between the 30C02 protein expressed in the parasitic life stages (within host plant roots) of both *H. schachtii* (JF896102) and *H. glycines* (JF896103), was determined using the Clustal W program (Thompson *et al.*, 1994); the underlined sequence represents the predicted secretion signal peptide motif (Bendtsen *et al.*, 2004). Identical amino acids are indicated by asterisks.

from the *Arabidopsis* Biological Resource Center and propagated to homozygosity.

RNA isolation and quantitative RT-PCR

Total RNA from *Arabidopsis* roots was isolated using an RNeasy Plant Mini kit (Qiagen; Valencia, CA) following the manufacturer's instructions. Prior to RT-PCR, total RNA was treated with RNase-free DNase I (Ambion) to eliminate any contaminating genomic DNA. First-strand cDNA was synthesized from 2–3 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and oligo-dT_{18} primers following the manufacturer's instructions. The RT-PCR was run for 35 cycles and consisted of 94 °C for 2 min, 56 °C for 1 min, and 72 °C for 1 min. The cycles were preceded by a 94 °C denaturation period for 4 min and followed by a 72 °C final extension period for 10 min.

All qRT-PCR runs were performed in a DNA Engine Mx3000P (Agilent Technologies; Santa Clara, CA). A single 20 μl PCR included 1× Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies), 2 μl of cDNA template and 5 μM of each forward and reverse primer. The PCR cycling parameters were set at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72 °C for 45 s. Dissociation melting-curve analyses, in which all products generated during the qPCR amplification reaction were melted at 95°C for 1 min, annealed at 55°C for 30 s, and subjected to gradual increases in temperature, were conducted to discount the effects of primer–dimer formation and contamination. The qRT-PCRs were performed in triplicate and the negative controls included water and mRNA extracted from the nematodes to check for DNA contamination in the analysed samples. All qRT-PCR samples were normalized against the *Arabidopsis* actin-8 gene (GenBank accession no. ATU42007) or the *H. schachtii* nematode actin gene (GenBank accession no. AY443352), as appropriate. The fold change relative to control plants was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). A 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). Reactions were repeated at least three times, and a representative result was displayed for individual assays. The sequences of the primers used in the qRT-PCR are available in Supplementary Table S1 (in *JXB* online) and Hamamouch *et al.* (2011).

Small-RNA sequencing and analysis from RNAi plants

Total RNA was isolated from a pool of *Arabidopsis* seedling roots from three independently transformed lines (L2-6, L1-5, and L6-4) containing the *Hg30C02* RNAi construct. Library preparation was performed according to the Illumina Small RNA Version 1.5 Sample Prep kit and sequencing by synthesis using the Illumina GAIIx at the Keck Center of the University of Illinois (Tuteja *et al.*, 2009) to obtain 11.2 million 40 nt reads. After trimming the 3′ adapter sequence (ATCTCGTATGCCGTCTTCTGCTTG) and retaining reads greater than 16 nt, the sequences were compared to obtain the unique sequences and the number of occurrences of each distinct sequence. Alignments of small RNAs to the 492 bp *Hg30C02* sequence were performed using Bowtie (Langmead *et al.*, 2009).

Protein interaction assays

The MATCHMAKER yeast two-hybrid system II (Clontech; CA) was used to identify interacting plant and nematode proteins. The cDNA (JF896103) encoding the mature peptide of 30C02 without the predicted nematode signal peptide was cloned in frame with the GAL4-binding domain of pGBKT7 and expressed as bait to screen an *A. thaliana* root cDNA library constructed in the GAL4 activation domain of pGADT7 from mRNA of *Arabidopsis* root tissues at 3, 7, and 10 d after *H. schachtii* infection (Hewezi *et al.*, 2008). Positive yeast two-hybrid matings were selected on a series of selective media as described in the protocol of the MATCHMAKER yeast two-hybrid system II and subjected to subsequent co-transformation of isolated clones to validate positive protein–protein interactions.

Bifluorescence complementation of the 30C02 and β-1, 3-endoglucanase interaction

Bifluorescence complementation analyses followed the procedure developed by Citovsky *et al.* (2006) to demonstrate protein interactions within plant cells. The *Hg30C02* cDNA without the signal peptide was PCR amplified using the following forward primer (5′-TACTCGAGTGCAACGGAAGGAAGTATATTTCGC-3′) and reverse primer (5′-GAAGCTTCTAAAAAAGTGTTCTG-CTGGTGAA-3′) containing *XhoI* and *HindIII* restriction sites (underlined), respectively, and cloned into *XhoI* and *HindIII* sites of pSAT4-cEYFPC1B to generate pSAT4-cEYFP$_{C1B}$-30C02. Meanwhile, the full-length beta-1,3-endoglucanase cDNA without signal peptide was PCR amplified using forward primer (5′-TACTCGAGTGGAATCAGTAGGTGTATGCTATGG-3′) and (5′-GAAGCTTTCACTCAACCGCCGTACCGTCT-3′) reverse primer containing *XhoI* and *HindIII* restriction sites (underlined), respectively, and cloned into *XhoI* and *HindIII* sites of pSAT4-nEYFP$_{C1}$ to generate pSAT4-cEYFP$_{C1B}$-β-1,3-endoglucanase. Tungsten particle (M10; Sylvania Chemicals/Metals; Towanda, PA) preparation and DNA precipitation on particles were performed as described previously (Vain *et al.*, 1993). DNA-coated tungsten particles were bombarded by biolistics into onion epidermal cells at 60 p.s.i. at 16 cm distance using a particle inflow gun (Finer *et al.*, 1992). Bombarded tissues were incubated at room temperature overnight before assessing protein interactions as yellow fluorescent protein (YFP) signals within onion cells. Bright-field and fluorescence images were observed using a Motic AE31 microscope (Motic Instruments; Richmond, BC, Canada) with an appropriate filter to observe YFP fluorescence, and images were taken using a SPOT digital camera (Diagnostic Instruments; Sterling Heights, MI).

Results

30C02 *exists in the* H. schachtii *genome*

A probe derived from *Hg30C02* was DIG-labelled and hybridized in a DNA gel blot to *Eco*RI-digested genomic DNA from *H. schachtii* and *H. glycines*. DIG-labelled *Hg30C02* hybridized to a single band of about 5 kb in the *H. glycines* genome and to at least five DNA fragments ranging from 0.7 to 6.0 kb in the *H. schachtii* genome (Fig. 1). No *Hg30C02* hybridization signal was detected in the genome of the root-knot nematode *M. incognita* or in WT *A. thaliana*.

Isolation of the expressed H. schachtii 30C02 *gene and sequence analysis*

Primer sequences designed from the coding region of the original *H. glycines 30C02* gene (Gao *et al.*, 2003) were used in RT-PCR to successfully amplify cDNA of *Hg30C02* (JF896103) and a cDNA orthologue from *H. schachtii*, *Hs30C02* (JF896102), using RNA extracted from mixed parasitic stages of *H. glycines* and *H. schachtii*, respectively. Sequence analyses of 20 independent cDNA clones found no polymorphisms within *Hs30C02* expressed in mixed parasitic stages of *H. schachtii*, despite the apparent existence of

multiple family members observed on DNA gel blots. Expressed *Hs30C02* (JF896102) contained a predicted open reading frame of 128 aa with a predicted mass of 13.46 kDa. Signal P 3.0 (Bendtsen *et al.*, 2004) predicted a signal peptide for Hs30C02 between position 16 (leucine) and 17 (glutamine) (Fig. 2), indicating that the proteins may be targeted for secretion outside the nematode gland cell into plant cells, a characteristic of phytoparasitic nematode effector proteins (Davis *et al.*, 2008). Protein domain searches did not identify any motif that could predict the function of 30C02, nor was 30C02 identified in the root-knot nematode *M. incognita* and *Meloidogyne hapla* genomes (Abad *et al.*, 2008; Opperman *et al.*, 2008). Comparison of *Hs30C02* and *Hg30C02* nucleotide sequences indicated that the two cDNA sequences shared 99% nucleotide identity (data not shown) and 98% predicted amino acid identity (Fig. 2). Thus, *Hs30C02* and *Hg30C02* expressed in parasitic stages were considered to be homologues, allowing *Hg30C02* to be used for functional analyses in the *H. schachtii*–*Arabidopsis* pathosystem.

