Effective and specific in planta RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success

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Received 19 August 2008; Revised 25 October 2008; Accepted 27 October 2008

Abstract
Cyst nematodes are highly evolved sedentary plant endoparasites that use parasitism proteins injected through the stylet into host tissues to successfully parasitize plants. These secretory proteins likely are essential for parasitism as they are involved in a variety of parasitic events leading to the establishment of specialized feeding cells required by the nematode to obtain nourishment. With the advent of RNA interference (RNAi) technology and the demonstration of host-induced gene silencing in parasites, a new strategy to control pests and pathogens has become available, particularly in root-knot nematodes. Plant host-induced silencing of cyst nematode genes so far has had only limited success but similarly should disrupt the parasitic cycle and render the host plant resistant. Additional in planta RNAi data for cyst nematodes are being provided by targeting four parasitism genes through host-induced RNAi gene silencing in transgenic Arabidopsis thaliana, which is a host for the sugar beet cyst nematode Heterodera schachtii. Here it is reported that mRNA abundances of targeted nematode genes were specifically reduced in nematodes feeding on plants expressing corresponding RNAi constructs. Furthermore, this host-induced RNAi of all four nematode parasitism genes led to a reduction in the number of mature nematode females. Although no complete resistance was observed, the reduction of developing females ranged from 23% to 64% in different RNAi lines. These observations demonstrate the relevance of the targeted parasitism genes during the nematode life cycle and, potentially more importantly, suggest that a viable level of resistance in crop plants may be accomplished in the future using this technology against cyst nematodes.

Key words: beet cyst nematode (BCN), soybean cyst nematode (SCN), host induced, in planta RNAi, resistance, RNAi, transgenic.

Introduction
Plant-parasitic nematodes are devastating pathogens that infect most cultivated plant species and cause considerable loss of food and fibre crops in terms of both yield and quality. The total annual yield losses caused by plant-parasitic nematodes are estimated to be >US$125 billion worldwide (Chitwood, 2003). Sedentary endoparasitic phytonematodes are highly evolved pests and have developed unique ways to infect their hosts. These obligate parasites start their life cycles as non-feeding, mobile infective second-stage juveniles (J2) in soil that are able to locate and then penetrate into host roots. J2 migrate inside the root to reach the vascular cylinder, where they become sedentary and induce the formation of feeding cells from selected root cells. To support their sedentary life cycle, nematodes inject parasitism proteins into initial feeding cells, which alters normal root cell fate and results in a cell redifferentiation into...
metabolically active, multinucleate specialized feeding cells known as giant-cells (root-knot nematodes) or syncytia (cyst nematodes). The parasitism proteins appear to provide many of the stimuli that result in the dramatically altered host gene expression observed in nematode-infected plant roots (Gheysen and Fenoll, 2002; Putthoff et al., 2003; Jammes et al., 2005; Ithal et al., 2007a, b). Secreted nematode parasitism proteins, directly or indirectly, appear to modulate the host cell cycle (Gheysen and Fenoll, 2002), reorganize the cytoskeleton (Favery et al., 2004; Caillaud et al., 2008), regulate signalling and metabolic pathways (Favery et al., 1998; Doyle and Lambert, 2003), phytohormone balances (Wubben et al., 2001; Karczmarek et al., 2004), endoreplication (Niebel et al., 1996), plant growth and development (Wang et al., 2005; Huang et al., 2006a), and defence responses, and target protein degradation (Gao et al., 2003), or directly modify gene expression by entering the nucleus (Goverse et al., 2000; Gheysen and Fenoll, 2002; Jasmer et al., 2003; Tytgat et al., 2004; Baum et al., 2007; Elling et al., 2007a).

More than 50 parasitism genes have been identified in the soybean cyst nematode (SCN), Heterodera glycines (Gao et al., 2001; Wang et al., 2001). While some of the corresponding parasitism proteins show significant database similarities with known eukaryotic proteins, the majority are pioneer proteins whose functions are difficult to determine because protocols for reverse genetics approaches in phytoparasitic nematodes have been lacking (Baum et al., 2007). Understanding the role of individual parasitism proteins will provide insights into the mechanisms of parasitism and will assist in finding novel control mechanisms.

