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ORIGINAL ARTICLE

# Host-delivered RNAi: an effective strategy to silence genes in plant parasitic nematodes

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**Abstract** Root-knot nematodes (*Meloidogyne* spp.) are obligate, sedentary endoparasites that infect many plant species causing large economic losses worldwide. Available nematicides are being banned due to their toxicity or ozone-depleting properties and alternative control strategies are urgently required. We have produced transgenic tobacco (*Nicotiana tabacum*) plants expressing different dsRNA hairpin structures targeting a root-knot nematode (*Meloidogyne javanica*) putative transcription factor, *MjTis11*. We provide evidence that *MjTis11* was consistently silenced in nematodes feeding on the roots of transgenic plants. The observed silencing was specific for *MjTis11*, with other sequence-unrelated genes being unaffected in the nematodes. Those transgenic plants able to induce silencing of *MjTis11*, also showed the presence of small interfering RNAs. Even though down-regulation of *MjTis11* did not result in a lethal phenotype, this study demonstrates the feasibility of silencing root-knot nematode genes by expressing dsRNA in the host plant. Host-delivered RNA interference-triggered (HD-RNAi) silencing of parasite genes provides a novel disease resistance strategy with wide biotechnological applications. The potential of HD-RNAi is not restricted to parasitic nematodes but could be adapted to control other plant-feeding pests.

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**Abbreviations**

| | |
|---|---|
| dsRNA | Double-stranded RNA |
| HD-RNAi | Host-delivered RNA interference |
| RKN | Root knot nematode |
| RNAi | RNA interference |
| qRT-PCR | Quantitative real time PCR |
| siRNA | Small interfering RNA |

## Introduction

Parasitism of plants by nematode infestation causes extensive damage to turf grasses, ornamental plants and food crops each year. Over 20% of the annual yield losses in major crops are caused by plant-parasitic nematodes with an economic loss estimated to be in excess of US $ 77 billion worldwide (Jung and Wyss 1999). Most of the damage is caused by the sedentary endoparasitic nematodes of the Tylenchoidea superfamily . This superfamily contains the cyst nematodes (part of the Heteroderidae family) and the root-knot nematodes (part of the Meloidogynidae family). Symptoms of diseased plants include stunting, wilting and enhanced susceptibility to other diseases. Some cultivars from species such as tomato, potato and soybean contain nematode resistance genes that can be effective against several nematode species, but the protection is usually quite narrow, controlling only specific pathotypes of a species. There are also many crops for which resistance loci are not available (e.g. sugar beet and pineapple). Root-knot nematodes (RKN) have the ability to infect thousands of plant species (Abad et al. 2003) making crop rotation of little value as a control strategy. Consequently, efforts to

eliminate or minimize damage caused by nematodes have typically involved the use of soil fumigation. Although the use of nematicides such as methyl bromide, organophosphates and carbamates is effective in reducing nematode population levels, their cost and toxicity to humans and the environment make them unsound as a control measure in plant production. Indeed, the use of many available nematicides is being banned due to their toxicity or ozone-depleting properties, and alternative control strategies are urgently required.

Plant root-knot nematodes hatch as motile and non-feeding second-stage juveniles (J2). They are attracted to root tips, where they penetrate the epidermis in the elongation zone and migrate intercellularly towards the stele. RKNs become sedentary after establishing a permanent feeding site in surrounding phloem parenchyma cells by stimulating and maintaining giant cell formation (Jung and Wyss 1999; Gheysen and Fenoll 2002; Abad et al. 2003; Williamson and Gleason 2003; Davis et al. 2004). These cells are multinucleated and undergo extensive remodelling of the cell wall, adopting characteristics of transfer cells. Giant cells act as sinks, diverting plant nutrients to provide metabolic energy for the nematode that feeds exclusively from them. Concurrently with giant cell formation, nearby pericycle and cortical cells enlarge and divide forming the root-knot or gall that is characteristic of infection by this group of nematodes.