Developmental expression of 30C02 *within* H. schachtii

Localized expression of *30C02* transcripts specifically within the single dorsal oesophageal gland secretory cell of different parasitic stages of *H. glycines* has been confirmed previously (Gao *et al.*, 2003) and was identical in *H. schachtii* (Fig. 3A). The developmental expression of *30C02* during *H. schachtii* pre-parasitic and parasitic stages was quantified here using qRT-PCR. *Hs30C02* mRNA was detected at maximum level in late (3–5 d p.i.) parasitic J2s and reduced in expression in later sedentary stages (Fig. 3B), suggesting a primary role of 30C02 during the early stages of nematode parasitism.

Expression of 30C02 in Arabidopsis *increases susceptibility to* H. schachtii

To gain a first insight into the effect of 30C02 in host plants, *Hg30C02* was constitutively expressed in *Arabidopsis* plants under the control of the cauliflower mosaic virus 35S promoter with and without the predicted nematode signal peptide sequence. The presence of the signal peptide should target the protein to the extracellular space of the plant cells (lines L6-3 and L13-12), whereas removal of the signal peptide should localize 30C02 within the plant cell cytoplasm (lines L1-5 and L2-1). The presence of expressed *Hg30C2* transcripts in the transformed *Arabidopsis* lines was confirmed by RT-PCR analysis (Fig. 4A). Phenotype analysis of transgenic plants indicated that *Hg30C02* expression had no apparent effect on plant growth and development (data not shown). However, plants that expressed *Hg30C02* with or without the nematode predicted signal peptide were significantly ($P \leqslant 0.05$) more susceptible to *H. schachtii* infection than WT, as evidenced by an increase in the number of developed cyst nematode adult females (Fig. 4B). Transgenic *Arabidopsis* plants that constitutively expressed *Hg30C02* were also infected with a different sedentary phytoparasitic nematode species, *M. incognita* (root-knot nematode), and showed no significant difference in the infection level when compared with WT plants (Fig. 4C). These data suggested that the secreted 30C02 effector protein specifically promotes host susceptibility to infection by cyst nematodes.

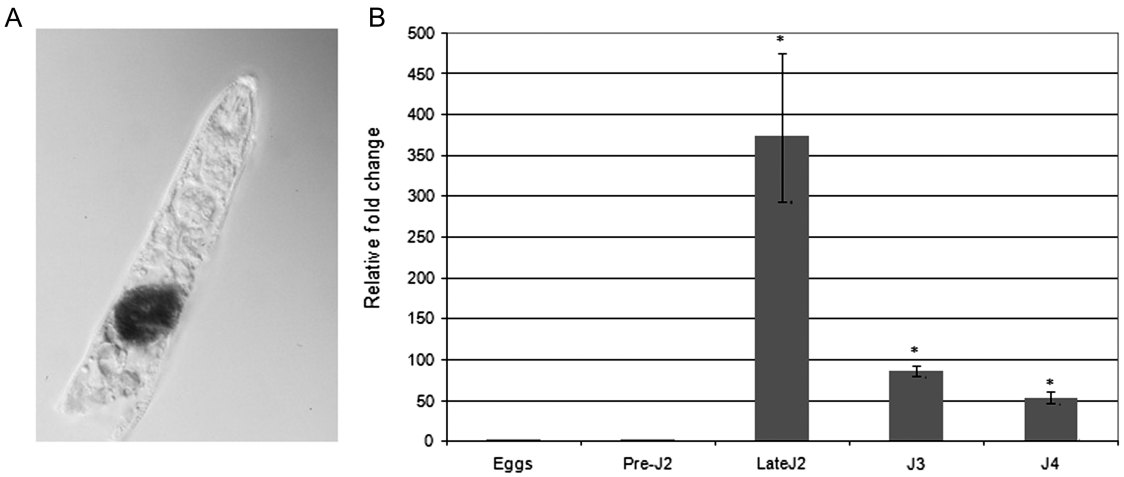

Fig. 3. Expression of the gene encoding the 30C02 effector protein in cyst nematodes. (A) Micrograph of a mRNA *in situ* hybridization of a DIG-labelled probe of *Hg30C02* specifically within the single enlarged dorsal oesophageal gland cell of a cyst nematode third-stage juvenile (J3) that was excised from a host root. (B) Quantitative expression of *Hs30C02* within the developmental life stages of the beet cyst nematode *H. schachtii* determined by qRT-PCR. The relative fold-change values were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and represent changes in mRNA level in nematode pre-parasitic J2 (pre-J2), late parasitic J2 (late J2), J3, and J4 relative to that of eggs (pre-J2). The *H. schachtii* actin gene (AY443352) was used as an internal control to normalize gene expression levels for all samples.