With the recent discovery of gene expression control via double-stranded RNA (dsRNA) molecules, biologists are exploring genes and development from a new perspective (Baulcombe, 2005). Understanding of this ubiquitous phenomenon has revealed RNA interference (RNAi) as a powerful tool to manipulate gene expression and analyse gene function (Fire et al., 2000; Gheysen and Vanholme, 2000; Lambert, 2003), phytohormone balances (Wubben et al., 2001; Karczmarek et al., 2004), endoreplication (Niebel et al., 1996), plant growth and development (Wang et al., 2005; Huang et al., 2006a), and defence responses, and target protein degradation (Gao et al., 2003), or directly modify gene expression by entering the nucleus (Goverse et al., 2000; Gheysen and Fenoll, 2002; Jasmer et al., 2003; Tytgat et al., 2004; Baum et al., 2007; Elling et al., 2007a).

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The success of the above in vitro dsRNA ingestion and down-regulation of the target genes inspired the in planta delivery of dsRNA to the feeding nematodes. Although both root-knot and cyst nematodes were found to be responsive to in vitro dsRNA uptake and down-regulation of the target gene, the most convincing success of in planta delivery of dsRNA to feeding nematodes came from root-knot nematodes (Huang et al., 2006b; Yadav et al., 2006). Huang et al. (2006b) reported significant reduction of gall formation by different root-knot nematode species on transgenic Arabidopsis plants expressing dsRNA for the parasitism gene 16D10, which is unique to root-knot nematodes. Similarly, tobacco RNAi lines expressing dsRNA for two root-knot nematode housekeeping genes (a splicing factor and an integrase gene) induced down-regulation of the target genes and reduced parasitic success (Yadav et al., 2006). Down-regulation of the putative transcription factor MjTis11 in the root-knot nematode Meloidogyne javanica by feeding on transgenic RNAi tobacco lines was also reported (Fairbairn et al., 2007), but there was no significant reduction in number of developing nematodes on transgenic plants. While these studies demonstrate the in planta delivery of dsRNA and its success in reducing the abilities of root-knot nematodes to colonize hosts, there are only very limited data available regarding in planta delivery of dsRNA to cyst nematodes. To date, Steeves et al. (2006) reported a reduction of developing SCN females using soybean plant-induced RNAi. That study demonstrated reduction in cyst formation and reduction in the number of eggs/cyst on transgenic plants by host-induced silencing of the major sperm protein gene. However, these data were criticized for being generated using only primary transgenic plants (T0) to screen for resistance and for being pooled from two different varieties with different degrees of susceptibility (Steeves et al., 2006; Gheysen and Vanholme, 2007). More recently, a virus-induced gene silencing approach (VIGS) has been used to attempt delivery of siRNA to feeding H. schachtii cyst nematodes (Valentine et al., 2007). The study did demonstrate the selective uptake of syncytium-expressed monomeric red fluorescent protein (mRFP) and down-regulation of the target gene, glyceraldehyde-3-phosphate dehydrogenase (GADPH), in the feeding cyst nematodes. However, this down-regulation was without effect on the nematode life cycle, and only a small fraction of nematodes could be demonstrated to take up the fluorescent protein. In the current report, it is clearly demonstrated that specific in planta RNAi against cyst nematode parasitism genes was accomplished and resulted in significant down-regulation of target genes and effectively reduced nematode parasitic success.

Materials and methods

Vector construction for RNAi knockout experiments

RNAi vector pHannibal (Wesley et al., 2001) was used to generate RNAi constructs. The sense and antisense cDNA sequences of the four nematode parasitism genes (Gao et al., 2001) 3B05 (AF469058), 4G06 (AF469060), 8H07

Materials and methods

Vector construction for RNAi knockout experiments

RNAi vector pHannibal (Wesley et al., 2001) was used to generate RNAi constructs. The sense and antisense cDNA sequences of the four nematode parasitism genes (Gao et al., 2001) 3B05 (AF469058), 4G06 (AF469060), 8H07
(AF500024), and 10A06 (AF502391) were amplified from full-length *H. glycines* cDNA clones and placed under the control of a constitutively expressing 35S promoter in pHannibal. The sense fragment was amplified using gene-specific primers having restriction sites XhoI (forward primer) and EcoRI (reverse primer) and inserted as an XhoI–EcoRI fragment into pHannibal. The antisense fragment was amplified using gene-specific primers having restriction sites XbaI (forward primer) and HindIII (reverse primer) and was inserted as an inverted fragment as HindIII–XbaI into pHannibal. All RNAi constructs were made using the same strategy except for gene 4G06 where a ClaI restriction site was used in the antisense reverse primer for amplification. The pHannibal vectors were subcloned at NotI sites into a binary vector pART27.