The discovery that feeding dsRNA to the nematode *Caenorhabditis elegans* inactivates endogenous genes (Timmons and Fire 1998; Timmons et al. 2001) by an RNA interference (RNAi) mechanism (Fire et al. 1998; Hannon 2002) suggests that a similar mechanism could also be present in other nematode species. We therefore devised a strategy to inactivate nematode genes using giant cells as delivery vehicles to provide dsRNA molecules to the feeding nematode. For such a strategy to be successful, root-knot nematodes must have a similar RNAi response as the distantly related free-living bacteriovore *C. elegans*. Recent work has demonstrated such a response after soaking J2 juveniles of potato and soybean cyst nematodes (Urwin et al. 2002; Chen et al. 2005) as well as the RKN *M. incognita* (Bakhetia et al. 2005a; Rosso et al. 2005) in a solution containing dsRNA. Also recently, a number of manuscripts have appeared using different RNAi-based approaches to control nematode infestation in plants (Huang et al. 2006; Steeves et al. 2006; Yadav et al. 2006; Gheysen and Vanholme 2007).

The aim of this study is to demonstrate the feasibility of down-regulating endogenous root-knot nematode transcripts by expressing dsRNA with sequence identity to the nematode gene in plant roots. We also study whether the silencing is sequence-specific, affecting only the targeted gene and not other unrelated nematode genes.

## Materials and methods

### Construction of plasmids

*Meloidogyne javanica MjTis11* EST clone (rk10c12.y1, GenBank accession BE578298) was obtained from the Washington University Parasitic Nematode EST Project (McCarter et al. 2000). Complete DNA sequencing of the clone was carried out and sequence analysed using the University of Wisconsin Genetics Computer Group (UWGCG) software version 8.1 (Devereux et al. 1984).

RNAi constructs were prepared by adding appropriate restriction sites to the ends of the primers used to perform PCR amplification with Elongase DNA polymerase (Invitrogen) as described by Chakravorty and Botella (2007), followed by subcloning into the vector pHannibal (Wesley et al. 2001, for the intron containing hairpin structures) or a derivative of this vector that had the intron replaced with a fragment of the *uidA* gene of the same size (741 bp). In all cases, PCR-amplified DNA sequences were sequenced to confirm that no errors had been introduced during amplification. The expression cassette from pHannibal was then subcloned into the binary vector pUQC477 (Fig. 3). Binary vectors were transferred by triparental mating into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al. 1983), which was then used for plant transformation.

### Plant transformation

The rapid flowering tobacco (*Nicotiana tabacum* L.) variety Ti68 (McDaniel et al. 1996) was transformed and regenerated according to a modified leaf-disk method (Horsch et al. 1985). Independent primary transgenic lines were propagated in tissue culture and rooted clonal replicates transferred to soil and acclimatized in a growth cabinet before transfer to the glasshouse. Possible effects of tissue culture on nematode feeding were addressed by producing control lines (TisA) through normal transformation procedures.

### Nematode challenge

Eggs of *M. javanica* were recovered from stock cultures maintained on tomato plants. Regenerated transgenic tobacco plants were transferred to 400 ml pots containing sand-based compost and osmocote nutrient granules. Two weeks after potting, the plants were inoculated using 10,000 *M. javanica* eggs and grown in a glasshouse. For each experiment, plants were positioned in a randomized way in glasshouse benches and infected with the same batch of nematode eggs. Plants were harvested 6 weeks after inoculation. Depending on the experiment, a total of 13–16 independent transgenic lines were analysed.

### Molecular analyses

At the time of collection, 6 weeks after inoculation, all roots were heavily infected (level 5). Roots from transgenic 35S promoter, TobRB7 promoter and WT plants were briefly bleached during harvesting to remove nematode eggs and newly hatched J2 juveniles. The majority of nematodes left inside the root tissues were mature females and late stage females. Acid fuschin staining confirmed that these stages were the predominant type in the harvested roots. Whole roots were used for cDNA production; therefore, the cDNA pool contained nematode and tobacco root cDNAs. However, nematode cDNAs were relatively abundant in the sample as supported by the Ct value for nematode actin being approximately 25.