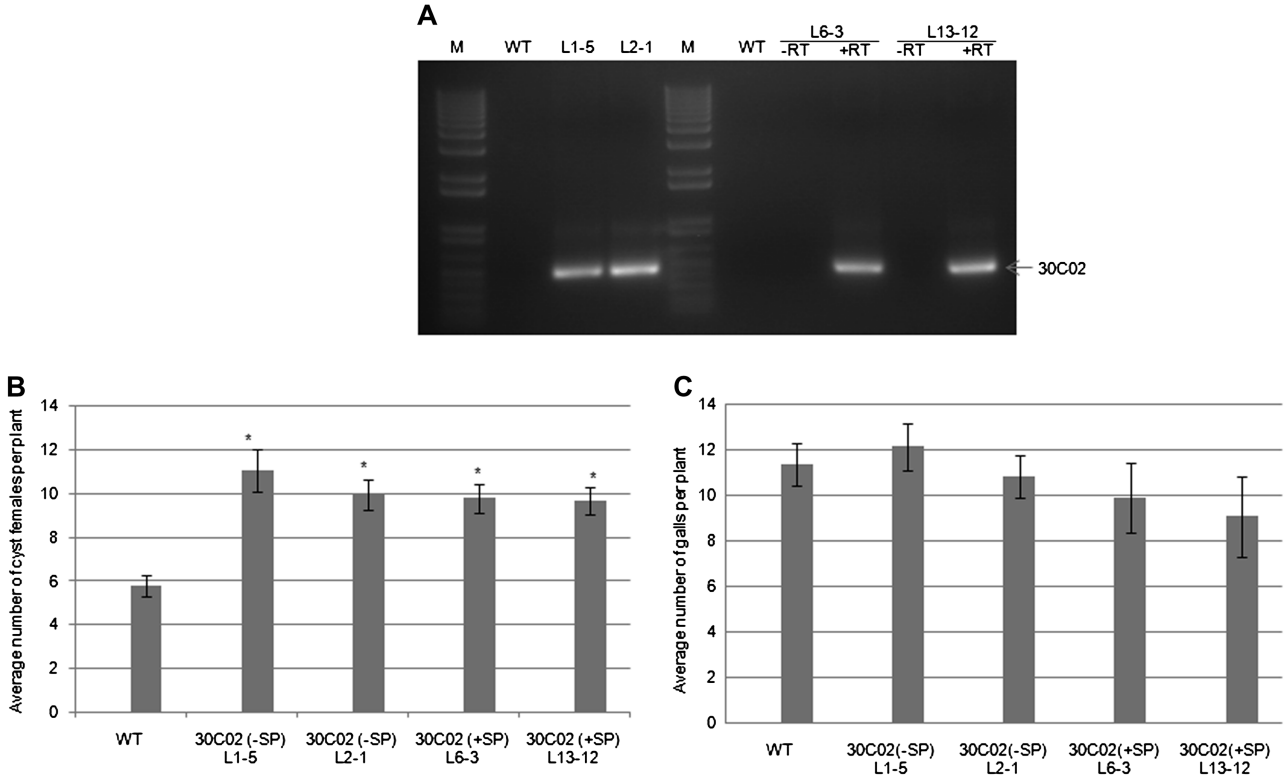

Fig. 4. Constitutive expression of *Hg30C02* in transgenic *A. thaliana* increases plant susceptibility to infection by the beet cyst nematode *H. schachtii*. (A) RT-PCR confirmed the expression of *Hg30C02* without signal peptide in transgenic *Arabidopsis* lines L1-5 and L2-1 compared with WT control, and RT-PCR amplification of expressed *Hg30C02* with signal peptide from transgenic lines L6-3 and L13-12 in the absence (–RT) and presence (+RT) of reverse transcriptase, respectively. (B) A significant (*, $P \leqslant 0.05$) increase in the mean number of *H. schachtii* females per plant was observed in the roots of transgenic *Arabidopsis* lines that expressed *Hg30C02* compared with females developed on the WT control plants. (C) No significant ($P \leqslant 0.05$) difference in root infection (gall number) by the root-knot nematode *M. incognita* was observed in roots of the same *Hg30C02* transgenic *Arabidopsis* lines compared with WT plants.

Hg30C02 specifically interacts with a plant β*-1, 3-endoglucanase*

To identify host protein(s) that interacted with 30C02, full-length Hg30C02 without the signal peptide was used as a bait in yeast two-hybrid assays to screen a cDNA prey library prepared from mRNA of *Arabidopsis* roots that had been infected with *H. schachtii* (Hewezi *et al.*, 2008). Yeast two-hybrid assays identified a specific *Arabidopsis* β-1, 3-endoglucanase (AT4G16260) as an interacting partner with Hg30C02. The AT4G16260 β-1,3-endoglucanse had the structure and activity of a pathogenesis-related (PR) protein (Mahalingam *et al.*, 2003; Doxey *et al.*, 2007; Lashbrook and Cai, 2008). The physical interaction between full-length AT4G16260 β,1-3-endoglucanase–GAL4 and Hg30C02 was confirmed in yeast cells grown on selective medium (Fig. 5A) and subsequent co-transformation assays of independent clones isolated from the matings.

To test whether this protein–protein interaction could occur within a plant cell, bimolecular fluorescence complementation assays (Citovsky *et al.*, 2006) were performed. Hg30C02 and AT4G16260 β-1,3-endoglucanase without signal peptides were separately fused to the N-terminal and C-terminal halves of YFP, respectively, and co-expressed in onion epidermal cells after biolistic transformation. The interaction between the 30C02 and β-1,3-endoglucanase proteins reconstituted the activity of YFP in the cytoplasm of transformed onion cells (Fig. 5B, C).

To further investigate potential pathways that may have been affected by the expression of Hg30C02 in plants, and that may underlie the observed increased susceptibility to *H. schachtii* of *Arabidopsis* that expressed *Hg30C02*, we used qRT-PCR to measure the transcript levels of *At4g16260*, a representative set (Hamamouch *et al.*, 2011) of genes that encode PR proteins (PR1, PR2, PR3, PR4, PR5, and PDF1.2), PAD4, which is required for synthesis of the phytoalexin camalexin, and isochorismate synthase, which is required for salicylic acid synthesis. Neither *At4g16260* nor any of the PR genes monitored exhibited a significant quantitative transcriptional change in transgenic plants that constitutively expressed *Hg30C02* compared with WT plants (data not shown).

Expression of the At4g16260 β*-1,3-endoglucanase gene during* H. schachtii *infection*

To examine the expression level of AT4G16260 β-1, 3-endoglucanase during infection by *H. schachtii*, we used