**Primer design for the target genes:** The gene regions were selected on the basis of regions either unique (URs) to that particular gene or conserved regions (CRs) across the gene families (Fig. 1). The regions and primers are shown in Table 1. The restriction sites used in the primers to facilitate the cloning of the PCR product into pHannibal vector are shown bold (XhoI, CTCGAG; EcoRI, GAATTC; XbaI, TCTAGA; HindIII, AAGCTT; ClaI, ATCGAT) (Table 1).

**PCR amplification:** The targeted region of the gene was amplified using an MJ Research PTC-100 (Water Town, MA, USA) thermal cycler. The PCR conditions included initial melting temperature of 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min. This was followed by a final extension time of 7 min at 72 °C. The PCR composition included 0.025 U/μl Taq polymerase (New England Biolabs, MA, USA) and Invitrogen (Carlsbad, CA, USA) reagents including 1.5 mM MgCl2 and 200 μM each of dATP, dTTP, dGTP, and dCTP nucleotides. The template plasmid DNA concentration was 1–10 ng μl⁻¹.

**DNA digestion, ligation, DNA minipreparation, and sequencing**

DNA was digested using restriction enzymes from New England Biolabs and Invitrogen employing protocols suggested by the manufacturers. Similarly, ligation of DNA molecules was performed essentially as described by the manufacturer (Invitrogen) using T4 DNA ligase. Plasmid DNA minipreparation was done using Qiagen kits (QIAgen, Valencia, CA, USA) following the instructions described by the manufacturers. All constructs were confirmed by DNA sequencing at the Iowa State University DNA Synthesis and Sequencing facility.

**Generation of transgenic RNAi plants**

The binary vector was mobilized into chemical-competent *Agrobacterium* strain C58 using a standard heat shock method and plated on LB medium containing kanamycin (50 μg ml⁻¹). Arabidopsis plants were transformed using the floral dip method following the method described by Clough and Bent (1998).

**Segregation analysis and obtaining homozygous lines**

A total of 300–500 seeds from 10 individual plants from each construct were planted on selectable medium [MS medium (Murashige and Skoog, 1962) supplemented with 500 mg l⁻¹ MES, 0.1% sucrose (pH 5.7), solidified with 8 g l⁻¹ phytoagar (Research Products International, Mt Prospect, IL, USA)]. Petri dishes were incubated at 25 °C with a 16/8 h light/dark cycle. Kanamycin-resistant plants growing on selectable medium were carefully removed and planted in pots containing a soil mixture and then transferred to a growth room for further growing till maturity. T1 seeds were collected from individual plants and plated on selectable medium to analyse further the segregation of the resistant phenotype. T2 kanamycin-resistant seeds were harvested from non-segregating families and further plated to confirm the kanamycin-resistant phenotypes. T3 homozygous lines were used in all assays described here.

**Characterization of homozygous transgenic plants**

Homozygous T3 plants were characterized using reverse transcription-PCR (RT-PCR) and siRNA northern analysis. Total RNA was isolated using the protocols as described by Verwoerd et al. (1989). The primers designed from the intron of the RNAi construct were used to amplify introns from the pre-processed mRNA. A northern blot was used to detect siRNAs in the transgenic plants for the genes used in RNAi experiments (Fig. 2). For northern analysis, 35 μg of total RNA was separated on a 15% pre-cast polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and electroblotted on a GeneScreen plus nylon membrane (Perkin Elmer, MA, USA) at 300 mA for 5 h. Starfire™ probes were designed and labelled essentially following the manufacturer’s instructions (Integrated DNA Technologies, Coralville, IA, USA). The sequences of probes are given in Table 2. Labelled probes were purified using a NucAway™

![Fig. 1. The regions of parasitism genes used in RNAi experiments. The conserved (CR) and unique regions (UR) of 8H07 are also shown. Numbers indicate nucleotide positions.](image-url)
spin column (Ambion, Austin, TX, USA). The electro-
blotted membranes were incubated at 35 °C for 1 h in pre-
hybridization buffer (7% SDS, 0.2 M Na2PO4 pH 7.0). The
purified probe was denatured at 95 °C for 4 min, snap-
cooled on ice for 30 s, and added to the hybridization tubes.
Each probe was hybridized overnight at 35 °C with slow
rotation of the tubes in a hybridization oven. Following
hybridization, membranes were washed three times at 35 °C
in 2× SSC and 0.1% SDS. Hybridized membranes were
exposed to phosphor imager screens for 72 h and then read
using a Bio-Rad PharosFx Plus Molecular Imager. For re-
probing, the hybridized membranes were stripped by adding
boiling 0.1% SDS and then letting it cool to room temperature.