For real-time quantitative PCR experiments (qRT-PCR), 1 µg of total RNA was reverse transcribed using Superscript III (Invitrogen) following the manufacturer's instructions. The resulting cDNA was subsequently diluted to a total volume of 250 µL. *M. javanica* target genes were amplified using gene-specific primers for *MjTis11* (forward, 5′-CTTGGGTTTAATTACCCAAGTTTGAGAT-3′ and reverse, 5′-TCCACGCGGACAATAACCTTTA-3′), *MjGAPDH*, coding for glyceraldehydes-3-phosphate dehydrogenase (forward, 5′-TGCTTCCTGCACTACTAACTG TCTTG-3′ and reverse, 5′-CAGTAACAGCGTGCACAG TAGTCAT-3′), *Mjα-tubulin* (forward, 5′-CCGACAACT GCCAAGGACTT-3′ and reverse, 5′-CAACCGAAAGAC GCTCCATAA-3′) and *Mjβ-actin* (forward, 5′-TGTCAAT GTCGCACTTCATGATC-3′ and reverse, 5′-CCGTTGC CCAGAATCTCTCTT-3′) designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA, USA) and consensus sequences of *Mjα-tubulin* and *Mjβ-actin* assembled from several public expressed sequence tags. qRT-PCR was carried out as described by Moyle et al. (2005) using the ABI PRISM 7700 sequence detector and SYBR Green Master mix (Applied Biosystems) using primers at a final concentration of 0.1 µM each and 2.5 µL (the equivalent of 10 or 0.1 ng total RNA) of cDNA as template. PCR-cycling conditions comprised an initial polymerase activation step at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Real-time DNA amplification was monitored and analysed using the Sequence Detector 1.7 software (Applied Biosystems). Quantification of gene expression relative to the *β*-actin reference gene was calculated using the Pfaffl equation (Pfaffl 2001), the amplification rate and cycle threshold (Ct). This equation takes into account differing primer efficiencies. Cycle threshold values were corrected for genomic DNA contamination in the RNA sample. An infected wild-type root sample, referred to as WTCAL in the text, was used as a calibrator to allow comparisons across samples.

For siRNA detection, total RNA was extracted from 2–3 g of ground leaf tissue using TRIzol reagent (Life Technologies/Gibco BRL) and a extraction buffer containing 0.5% SDS, 1 M NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH8.0. High molecular weight RNAs (mRNA and rRNA) were precipitated by adding 1 volume 20% PEG (MW 8000), 1 M NaCl, incubating at 4°C for 30 min and centrifuging for 30 min at room temperature. Low molecular weight (LMW) RNAs including siRNAs were ethanol precipitated from the PEG/NaCl supernatant by adding 3 volumes of ethanol and centrifuging for 30 min at 4°C. After washing the pellet with 80% ethanol, an aliquot was redissolved in water for quantification and the remainder redissolved in formamide for gel electrophoresis. Purified LMW RNAs (2–10 µg) were separated by denaturing (7 M urea) polyacrylamide (15%, 19:1) gel electrophoresis in 1 × tris borate EDTA buffer. The separated LMW RNAs were transferred to Hybond NX membrane (Amersham) by electroblotting. Antisense riboprobes were prepared from linearized plasmids containing the *MjTis11* or *uidA* gene with 5′[α-$^{32}$P] rCTP using SP6 RNA polymerase and the "Riboprobe" system from Promega as per manufacturer's instructions. Hybridization conditions against the blotted LMW RNAs were performed as described by Trusov et al. (2006). All hybridization signals were detected by phosphorimaging as described by Purnell et al. (2005).

### Statistical analysis

JMP IN 4.0 software (SAS Institute Inc.) was used for statistical analysis of the qRT-PCR data. The normality assumption was tested using a normal quantile plot and a Shapiro-Wilk W goodness of fit test. Each set of independent transgenic plants had a *P* value of 0.03 or less, indicating a normal distribution. The Student's *t* test with an alpha level of 0.05 was used to compare relative transcript level means. For the calculation of the *t* test, all data from nematodes feeding on a specific genotype (such as TisS plants) was considered together.