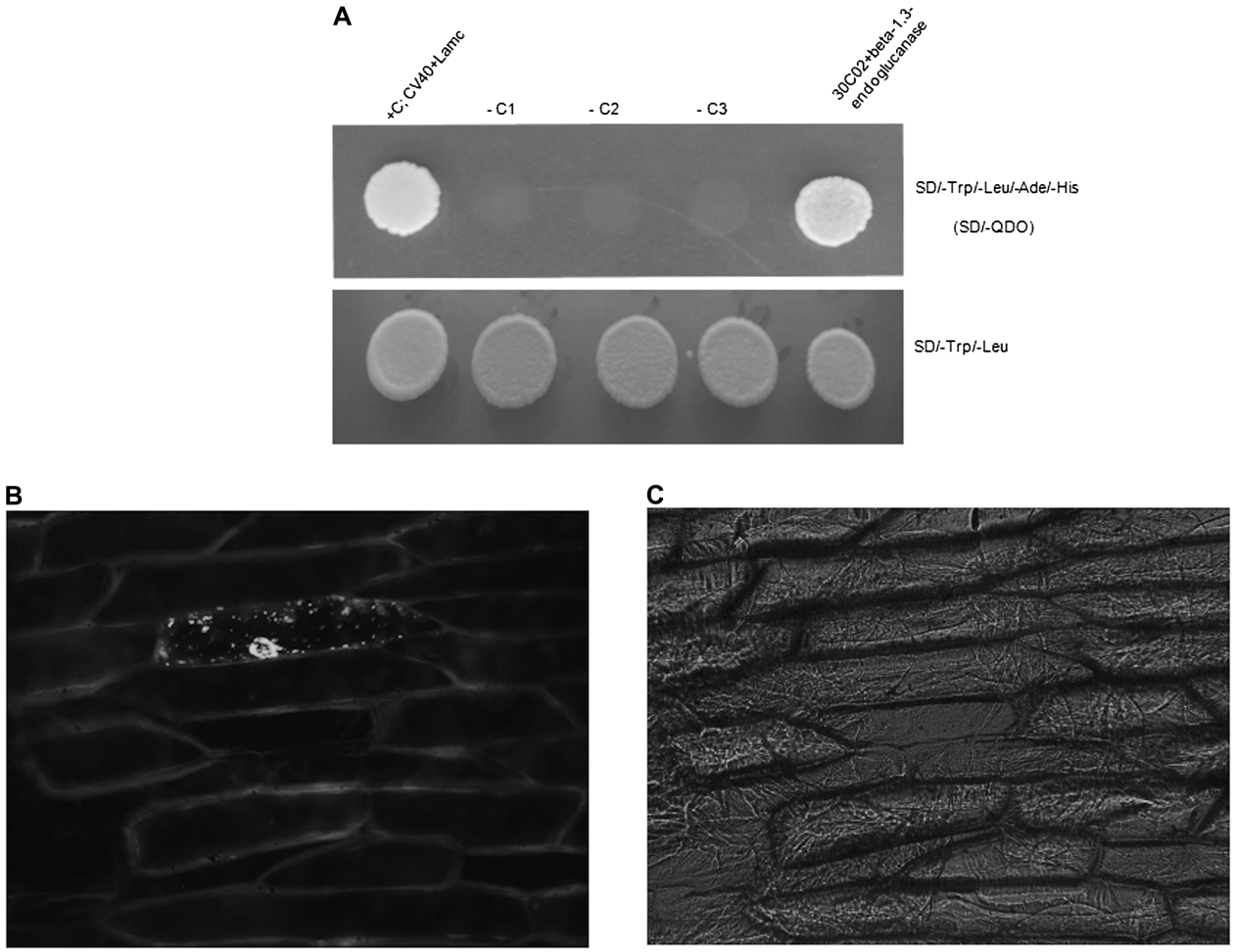

Fig. 5. Protein–protein interactions of Hs30C02 with the *Arabidopsis* β-1,3-endoglucanase (AT4G16260) in yeast and onion cells. (A) Yeast two-hybrid interaction between Hg30C02 and AT4G16260 *Arabidopsis* β-1,3-endoglucanase visualized by differential growth on non-selective medium (SD/–Trp/–Leu) and on selective medium (SD/–DQO). Only yeast cells containing the β-1,3-endoglucanase prey plus the 30C02 bait (upper right) or the positive control (+C) interaction of CV40 plus LamC (upper left) grew on the selective medium. No interaction of the *Arabidopsis* β-1,3-endoglucanase with the empty vector control (–C1) or LamC (–C2), or interaction of 30C02 with empty vector control (–C3), was detected on the selective medium. (B) The interaction between Hg30C02 and AT4G16260 *Arabidopsis* β-1,3-endoglucanase proteins within a transformed onion epidermal cell visualized by bifluorescence complementation. (C) A bright-field image of the onion epidermal cells presented in (B). The images in (B) and (C) were taken 24 h after transformation of onion cells by particle bombardment. (This figure is available in colour at *JXB* online.)

qRT-PCR to measure the transcript levels of *At4g16260* β-1,3-endoglucanase in the roots of *Arabidopsis* plants at 5, 9, and 14 d after nematode infection. We observed that expression of *At4g16260* was increased at 5 d p.i. and decreased thereafter (Fig. 6A). However, this analysis reflected the whole root (and possibly systemic) response and did not reflect changes at the nematode feeding sites. To address this question, we used qRT-PCR to measure the transcript levels of *At4g16260* in excised nematode feeding sites at 5, 9, and 14 d p.i. The results indicated that expression of the *At4g16260* β-1,3-endoglucanase gene increased significantly to a maximum at 5 d p.i. and was downregulated by 9 and 14 d p.i. (Fig. 6B), suggesting that during the early stages of nematode infection, the plant increased expression of AT4G16260 β-1,3-endoglucanase as a potential defence response.

The role of AT4G16260 β-1,3-endoglucanase in nematode infection

To investigate the role of AT4G16260 β-1,3-endoglucanase in *H. schachtii* parasitism of *Arabidopsis* roots, homozygous transgenic *Arabidopsis* plants that constitutively expressed the *At4g16260* β-1,3-endoglucanase cDNA and a homozygous *Arabidopsis* β-1,3-endoglucanase *At4g16260* T-DNA mutant line (Salk_031479) that does not express the β-1,3-endoglucanase were assayed for infection by *H. schachtii*. Constitutive overexpression of the *At4g16260* cDNA was confirmed by RT-PCR, and the homozygous *At4g16260* T-DNA mutant line (Salk_031479) was confirmed by genomic PCR of the T-DNA insert (data not shown). The results indicated that plants that overexpressed the *At4g16260* β-1,3-endoglucanase cDNA were less susceptible to nematode

infection and had fewer developed cyst females than WT plants (Fig. 7A). In contrast, the *At4g16260* β-1,3-endoglucanase T-DNA knockout line exhibited increased susceptibility to *H. schachtii* infection compared with WT plants (Fig. 7B). Transgenic plants that overexpressed *At4g16260* β-1,3-endoglucanase and T-DNA knockout lines did not show any apparent phenotypic variation compared with WT plants.

Host-derived RNAi of Hg30C02

To examine whether 30C02 was critical for *H. schachtii* parasitism, plant host-derived RNAi (reviewed by Gheysen and Vanholme, 2007; Rosso *et al.*, 2009) was used to silence expression of the *Hg30C02* parasitism gene within the nematode, and the subsequent effect on nematode parasitism was evaluated. *Arabidopsis* lines harbouring the *Hg30C02* RNAi constructs were confirmed for constitutive expression of the *30C02* dsRNA using RT-PCR amplification of the PDK intron (Fig. 8A) present in the pHANNIBAL vector (Wesley *et al.*, 2001). No plant morphological differences were observed between transgenic *Arabidopsis 30C02*-RNAi lines and the non-transformed control plants or plants transformed with an RNAi (dsRNA) construct of GFP (a non-nematode gene). Transgenic plants that expressed RNAi constructs were inoculated with *H. schachtii*, and at 3–4 weeks post-inoculation, a decrease of up to 92% in the number of females was observed in *Hg30C02* dsRNA-expressing lines compared with the control plants (Fig. 8B), indicating that 30C02 is an essential effector protein for parasitism by cyst nematodes.

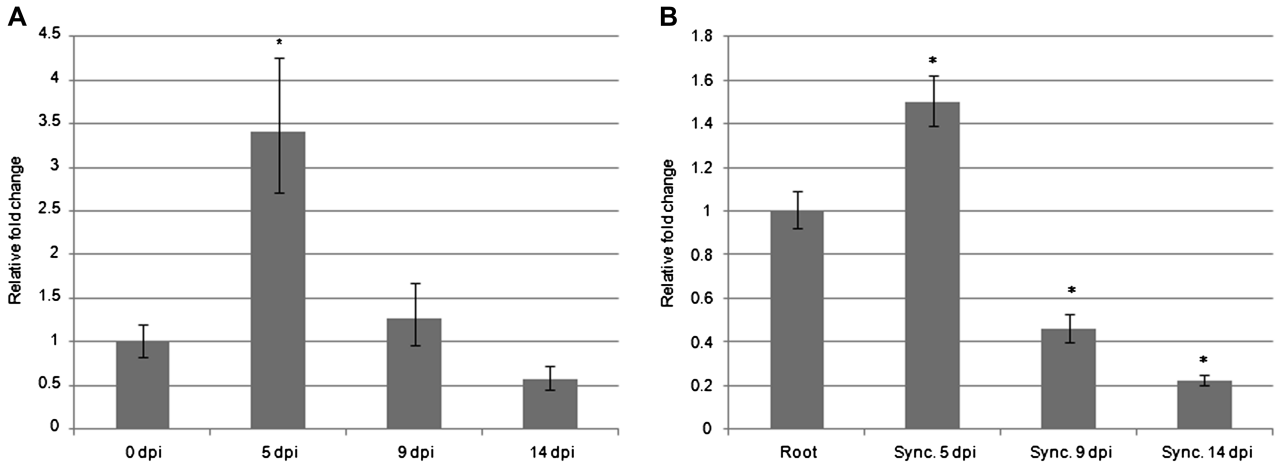

Fig. 6. Relative expression of *At4g16260* β-1,3-endoglucanase in WT (Col-1) *Arabidopsis* roots peaked at 5 d after infection by the beet cyst nematode *H. schachtii*. (A) qRT-PCR of *At4g16260* β-1,3-endoglucananse in whole WT *Arabidopsis* root systems at 5, 9, and 14 d p.i. (dpi) after infection with *H. schachtii*. (B) Expression of *At4g16260* β-1,3-endoglucananse in *H. schachtii* feeding sites (syncytia) excised from whole roots at the same time points. The relative fold-change values were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and represent changes in mRNA level relative to 0 d p.i. The *A. thaliana* actin-8 gene (ATU42007) was used as an internal control to normalize gene expression levels for all samples.