**Total RNA extraction from nematodes and quantitative
real-time PCR**

Nematodes feeding on the roots of wild-type and transgenic
RNAi plants were isolated at 4 and 7 days post-infection by
macerating infected roots in a blender for 5 min followed by
sieving and separation on a sucrose gradient. The isolated
nematodes were immediately frozen in liquid nitrogen and
then stored at −80 °C. Total RNA was isolated using the
protocol described by Verwoerd et al. (1989). The isolated
total RNA was treated with DNase I.

**Real-time RT-PCR**

Transcript abundance of nematode parasitism genes was
analysed by quantitative real-time RT-PCR. Gene-specific
primers from regions outside of those used in RNAi target
regions were designed (Table 3). A 10 ng aliquot of DNase
I-treated RNA was used for cDNA synthesis and PCR
amplification using a one-step RT-PCR kit (Bio-Rad)
according to the manufacturer’s protocol. The PCRs were
run in an iCycler (Bio-Rad) using the following program:
50 °C for 10 min, 95 °C for 5 min, and 40 cycles of 95 °C for
30 and 60 °C for 30 s. Following PCR amplification, the
reactions were subjected to temperature ramping to create
the dissociation curve, measured as changes in fluorescence
measurements as a function of temperature, by which the
non-specific products can be detected. The dissociation
program was 95 °C for 1 min, 55 °C for 10 s followed by

---

**Table 1. The list of primer sequences used in amplifying the target genes**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences</th>
<th>Target PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10A06-CR-S-Forward</td>
<td>ATCTCGAGTTTGATGGCTGCTTATCACTGACCTT</td>
<td>103</td>
</tr>
<tr>
<td>10A06-CR-S-Reverse</td>
<td>ATGAATTCTTTTTTTTTAAAAACCCATCAACGCA</td>
<td></td>
</tr>
<tr>
<td>10A06-CR-AS-Forward</td>
<td>ATCTCTAGTTTGATGGCTTATCACTGACCTT</td>
<td></td>
</tr>
<tr>
<td>10A06-CR-AS-Reverse</td>
<td>ATAAAGCTTTTTTTTTAAAAACCCATCAACGCA</td>
<td>153</td>
</tr>
<tr>
<td>4G06-UR-S-Forward</td>
<td>ATCTCGAGGAAATGGGAAAGAGAAACA</td>
<td></td>
</tr>
<tr>
<td>4G06-UR-S-Reverse</td>
<td>ATGAATTCAAGATAACCCGGGAAAGG</td>
<td></td>
</tr>
<tr>
<td>4G06-UR-AS-Forward</td>
<td>ATCTCTAGGAAATGGGAAAGAGAAACA</td>
<td></td>
</tr>
<tr>
<td>4G06-UR-AS-Reverse</td>
<td>ATATCGATGAAATACCCGGGAAAGG</td>
<td></td>
</tr>
<tr>
<td>8H07-UR-S-Forward</td>
<td>ATCTCGAGCAGGAGTGGAGAGAGAA</td>
<td>82</td>
</tr>
<tr>
<td>8H07-UR-S-Reverse</td>
<td>ATGAATTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT</td>
<td></td>
</tr>
<tr>
<td>8H07-UR-AS-Forward</td>
<td>ATCTCTAGGAAATGGGAAAGAGAAACA</td>
<td></td>
</tr>
<tr>
<td>8H07-UR-AS-Reverse</td>
<td>ATATCGATGAAATACCCGGGAAAGG</td>
<td></td>
</tr>
<tr>
<td>8H07-CR-S-Forward</td>
<td>ATCTCGAGAAACCATATCCCAAATG</td>
<td>338</td>
</tr>
<tr>
<td>8H07-CR-S-Reverse</td>
<td>ATGAATTCTATTTGGTGTGATTTGGTGTGCTG</td>
<td></td>
</tr>
<tr>
<td>8H07-CR-AS-Forward</td>
<td>ATCTCTAGGAAACCATATCCCAAATG</td>
<td></td>
</tr>
<tr>
<td>8H07-CR-AS-Reverse</td>
<td>ATTCTCTAGGAAACCATATCCCAAATG</td>
<td></td>
</tr>
<tr>
<td>3B05-S-Forward</td>
<td>ATCTCGAGATGCTATTGGTTTTATTGGATTGGCTG</td>
<td>233</td>
</tr>
<tr>
<td>3B05-S-Reverse</td>
<td>ATGAATTCCGAAATGGACCCATTTT</td>
<td></td>
</tr>
<tr>
<td>3B05-AS-Forward</td>
<td>ATCTCTAGGAAATGGGAAAGAGAAACA</td>
<td></td>
</tr>
<tr>
<td>3B05-AS-Reverse</td>
<td>ATATCGATGAAATACCCGGGAAAGG</td>
<td></td>
</tr>
</tbody>
</table>