## Results

### *MjTisII* is mainly expressed at the egg and mature female stages

Functional genomic screens of *C. elegans* using dsRNA feeding techniques have shown that most genes resulting in a lethal phenotype are highly expressed in embryos rather than adult tissues (Fraser et al. 2000). We chose a *M. javanica* Tis11 zinc finger $CX_8CX_5CX_3H$ type putative transcription factor (*MjTis11*, GenBank #BE578298) that is mainly expressed in eggs and in egg-producing adult females (Fig. 1). *In-silico* studies showed that MjTIS11 has limited similarity to the *C. elegans* MEX-1, PIE-1 and

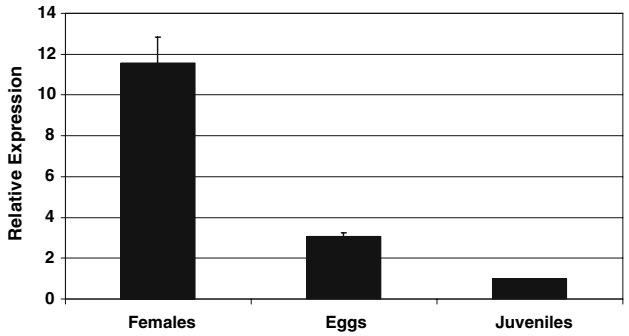


**Fig. 1** Expression levels of *MjTis11* in *M. javanica*. Real-time qRT-PCR analysis of *M. javanica* putative transcription factor *MjTis11* transcript levels at different stages of the nematode life cycle. *MjTis11* mRNA abundance was normalized to actin levels and shown relative to mRNA levels at the juvenile stage. Each *bar* represents the mean of duplicate assays repeated twice. Standard errors are shown for females and eggs, but not for juveniles since mRNA levels are shown relative to this stage. All differences are statistically significant ($P < 0.001$)

POS-1 proteins (Fig. 2), which are involved in determining cell fate during the early stages of embryogenesis. RNAi-mediated silencing of these genes result in an embryo lethal phenotype in *C. elegans* (http://www.wormbase.org). Extensive database searches showed that *MjTis11* has no apparent homology with any known plant genes, thereby minimizing any potential RNAi off-target effects (Bakhetia et al. 2005b). In addition, PCR experiments using the specific primers designed for *MjTis11* failed to amplify any product using either tobacco cDNA samples from several tissues or tobacco genomic DNA (results not shown).

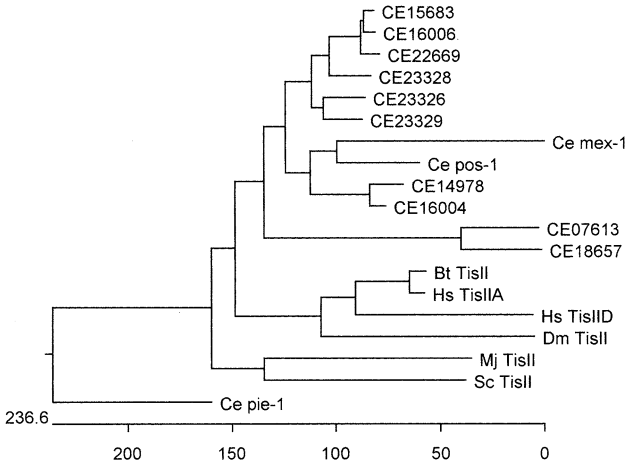


**Fig. 2** Phylogenic analysis of CCCH type putative zinc finger transcription factors. Phylogenic cladogram of relationships between CCCH type putative zinc finger transcription factors from *C. elegans* [WormBase protein IDs CE15683, CE16006, CE22669, CE23328, CE23326, CE23329, CE14978, CE16004, CE07613, CE18657, CE28097 (Ce mex-1), CE04629 (Ce pos-1), CE28134 (Ce pie-1)], cow [Bt TisII (GenPept #P53781)], human [Hs TisII, Hs TisIID (GenPept #P26651 and #P47974, respectively)], fly [Dm TisII (GenPept #P47980)], yeast [Sc TisII (GenPept #NP_013237)] and *Meloidogyne javanica* (Mj TisII). Branch distances are proportional to sequence divergence

## Reproducible down-regulation of *MjTisII* in nematodes feeding on transgenic tobacco roots

A total of four RNAi constructs were prepared for plant transformation (Fig. 3). Two of these consisted of an inverted repeat of a 772 bp fragment of the *MjTis11* cDNA, separated by either a spacer region (TisS) or an intron (TisI), and driven by the constitutive 35S cauliflower mosaic virus promoter (CaMV35S). The remaining two also contained an inverted repeat of the *MjTis11* cDNA fragment, but driven by the gall-specific Δ0.3 TobRB7 promoter (TisTS and TisTI; Opperman et al. 1994). A control construct (TisA) was also prepared containing the *MjTis11* cDNA in antisense orientation driven by the CaMV35S promoter.