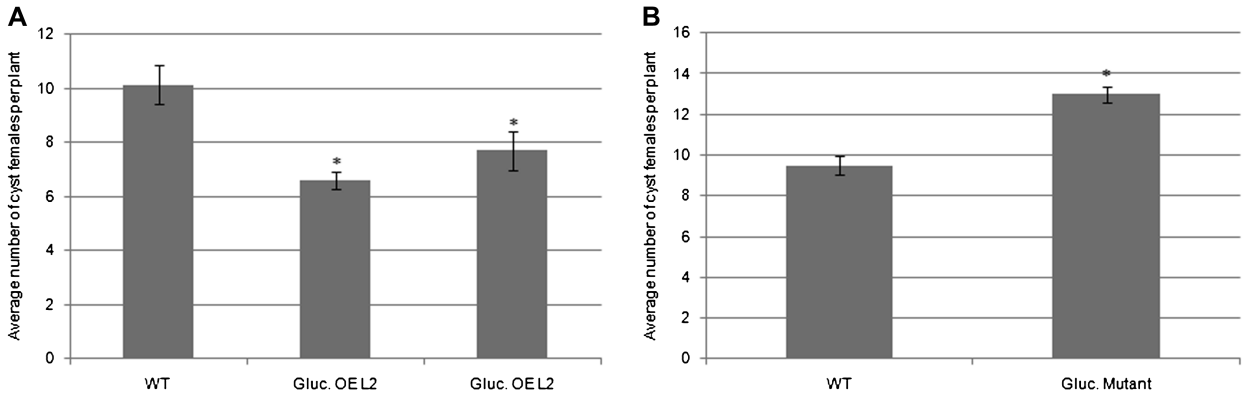

Fig. 7. Effect of *At4g16260* β-1,3-endoglucanase overexpression in transgenic *Arabidopsis* and an *At4g16260* T-DNA knockout mutant on *H. schachtii* infection of *Arabidopsis* roots. (A) Two transgenic *Arabidopsis* lines that constitutively overexpressed (OE) *At4g16260* showed significantly reduced infection by *H. schachtii*. (B) An *At4g16260* T-DNA knockout mutant (Salk_031479) showed enhanced susceptibility to *H. schachtii*. Constitutive overexpression of the *At4g16260* cDNA was confirmed by RT-PCR, and the homozygous *At4g16260* T-DNA mutant line (Salk_031479) was confirmed by genomic PCR of the T-DNA insert (data not shown). Data are presented as means ±SE. Mean values that were significantly different ($P<0.05$) from WT as determined by paired *t*-test are denoted by asterisks.

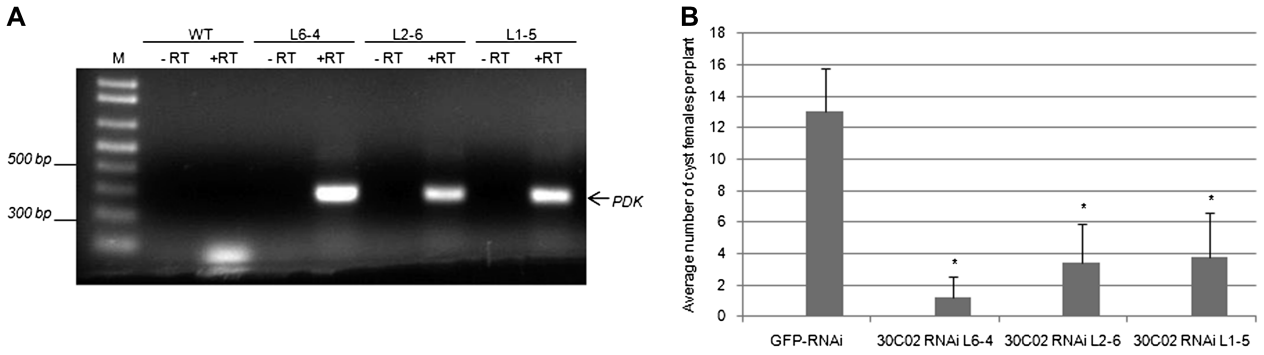

Fig. 8. Effect of *Arabidopsis*-derived RNAi of *30C02* on *H. schachtii* parasitism. (A) RT-PCR of the single-stranded PDK intron (Wesley *et al*., 2001) of the hairpin *30C02* dsRNA was used to confirm expression of *35S*::*Hg30C02* dsRNA and *35S*::*GFP* dsRNA (not shown) constructs in the roots of three independent transgenic *Arabidopsis* lines (L6-4, L2-6, and L1-5). Results are shown for RT-PCR without (-RT) or with (+RT) reverse transcriptase. (B) Transgenic *Arabidopsis* lines that expressed *30C02* dsRNA showed significant resistance to *H. schachtii* infection, as evidenced by the low number of females per plant developed in transgenic plants at 3 weeks post-inoculation compared with the plants that expressed the GFP dsRNA control construct. Bars indicate means ±SE (n=24). Asterisks represents a significant difference (t-test, $P \leqslant 0.05$) compared with the GFP RNAi control.

To relate the observed response of nematodes on *30C02* RNAi plants to the production of corresponding small interfering RNA (siRNA), the *30C02* small-RNA profile in roots of *30C02* RNAi plants was assayed using Illumina sequencing by the method of Tuteja *et al.* (2009). Of 11.2 million raw sequence reads, 1298 unique small-RNA sequences with lengths of 19–25 nt and a total abundance of 39 522 occurrences could be aligned to the 492 bp *Hg30C02* sequence with 100% identity (Fig. 9A). The 21 nt class formed 75% of the total small RNAs that matched the *30C02* target (Fig. 9B). As shown in Fig. 9A, these sequences matched both the positive and negative strands, as typically found for siRNAs generated from dsRNA by the RNAi pathway, similar to findings in other systems (de Paoli *et al.*, 2009; Tuteja *et al.*, 2009). The abundance of siRNA concentration was relatively even across the span of the *Hg30C02* coding sequence, with only a few foci demonstrating a modest increase in relative siRNA accumulation (Fig. 9A).

Discussion

Cyst nematodes employ an array of effector proteins to invade and induce feeding sites in host plant roots (Davis *et al.*, 2008; Gheysen and Mitchum, 2011). These proteins are produced within the nematode oesophageal gland cells and contain predicted signal peptides for secretion from the gland cells into host root cells via the nematode stylet. The *Hg30C02* parasitism gene of *H. glycines* encodes a putative novel effector protein with a predicted signal peptide for secretion into host cells (Gao *et al.*, 2003). A DNA gel blot assay confirmed the presence of several potential *30C02* family members in the *H. schachtii* genome, but only a single form of *30C02* was expressed in the dorsal oesophageal gland secretory cell of the parasitic stages of both *H. schachtii* and *H. glycines*. Expressed *Hs30C02* (JF896102) shared 98% predicted amino acid identity with Hg30C02 (JF896103). Thus, the two were considered as homologues and the *Arabidopsis–H.schachtii* pathosystem could therefore be used to study the role of Hg30C02 in nematode parasitism. The spike in expression of *30C02* in parasitic cyst nematode J2 at 3–5 d p.i. suggested a primary role of this effector protein in the early stages on the host–parasite interaction as the feeding site is established.