CR, conserved region; UR, unique region; S, sense; AS, antisense.

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**Fig. 2.** Homozygous 4G06 RNAi lines planted on modified Knob’s medium next to wild-type plants showing comparable growth of roots after 12 d of planting.
Table 2. List of probe sequences used for northern blotting

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>8H07QR</td>
<td>GGAAGAAAGGAG TGGGAAAGAT CCGACGAGGCA AGCTGAGGGA CGCCTATGCAA</td>
</tr>
<tr>
<td>4G06</td>
<td>CACCGGAAAG ACAAAGAAA GCAACAAAAA GCTTGATCAG AATGTAAGAG</td>
</tr>
<tr>
<td>10A06</td>
<td>ATGCATGCT ATATCCTAGAC CTTGAAATTG AGCAAGATC CTATCGCTTT</td>
</tr>
<tr>
<td>3B05</td>
<td>GTAACGCTAAGGTAACGAGTGAACAGTTGGAAAACAATGGAATAATGGAACACAATT</td>
</tr>
<tr>
<td>U6</td>
<td>TCA TCC TG TGG AGG GGG CA</td>
</tr>
</tbody>
</table>

Table 3. List of primers used in quantitative real time RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>8H07QF1</td>
<td>5'-ACA AAT GCA GCA ACA ACA GAA TCA TGG CAG-3'</td>
</tr>
<tr>
<td>8H07QR1</td>
<td>5'-CTT CCT CGC CAT TCA TCA TGC TG-3'</td>
</tr>
<tr>
<td>4G06QF</td>
<td>5'-TTGC CCA TTCTC AATT GCTTCTCCG-3'</td>
</tr>
<tr>
<td>4G06QR</td>
<td>5'-CGT CAA TGT CTT CAC GAA AAT TTG CAT GC-3'</td>
</tr>
<tr>
<td>3B05QF</td>
<td>5'-ATT TGA TGC ACA ATT AAC ACT GCT TGG-3'</td>
</tr>
<tr>
<td>3B05QR</td>
<td>5'-CAT TGT TTG GTG GGA ATA TTG TTC GTG-3'</td>
</tr>
</tbody>
</table>

Results

In preliminary studies, it was determined that *H. schachtii* homologues of *H. glycines* parasitism genes show a high degree of at least 90% nucleotide identity between these two species (Supplementary Fig. S1 available at JXB online). This observation allowed the previously reported *H. glycines* parasitism gene sequences (Gao et al., 2003) to be used directly for the transgenic RNAi approach using the *Arabidopsis thaliana–H. schachtii* pathosystem, which ensured timely progress without having to identify full-length *H. schachtii* sequences. The feasibility of this approach was confirmed by the high degree of sequence identity recently revealed for the parasitism genes in question used in this study (Supplementary Fig. S1).

In planta RNAi in cyst nematodes

In planta RNAi was performed using *Arabidopsis thaliana* line and for three independent experiments. Each experiment was analysed individually by a modified *t*-test using the statistical software package SAS.

**Nematode infection assays**

**Plant material:** Seeds of wild-type *C24* and T3 RNAi lines were surface sterilized with 10% sodium hypochlorite and 0.01% Tween-20 for 3 min followed by 1 min with 70% ethanol. Seeds were washed three times with sterile distilled water to remove residual sterilization solutions. Wild-type *C24* and transgenic RNAi seeds were aseptically transferred, one plant per well, into 12-well culture plates (Costar, Corning, NY, USA) containing 1.5 ml of modified Knops medium (Sijmon et al., 1991) solidified with 0.8% Daishin agar (Brunschwig Chemie BV, Amsterdam, The Netherlands). The plates were sealed with parafilm and allowed to grow in a growth chamber with 16 h light/8 h dark days. Seedlings were inoculated 10 d post-germination.

