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 defense 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; Wrath 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, digoxigenin; 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.
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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., 2006b; 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., 2006a; 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., 2006b; 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 EcoRI, 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 digoxigenin (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’-ATGAGGAAAACCTCCATTGGC-3’ and 3’-CGTTTCAACTTCGAAGAGTCC-5’).
M. incognita and H. glycines are present in five copies of 30C02. M. incognita and the treated specimens were observed by light microscopy. Detected with alkaline phosphatase-conjugated anti-DIG antibody, cDNA probes. Hybridization signals within the nematodes were indicated by asterisks. 1994); the underlined sequence represents the predicted secretion signal peptide motif (Bendtsen 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.

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 indicated by asterisks. 1994); the underlined sequence represents the predicted secretion signal peptide motif (Bendtsen 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 indicated by asterisks.

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 SacII and SacI, and subcloned into pBlack plasmid digested with SacII and SacI. The 35S promoter was excised from pBI121 using HindIII and BamHI, and then subcloned into pBlack plasmid upstream of the 30C02 coding sequence. The identity, orientation, and junctions of the resulting 3SS::30C02 construct were confirmed by PCR and sequencing. The 3SS-GUS gene of pBI121 plasmid (Chen et al., 2003) was digested with HindIII and SacI, and replaced with the 3SS::30C02 construct resulting in the pBl-30C02 vector.

For RNAi vectors, full-length Hg30C02 was isolated from pGEM-T Easy by EcoRI restriction digestion and subcloned in the antisense orientation in pHANNIBAL vector (Wesley et al., 2001) digested with EcoRI enzyme. The sense strand of 30C02 was amplified using the primers 5′-TAGATCCATTGCCACCCAGTTATTCTCCC-3′ and 5′-ATTACTAGGGATTGGATTTCTCTCC-3′, which introduced HindIII and XbaI 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 NsiI and cloned into the pART27 binary vector (Gleave, 1992) for subsequent Agrobacterium and plant transformation, resulting in pART27-30C02 and pART27-GFP, respectively.

Generation of transgenic Arabidopsis plants

The binary vectors pART27-30C02, pART27-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-endoglucanase T-DNA mutant line (Salk_031479) was obtained.

Molecular cloning and vector construction

The binary vectors pART27-30C02, pART27-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-endoglucanase T-DNA mutant line (Salk_031479) was obtained.

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.

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-dT6 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−ΔΔCt 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 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 adapter sequence (ATCTCGATATGCCGTCTTCTGCTTG) and a representative result was displayed for individual assays. The RT-PCR was run for 35 cycles and consisted of 94°C for 1 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−ΔΔCt 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 online) and Hamamouch et al. (2011).

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’-TACTCGAGTGCAACCGAGAGGATAATTTCG-3’) and reverse primer (5’-GAAGCTTCTAAAAAAGTGTTCTGCTGGTGAAA-3’) containing Xhol and HindIII restriction sites (underlined), respectively, and cloned into Xhol and HindIII sites of pSAT4-cEYFP1B 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’-TACTCGAGTGGAACGGAAG GAAGTATATTTCGC-3’) and (5’-GAAGCTTCTACAACCGCGCTACCGTCT-3’) reverse primer containing Xhol and HindIII restriction sites (underlined), respectively, and cloned into Xhol and HindIII sites of pSAT4- nEYFPC1 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 EcoRI-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 Hg30C02 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<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 best cyst nematode H. schachtii determined by qRT-PCR. The relative fold-change values were calculated using the 2−ΔΔCT 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.
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-endoglucanase 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
qRT-PCR to measure the transcript levels of \textit{At4g16260} \textbeta-1,3-endoglucanase in the roots of \textit{Arabidopsis} plants at 5, 9, and 14 d after nematode infection. We observed that expression of \textit{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 \textit{At4g16260} in excised nematode feeding sites at 5, 9, and 14 d p.i. The results indicated that expression of the \textit{At4g16260} \textbeta-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 \textit{AT4G16260} \textbeta-1,3-endoglucanase as a potential defence response.

\textbf{The role of \textit{AT4G16260} \textbeta-1,3-endoglucanase in nematode infection}

To investigate the role of \textit{AT4G16260} \textbeta-1,3-endoglucanase in \textit{H. schachtii} parasitism of \textit{Arabidopsis} roots, homozygous transgenic \textit{Arabidopsis} plants that constitutively expressed the \textit{At4g16260} \textbeta-1,3-endoglucanase cDNA and a homozygous \textit{Arabidopsis} \textbeta-1,3-endoglucanase \textit{At4g16260} T-DNA mutant line (Salk_031479) that does not express the \textbeta-1,3-endoglucanase were assayed for infection by \textit{H. schachtii}. Constitutive overexpression of the \textit{At4g16260} cDNA was confirmed by RT-PCR, and the homozygous \textit{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 \textit{At4g16260} \textbeta-1,3-endoglucanase cDNA were less susceptible to nematode infection.
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-endoglucanase 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-endoglucanase 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^−ΔΔCT 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.
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).

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.
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
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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-endoglucanase (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 down-regulated 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., 2006a; 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.

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