Constitutive expression of the *30C02* gene in *Arabidopsis* plant did not produce any observable effect on host root or shoot growth, but it rendered plants more susceptible to infection by *H. schachtii*. The expressed *30C02* gene had no effect on parasitism by the root-knot nematode *M. incognita*, suggesting that 30C02 has a specific role in cyst nematode feeding site formation. Increased host susceptibility to *H. schachtii* infection following overexpression of nematode parasitism genes has been documented in previous studies (Hewezi *et al.*, 2008, 2010; Patel *et al.*, 2010), suggesting that an excess of some effector proteins can enhance a compatible host–parasite interaction. Modulation of plant stress responses (Patel *et al.*, 2010) and defence responses (Hewezi *et al.*, 2010) have been implicated in nematode parasitic success in the plants that overexpress specific cyst parasitism genes. In a previous study, we showed that expression levels of *PR1*, *PR2*, and *PR5*, which are often used as markers for salicylic acid-dependent systemic acquired resistance, increased in *Arabidopsis* roots at 9 d after infection with *H. schachtii*, while expression level of *PR3*, *PR4*, and *PDF1.2*, which are commonly used as markers for characterization of jasmonate-dependent defence responses, did not change (Hamamouch *et al.*, 2011). The expression level of any of these *PR* genes did not change in roots of plants that overexpressed *Hg30C02*. Similarly, expression of *PAD4* and *ISC*, which are involved in synthesis of the phytoalexin camalexin and salicylic acid, respectively, did not change in plants that overexpressed *30C02*. These observations suggested that the 30C02 effector protein may target the activity of plant proteins involved in the host response, rather than the direct expression of host defence genes.

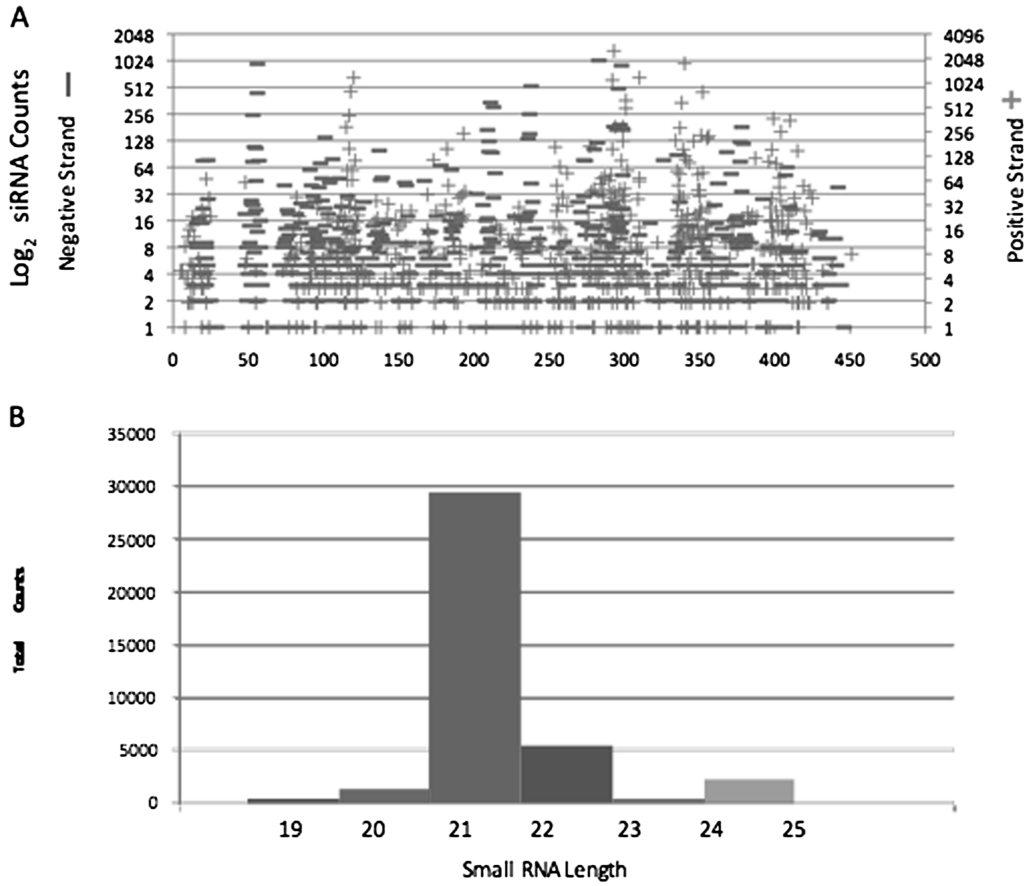

Fig. 9. Representation of abundance and alignment of siRNAs generated in *Arabidopsis* roots of plants transformed with the *30C02* RNAi constructs. (A) Counts and positions of all small RNAs with lengths of 19–25 nt that had a 100% match to either the positive (+) or negative (–) strands of the 492 bp *30C02* coding sequence are shown. Note the relatively even abundance of +/– siRNAs along the entire *30C02* coding sequence. Data represent the pooled small-RNA populations from the three independently transformed *35S*::*Hg30C02* dsRNA plant lines (L2-6, L1-5, and L6-4) used in Fig. 8. Small-RNA sequencing data from the roots of *Arabidopsis* lines transformed with other constructs, but not *30C02*, served as controls and contained very few sequences with 100% matches when aligned to the *30C02* sequences, indicating no significant similarity of *30C02* to the naturally occurring small RNAs in *Arabidopsis* seedling roots. (B) Abundance of each *30C02* small-RNA sequence with sizes of 19–25 nt showing a predominance of 21 nt small RNAs generated in the roots of the pooled *35S*::*Hg30C02* dsRNA *Arabidopsis* lines L2-6, L1-5, and L6-4. (This figure is available in colour at *JXB* online.)

In fact, yeast two-hybrid and bifluorescence complementation assays revealed that 30C02 specifically interacts with *Arabidopsis* AT4G16260 β-1,3-endoglucanase at the protein level. β-1,3-Endoglucanases are a class of hydrolytic enzyme that catalyse the cleavage of 1,3-β-D-glucosidic linkages in β-1,3-glucans (Lashbrook and Cai, 2008). They have received a considerable amount of attention due to their role in plant pathogen defence (van Loon *et al.*, 2006). As members of the PR2 group of PR proteins, β-1,3-endoglucanases are induced by pathogen infection and play an active role in hydrolysing β-1,3-glucan, a major structural component of fungal cell walls (Leubner-Metzger and Meins, 1999). For phytoparasitic nematodes, plant β-1,3-glucan deposition (callose) has been detected around the nematode stylet, syncytial cell walls and neighbouring plant cell walls (Hussey *et al.*, 1992; Grundler *et al.*, 1997). The AT4G16260 endoglucanase is active in cell-wall expansion in etiolated hypocotyls of *Arabidopsis* (Irshad *et al.*, 2008) and may play a similar role in developing syncytial cell walls. It is also possible that the nematode interacts with AT4G16260 β-1,3-endoglucanase to control callose formation or to limit the generation of signal molecules that may function as elicitors of defence responses. The highest levels of *30C02* expression in the nematode coincided with the highest levels of *At4g16260* β-1,3-endoglucanase expression during the plant–nematode interaction, suggesting a developmental mechanism for increased levels of 30C02 protein to counter the effects of increased AT4G16260 to promote successful parasitism in the early stages (3–5 d p.i.) of infection by cyst nematodes.