**Inoculations and assessment of nematode infection:** *Heterodera schachtii* were propagated on roots of greenhouse-grown canola, and *H. schachtii* eggs were collected by breaking open cysts and collecting the eggs on a stack consisting of a #60/#200/#500 (USA Standard Testing Sieve). Eggs were then cleaned up on a sucrose gradient. Eggs were placed in a modified Baermann pan (hatch chamber) with 3.14 mM ZnSO₄ for hatching at 26 °C. The J2s were surface-sterilized for 1 h in 0.001% hibitane followed by 7 min in 0.001% mercuric chloride and washed three times with sterile distilled water. Nematodes were suspended in 1.5% low-melting point agarose to allow even distribution of nematodes to each plant and to facilitate the penetration of the J2 into the solid growth medium. Approximately, 250 J2s were inoculated per plant. At 14 days post-inoculation (dpi) the plants were observed using a Zeiss Stemi 2000 dissecting microscope. Females per plant root system were counted and used as a measure of nematode susceptibility. RNAi transgenic plant roots and shoots were also compared visually with the wild type to note any phenotypic differences that may indirectly alter the infection of nematodes. Mean values of nematode females/plant were generated from a minimum of 15 replicates per *Arabidopsis* line and for three independent experiments. Each experiment was analysed individually by a modified *t*-test using the statistical software package SAS.

**Targeted parasitism genes**

Selected regions of cDNA sequences of the four SCN parasitism genes 3B05 (AF469058), 4G06 (AF469060), 8H07 (AF500024), and 10A06 (AF502391) (Gao et al., 2003) were chosen for dsRNA expression in transgenic *Arabidopsis* (Fig. 1). The 3B05 gene codes for a cellulose-binding protein (Gao et al., 2004) and the RNAi construct was designed to target the cellulose-binding domain. The 4G06 gene codes for a predicted protein with similarity to plant ubiquitins and that contains a novel C-terminal 19 amino acid extension peptide. Since ubiquitin is a well
conserved and abundant protein in plant cells, the RNAi target region was chosen to be specific for the unique C-terminal region. The 8H07 gene codes for a protein with similarity to the SKP1 protein of plants, which is a component of the plant SCF complex involved in polyubiquitination. Two different RNAi constructs were designed for this gene. The 8H07CR (conserved region) construct is specific to 8H07 regions conserved in SKP1 proteins. Nonetheless, the divergence at the nucleotide level is large enough to avoid non-specific silencing of plant SKP1 genes. On the other hand, the 8H07UR (unique region) construct is specific to a stretch of nucleotides unique to the nematode 8H07 cDNA. The 10A06 protein has similarity to plant ring H2 zinc finger proteins, i.e., another group of proteins with potential roles in the SCF complex of the ubiquitination pathway. For 10A06, the region covering the ring H2 zinc finger domain was used in the RNAi experiments. Because this region is sufficiently different from Arabidopsis ring H2 zinc finger domains, unintended silencing of host genes should be avoided. These five different constructs in relation to their target regions are shown in Fig. 1. As mentioned above, the approach requires the feeding of the nematode before RNAi can be induced in the nematode, and, therefore, the time of expression of the parasitism genes under study needs to be after feeding has commenced. The four genes chosen here all are dorsal gland-expressed genes with mRNA expression peaks at time points after feeding has commenced (Gao et al., 2003; Elling et al., 2007b). Therefore, these genes are suitable targets for the approach.

Transgenic Arabidopsis lines express the transgenes

Transgenic Arabidopsis plants expressing hairpin dsRNA were generated and five independent homozygous T3 Arabidopsis lines for each construct were obtained through kanamycin selection. Total RNA from selected homozygous T3 lines was subjected to RT-PCR experiments to amplify the intron region of the RNAi constructs in order to document the presence of the transgene transcripts (data not shown). Amplification of the desired bands confirmed the expression of the short hairpin RNA, a pre-processed mRNA required for siRNA production. Furthermore, the production of siRNA was subsequently confirmed in selected lines by RNA blot hybridizations (see below).