Studies have shown that expression of β-1,3-endoglucanase genes increases following attacks by several fungal pathogens (Doxey *et al.*, 2007), oomycete (Mahalingam *et al.*, 2003) and bacteria (Mahalingam *et al.*, 2003). Microarray analysis of *Arabidopsis* endoglucanases expressed in response to pathogens indicated that the *At4g16260* β-1,3-endoglucanase identified in this study displayed the most significant response

to fungal pathogens and therefore was most likely to play a defence role (Doxey *et al.*, 2007). The specific plant β-1, 3-endoglucanase affected and its timing of expression appear to be critical considerations, however, with respect to the plant–nematode interaction described here. The *At3g57260* β-1,3-endoglucanse (*PR2*) gene was recently found to spike in expression at 9 d after infection of *Arabidopsis* roots by *H. schachtii* (Hamamouch *et al.*, 2011), but, as mentioned above, expression of that same *PR2* gene was not influenced here by 30C02 overexpression in plants. Although upregulated at 5 d p.i., expression of the (30C02-interacting) *At4g16260* β-1,3-endoglucanase was greatly reduced in roots by 9 d after infection with *H. schachtii*, especially within syncytia. This latter finding is further supported by microarray analysis of *Arabidopsis* genes expressed following infection by *H. schachtii*, which indicated that the expression level of the *At4g16260* β-1,3-endoglucanase gene is downregulated in the syncytium by 15 d p.i. (Szakasits *et al.*, 2009).

Reduced infection by *H. schachtii* in *Arabidopsis* plants that overexpressed *At4g16260* β-1,3-endoglucanase, and conversely, increased susceptibility to *H. schachtii* in the *At4g16260* β-1,3-endoglucanase T-DNA knockout line indicated that this host β-1,3-endoglucanase is an important component of host plant response to *H. schachtii*. The ability to silence expression of the gene encoding the 30C02-interacting protein on the nematode side presented further evidence that the function of this effector may be important to nematode parasitic success. Investigations of plant host-derived RNAi targeted to selected cyst nematode parasitism genes have reported both reduced nematode infection levels and silencing of the target transcript in the nematode (Huang *et al.*, 2006*a*; Patel *et al.*, 2008, 2010; Sindhu *et al.*, 2009). In this study, *Arabidopsis*-derived RNAi targeted against the *Hs30C02* gene strongly reduced infection by *H. schachtii*, suggesting that the *30C02* gene and its product play an essential role in plant parasitism by cyst nematodes. The abundance and diversity of the small-RNA population generated in the roots of *Arabidopsis* plants transformed with the *30C02* RNAi construct are consistent with siRNA phenomena that have been shown to have physiological effects *in planta*, including transgenic petunia lines that exhibit co-suppression (de Paoli *et al.*, 2009) and naturally occurring downregulation of seed coat colour in soybean (Tuteja *et al.*, 2009).

In conclusion, we have demonstrated a specific interaction of the cyst nematode 30C02 effector protein with the *Arabidopsis* AT4G16260 β-1,3-endoglucanase, and have shown that silencing of nematode *30C02* or an increase in expression of *At4g16260* significantly reduces plant root infection by cyst nematodes (and vice versa). These data suggest that cyst nematodes act at two levels to suppress the potentially defence-related effects of plant β-1, 3-endoglucanase by: (i) introducing 30C02 into host cells to physically interact with AT4G16260 β-1,3-endoglucanase to neutralize its activity in the early stages of parasitism; and (ii) directly or indirectly reducing the expression of *At4g16260* in nematode feeding sites in the later stages of infection to promote successful plant parasitism.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Forward and reverse primers used in quantitative real-time RT-PCR to assess *30C02* gene expression in the beet cyst nematode *H. schachtii*, expression of selected *A. thaliana* genes, and internal actin controls to normalize gene expression.

Acknowledgements

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References

Abad P, Gouzy J, Aury JM, *et al*. 2008. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nature Biotechnology* **26,** 909–915.

Bendtsen JD, Jensen LJ, Blom N, von Heijne G, Brunak S. 2004. Feature-based prediction of non-classical and leaderless protein secretion. *Protein Engineering Design & Selection* **17,** 349–356.

Chen PY, Wang CK, Soong SC, To KY. 2003. Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. *Molecular Breeding* **11,** 287–293.

Citovsky V, Lee LY, Vyas S, Glick E, Chen MH, Vainstein A, Gafni Y, Gelvin SB, Tzfira T. 2006. Subcellular localization of interacting proteins by bimolecular fluorescence complementation *in planta*. *The Plant Journal* **362,** 1120–1131.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Davis EL, Hussey RS, Baum TJ, Bakker J, Schots A, Rosso MN, Abad P. 2000. Nematode parasitism genes. *Annual Review of Phytopathology* **38,** 365–396.

Davis EL, Hussey RS, Mitchum MG, Baum TJ. 2008. Parasitism proteins in nematode–plant interactions. *Current Opinion in Plant Biology* **11,** 360–366.

de Boer JM, Yan YT, Wang XH, Smant G, Hussey RS, Davis EL, Baum TJ. 1999. Developmental expression of secretory β-1, 4-endoglucanases in the subventral esophageal glands of *Heterodera glycines*. *Molecular Plant–Microbe Interactions* **12,** 663–669.

de Paoli E, Dorantes-Acosta A, Zhai J, Accerbi M, Jeong D-H, Park S, Meyers BC, Jorgensen RA, Green PJ. 2009. Distinct extremely abundant siRNAs associated with cosuppression in petunia. *RNA* **15,** 1965–1070.

Ding X, Shields J, Allen R, Hussey RS. 1998. A secretory cellulose-binding protein cDNA cloned from the root-knot nematode (*Meloidogyne incognita*). *Molecular Plant-Microbe Interactions* **11,** 952–959.

Doxey AC, Yaish MWF, Moffatt BA, Griffith M, McConkey BJ. 2007. Functional divergence in the Arabidopsis β-1,3-glucanase gene family inferred by phylogenetic reconstruction of expression states. *Molecular Biology & Evolution* **24,** 1045–1055.

Endo B. 1991. Ultrastructure of initial responses of susceptible and resistant soybean roots to infection by *Heterodera glycines*. *Revue de Nematologie* **14,** 73–94.

Finer JJ, Vain P, Jones MW, McMullen MD. 1992. Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Reports* **11,** 323–328.

Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mell CC. 1998. Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. *Nature* **391,** 806–811.

Gao BL, Allen R, Maier T, Davis EL, Baum TJ, Hussey RS. 2001. Identification of putative parasitism genes expressed in the esophageal gland cells of the soybean cyst nematode *Heterodera glycines*. *Molecular Plant–Microbe Interactions* **14,** 1247–1254.

Gao BL, Allen R, Maier T, Davis EL, Baum TJ, Hussey RS. 2003. The parasitome of the phytonematode *Heterodera glycines*. *Molecular Plant–Microbe Interactions* **16,** 720–726.

Gheysen G, Mitchum MG. 2011. How nematodes manipulate plant development pathways for infection. *Current Opinion in Plant Biology* **14,** 1–7.

Gheysen G, Vanholme B. 2007. RNAi from plants to nematodes. *Trends in Biotechnology* **25,** 89–92.

Gleave AP. 1992. A versatile binary vector system with a T-DNA organizational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Molecular Biology* **20,** 1203–1207.

Goellner M, Wang XH, Davis EL. 2001. Endo-β-1,4-glucanase expression in compatible plant–nematode interactions. *Plant Cell* **13,** 2241–2255.

Grundler FMW, Sobczak M, Lange S. 1997. Defence responses of *Arabidopsis thaliana* during invasion and feeding site induction by the plant-parasitic nematode *Heterodera glycines*. *Physiological and Molecular Plant Pathology* **50,** 419–429.

Hamamouch N, Li C, Seo PJ, Park CM, Davis EL. 2011. Expression of Arabidopsis pathogenesis-related genes during nematode infection. *Molecular Plant Pathology* **12,** 355–364.

Hewezi T, Howe PJ, Maier TR, Hussey RS, Mitchum MG, Davis EL, Baum TJ. 2008. Cellulose binding protein from the parasitic nematode *Heterodera schachtii* interacts with *Arabidopsis* pectin methylesterase: cooperative cell wall modification during parasitism. *Plant Cell* **20,** 3080–3093.

Hewezi T, Howe PJ, Maier TR, Hussey RS, Mitchum MG, Davis EL, Baum TJ. 2010. Arabidopsis spermidine synthase is targeted by an effector protein of the cyst nematode *Heterodera schachtii*. *Plant Physiology* **152,** 968–984.

Huang GZ, Allen R, Davis EL, Baum TJ, Hussey RS. 2006*a*. Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *Proceedings of the National Academy of Sciences U S A* **103,** 14302–14306.

Huang GZ, Dong RH, Allen R, Davis EL, Baum TJ, Hussey RS. 2006*b*. A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. *Molecular Plant–Microbe Interactions* **19,** 463–470.

Hussey RS. 1989. Disease-inducing secretions of plant-parasitic nematodes. *Annual Review of Phytopathology* **27,** 123–141.

Hussey RS, Barker KR. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp. *Plant Disease Reporter* **57,** 1025–1028.

Irshad M, Canut H, Borderies G, Pont-Lezica R, Jamet E. 2008. A new picture of cell wall protein dynamics in elongating cells of *Arabidopsis thaliana*: confirmed actors and newcomers. *BMC Plant Biology* **8, 94**.

Jones MGK. 1981. Host-cell responses to endo-parasitic nematode attack - structure and function of giant-cells and syncytia. *Annals of Applied Biology* **97,** 353–372.

Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10,** R25.

Lashbrook CC, Cai S. 2008. Cell wall remodeling in Arabidopsis stamen abscission zones. *Plant Signaling and Behavior* **3,** 733–736.

Lee C, Chronis D, Kenning C, Peret B, Hewezi T, Davis EL, Baum TJ, Hussey RS, Bennett M, Mitchum MG. 2011. The novel cyst nematode effector protein 19C07 interacts with the Arabidopsis auxin influx transporter LAX3 to control feeding site development. *Plant Physiology* **155,** 866–880.

Leubner-Metzger G, Meins FJ. 1999. Functions and regulation of plant β-1,3-glucanase (PR-2). In: Datta SK, Muthukrishnan S, eds. *Pathogenesis-related proteins in plants*. CRC Press, Boca Raton, FL, 49–76.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25,** 402–408.

Mahalingam R, Gomez-Buitrago A, Eckardt N, Shah N, Guevara-Garcia A, Day P, Raina R, Fedoroff NV. 2003. Characterizing the stress/defense transcriptome of *Arabidopsis*. *Genome Biology* **4,** R20.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Niblack TL, Lambert KN, Tylka GL. 2006. A model plant pathogen from the kingdom Animalia: *Heterodera glycines*, the soybean cyst nematode. *Annual Review Phytopathology* **44,** 283–303.

Opperman CH, Bird DM, Williamson VM, *et al*. 2008. Sequence and genetic map of *Meloidogyne hapla*: a compact nematode genome for plant parasitism. *Proceedings of the National Academy of Sciences U S A* **105,** 14802–14807.

Patel N, Hamamouch N, Li C, Hewezi T, Hussey RS, Baum TJ, Mitchum MG, Davis EL. 2010. A nematode effector protein similar to annexins in host plants. *Journal of Experimental Botany* **61,** 235–248.

Patel N, Hamamouch N, Li C, Hussey R, Mitchum M, Baum T, Wang XH, Davis EL. 2008. Similarity and functional analyses of expressed parasitism genes in *Heterodera schachtii* and *Heterodera glycines*. *Journal of Nematology* **40,** 299–310.

Rehman S, Postma W, Tytgat T, *et al*. 2009. A secreted SPRY domain-containing protein (SPRYSEC) from the plant-parasitic

nematode *Globodera rostochiensis* interacts with a CC-NB-LRR protein from a susceptible tomato. *Molecular Plant–Microbe Interactions* **22,** 330–340.

Replogle A, Wang J, Bleckmann A, Hussey R, Baum T, Sawa S, Davis E, Wang X, Simon R, Mitchum M. 2011. Nematode CLE signaling in Arabidopsis requires CLAVATA2 and CORYNE. *The Plant Journal* **65,** 430–440.

Rosso MN, Jones JT, Abad P. 2009. RNAi and functional genomics in plant parasitic nematodes. *Annual Review of Phytopathology* **47,** 207–232.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sijmons PC, Grundler FMW, von Mende N, Burrows PR, Wyss U. 1991. *Arabidopsis thaliana* as a new model for plant-parasitic nematodes. *The Plant Journal* **1,** 245–254.

Sindhu AS, Maier TR, Mitchum MG, Hussey RS, Davis EL, Baum TJ. 2009. Effective and specific *in planta* RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success. *Journal of Experimental Botany* **60,** 315–324.

Subbotin SA, Vierstraete A, De Ley P, Rowe J, Waeyenberge L, Moens M, Vanfleteren JR. 2001. Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Molecular Phylogenetics & Evolution* **21,** 1–16.

Szakasits D, Heinen P, Wieczorek K, Hofmann J, Wagner F, Kreil DP, Sykacek P, Grundler FMW, Bohlmann H. 2009. The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in Arabidopsis roots. *The Plant Journal* **57,** 771–784.

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673–4680.

Tuteja J, Zabala G, Varala K, Hudson M, Vodkin L. 2009. Endogenous, tissue specific short interfering RNAs silence the chalcone synthase gene family in *Glycine max* seed coats. *Plant Cell* **21,** 3063–3077.

Vain P, Keen N, Murillo J, Rathus C, Nemes C, Finer JJ. 1993. Development of the particle inflow gun. *Plant Cell Tissue & Organ Culture* **33,** 237–246.

van Loon LC, Rep M, Pieterse CMJ. 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology* **44,** 135–162.

Wang J, Lee C, Replogle A, Joshi S, Korkin D, Hussey RS, Baum TJ, Davis EL, Wang X, Mitchum MG. 2010. Dual roles for the variable domain in protein trafficking and host-specific recognition of *Heterodera glycines* CLE effector proteins. *New Phytologist* **187,** 1003–1017.

Wang J, Replogle A, Hussey RS, Baum TJ, Wang X, Davis EL, Mitchum MG. 2011. Identification of potential host plant mimics of CLV3/ESR (CLE)-like peptides from the plant parasitic nematode *Heterodera schachtii*. *Molecular Plant Pathology* **12,** 177–186.

Wang XH, Allen R, Ding XF, Goellner M, Maier T, De Boer JM, Baum TJ, Hussey RS, Davis EL. 2001. Signal peptide-selection of cDNA cloned directly from the esophageal gland cells of the soybean cyst nematode *Heterodera glycines*. *Molecular Plant–Microbe Interactions* **14,** 536–544.

Wang XH, Mitchum MG, Gao B, Li C, Diab H, Baum TJ, Hussey RS, Davis EL. 2005. A parasitism gene from a plant parasitic nematode with function similar to CLAVATA3/ESR (CLE) of *Arabidopsis thaliana*. *Molecular Plant Pathology* **6,** 187–191.

Wesley SV, Helliwell CA, Smith NA, *et al*. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant Journal* **27,** 581–590.

Williamson VM, Hussey RS. 1996. Nematode pathogenesis and resistance in plants. *Plant Cell* **8,** 1735–1745.

Wrather JA, Koenning SR. 2006. Estimates of disease effects on soybean yields in the United States 2003 to 2005. *Journal of Nematology* **38,** 173–180.

Wyss U, Zunke U. 1986. Observations on the behavior of second-stage juveniles of *Heterodera schachtii* inside host root. *Revue de Nematologie* **9,** 153–165.