Morphological comparison of wild-type and RNAi lines

In order to discern if the manipulations or the activation of systemic RNAi had adverse effects on plant development, selected transgenic lines from each RNAi construct were compared morphologically with wild-type control Arabidopsis lines. The shoot phenotypic characters of plant height, number of leaves, leaf size, flowering time, and appearance of floral structures on plants grown in potting media were assessed. No major visible morphological differences were observed between RNAi lines and wild-type plants. Similarly, root growth patterns and root length of RNAi lines and Arabidopsis wild-type plants were assessed on agar plant medium and no gross morphological differences in root growth patterns or length in different transgenic RNAi lines were detected (Fig. 2).

Altered susceptibility of RNAi lines

Delivering dsRNA and/or siRNA to the nematode through oral uptake should result in RNAi-mediated down-regulation of nematode target gene mRNA and should deprive the developing nematode of this gene’s function. Depending on gene importance and level of mRNA decrease, it can be expected that nematode parasitic success should be compromised. For all four parasitism genes studied here, at least two RNAi-positive T3 Arabidopsis lines (three in the case of 4G06 3B05 and 8H07UR) were inoculated with H. schachtii J2 nematodes and parasitic success was assessed by determining the number of female nematodes at 14 dpi and comparing them with the number of H. schachtii females developing on wild-type Arabidopsis plants. All homozygous RNAi lines from each construct were tested once, and one of the lines from each construct was tested in additional multiple independent experiments to produce more robust data on female counts on RNAi lines. All tested RNAi lines exhibited significant reductions in female numbers as compared with the wild-type controls, except for 3B05 RNAi lines where only one of the lines exhibited statistically significant female reductions ranging from 12% to 47%. All data of the different experiments are summarized in Fig. 3 using a sigma box plot. 10A06 RNAi lines exhibited up to 42% reduction in developing females. For 4G06 RNAi lines, all independent experiments exhibited statistically significant reductions in
developing females, showing a range from 23% to 64%. For construct 8H07\textsuperscript{UR}, all three independent RNAi lines exhibited statistically significant female reductions, with the highest effect reaching 64%. In the case of 8H07\textsuperscript{CR}, two lines were tested and both exhibited reduced numbers of developing females, with one line showing a reduction of >50%. It was also determined that the RNAi effects on nematode parasitic success were due to the target sequence of the dsRNA expressed in the transgenic plants because transgenic Arabidopsis lines that expressed an RNAi construct to either the green fluorescent protein (GFP) gene or a non-target root-knot nematode parasitism gene 16D10 (Huang et al., 2006\textsuperscript{b}) had no significant effect on the parasitic success of H. schachtii as compared with wild-type plants (data not shown).

Following this confirmation, the lines that had been used in multiple independent nematode screening experiments and showed consistent reduction in developing females [10A06 (3-1-5), 4G06 (6-1-4), 3B05 (9-2-4), 8H07 (15-1-3)] were further analysed by siRNA northern blots using a probe from the target region of the gene. The presence of specific siRNA bands of 21–23 nt in size confirmed the activation of the RNAi pathway for the target genes in question (Fig. 4). In other words, siRNA molecules were produced in the transgenic lines and were available for nematode uptake through feeding.

**Down-regulation of parasitism genes in nematodes feeding on RNAi lines**

Total RNA extracted from nematodes feeding on roots of Arabidopsis RNAi lines at 4 dpi was used in quantitative real-time PCR to analyse the transcript abundance of target parasitism genes in each RNA sample. For these quantitative RT-PCR experiments nematodes recovered from those RNAi lines that received multiple susceptibility tests (Fig. 3), i.e., 10A06 (3-1-5), 4G06 (6-1-4), 3B05 (9-2-4), and 8H07\textsuperscript{UR} (15-1-3), were used. While the RNA isolated from nematodes that infected the 10A06 (3-1-5) RNAi line was of poor quality and could not be used for quantitative PCR experiments, RNA from nematodes from RNAi lines for 4G06, 8H07\textsuperscript{UR}, and 3B05 produced robust data. The RNA transcript abundance data were obtained from two identical experiments with three replications each (see Materials and methods). For 4G06, a 2-fold down-regulation was observed, while 3B05 showed an ~1.5-fold decrease in transcript levels in nematodes excised from roots of RNAi plants (Fig. 5). On the other hand, the observed decrease in 8H07 transcript levels at 4 dpi was comparatively small. After a significant down-regulation of target genes at 4 dpi was established in these initial experiments, the same RNAi lines were used to conduct a third experiment at 7 dpi in which the mRNA abundance of the three RNAi target genes as well as of parasitism genes that were not targeted by RNAi in the requisite Arabidopsis line were assessed. Almost 2-fold decreases in transcript levels of the target parasitism genes were determined, whereas the non-target parasitism gene transcript levels were statistically not altered (Fig. 6). In other words, all tested parasitism genes exhibited specific mRNA down-regulation at 7 dpi in nematodes recovered from RNAi plants. The decrease in the transcript levels of 3B05 and 4G06 at 7dpi was statistically significant ($P <0.05$).

**Discussion**

The success of nematode parasitism greatly depends on the effective use of the available arsenal of parasitism proteins to overcome plant defences and to induce and maintain the functional feeding cells. Considering the pivotal role that parasitism genes provide for the survival of plant-parasitic nematodes, this study was devised to investigate the possibility of down-regulating individual parasitism genes through in planta RNAi, thereby reducing the success rate of cyst nematodes parasitizing their hosts. Furthermore, analysis of gene function by systematic mRNA ablation by RNAi has been a very viable option especially in scenarios where genetic knockouts or any other reverse genetics approaches are not available. The applications of RNAi technology have been a transforming event in biological research and it has become an essential tool for the functional analyses of genes.
As described above, in planta RNAi successes with the root-knot nematode dramatically outnumber those of cyst nematodes. The demonstration of the success of down-regulating target genes by stimulated in vitro ingestion of dsRNA implied that ingested dsRNA is able to escape degradation in both cyst and root-knot nematodes and is effective across several tissues such as the oesophageal glands, amphidial sheath cells, and intestinal and reproductive tissues (Bakhetia et al., 2007). Also, both root-knot and cyst nematodes use their stylets to withdraw nutrients from the feeding cells and, during every feeding session, these nematodes form a feeding tube within the cytoplasm of the feeding cell. Feeding tubes function as molecular sieves with characteristic size exclusion properties. The selective uptake of molecules by nematodes is not uniform across genera, and the size exclusion limit of root-knot nematode feeding tubes is considerably larger than that of cyst nematodes (Böckenhoff and Grundler, 1994; Urwin et al., 1997; Lilley et al., 2007). In support of these observations, the 28 kDa GFP protein was taken up by *Meloidogyne incognita* much more efficiently than by *Globodera rostochiensis* (Urwin et al., 1997; Goverse et al., 1998). This phenomenon raises the question of whether dsRNA or siRNA is excluded during the uptake process in cyst nematodes as there is only limited proof of in planta RNAi against cyst nematodes.

In the present study it is demonstrated that the *in planta* dsRNA/siRNA uptake in cyst nematodes in fact appears to be functional because specific down-regulation of target gene mRNAs was observed (Figs 5, 6) and because of the fact that nematodes feeding on RNAi plants were less successful in further development. Therefore, it appears that the cyst nematode feeding tube allows passage of RNA despite the lower molecular weight cut-off when compared with root-knot nematodes. However, it remains remarkable that no completely resistant *Arabidopsis* lines were observed in light of the fact that in experiments with root-knot nematodes such dramatic susceptibility changes appear to be the norm. This difference could potentially be explained by the fact that root-knot nematode parasitism may be more vulnerable to the loss of individual parasitism gene functions, whereas cyst nematode parasitism could be more robust and resilient to perturbations. Furthermore, the level of resistance achieved through RNAi of parasitism genes may also be dependent on the role an individual parasitism protein plays during the infection process. These hypotheses are currently being explored by targeting several cyst nematode parasitism genes at once in combined RNAi approaches. Regardless of the outcome of these studies, it is believed that *in planta* RNAi is also a useful reverse genetics tool in cyst nematodes and, furthermore, has promise to engineer crop plant cultivars in which levels of cyst nematode susceptibility are sufficiently reduced to allow their use as nematode management tools.

### Supplementary data

Supplementary data are available at *JXB* online.

_Figure S1._ The homology between the regions of soybean cyst nematode (SCN) and sugar beet cyst nematode (BCN) parasitism genes used for RNAi experiments.

### References


Elling AA, Davis EL, Hussey RS, Baum TJ. 2007a. Active uptake of cyst nematode parasitism proteins into the plant cell nucleus. *International Journal for Parasitology*.