In order to monitor endogenous *MjTis11* transcript levels in *M. javanica* nematodes, we used real-time quantitative PCR (qRT-PCR) with primers designed towards a 5′ region of *MjTis11* (*5'MjTis11*), upstream of the fragment used in our binary constructs. This allowed the endogenous *MjTis11* transcript levels in nematode tissues to be monitored independently of any *MjTis11* transcripts present in transgenic plant tissues (originating from the transcription of the inserted T-DNA). *5′MjTis11* levels were normalized to *M. javanica* actin (GenBank #AF532604) levels in order

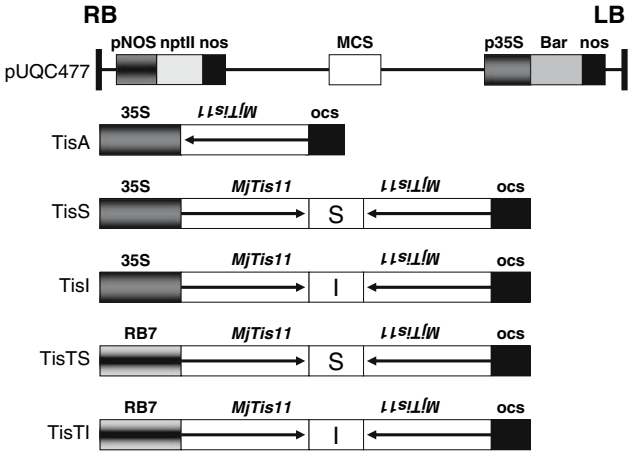


**Fig. 3** Schematic diagram showing the genetic constructs used in the production of transgenic tobacco plants. Schematic diagram of the different T-DNA cassettes used to produce the transgenic tobacco lines used in this study. The T-DNA components were the nopaline synthase promoter (*pNOS*) and terminator (*nos*), CaMV35S promoter (*35S*), Δ0.3 TobRB7 promoter (*RB7*), neomycin phosphatase (*nptII*), pdk intron (*I*), spacer region (*S*), phosphinothricin acetyltransferase (*bar*), octopine synthase terminator (*ocs*) and *MjTis11*. The five different cassettes TisA, TisS, TisI, TisTS and TisTI were cloned into the multiple cloning site (*MCS*) of the binary vector pUQC477

to compare data from different samples. In this way, the relative expression levels of *MjTis11* in nematodes could be determined independent of the severity of nematode infection or the initial amount of nematode tissue present in each sample. To ensure that the primers used did not result in non-specific amplification of a tobacco endogenous gene, PCR experiments were conducted with tobacco cDNA and genomic DNA with negative results.

In preliminary screenings, samples from infected roots of 13–15 independent transgenic lines for each of the five constructs as well as wild type (WT) were analysed. Our results showed a decrease in *5′MjTis11* transcript levels in nematodes feeding on the roots of transgenic tobacco lines expressing dsRNA *MjTis11* under the control of the CaMV35S promoter (lines TisS and TisI; Fig. 4a). None of the lines containing the TobRB7 promoter showed signs of *MjTis11* silencing. We have produced transgenic tobacco lines carrying the *β*-glucuronidase (GUS) reporter gene under the control of the Δ0.3 TobRB7 promoter. Detailed analysis of several transgenic lines showed that (1) the majority of galls displayed no observable GUS staining, and (2) for the small percentage of galls showing some GUS activity, the intensity of the stain was very light (results not shown). The lack of silencing observed in the TobRB7 promoter lines could therefore be attributed to the weakness of the TobRB7 promoter. Of the two constructs showing some silencing effect in nematodes, the RNAi construct with the spacer (TisS) was much more effective than the construct containing the intron (TisI). This was somewhat surprising since the use of an intron rather than a spacer has been reported to improve the efficiency of gene silencing (Smith et al. 2000).

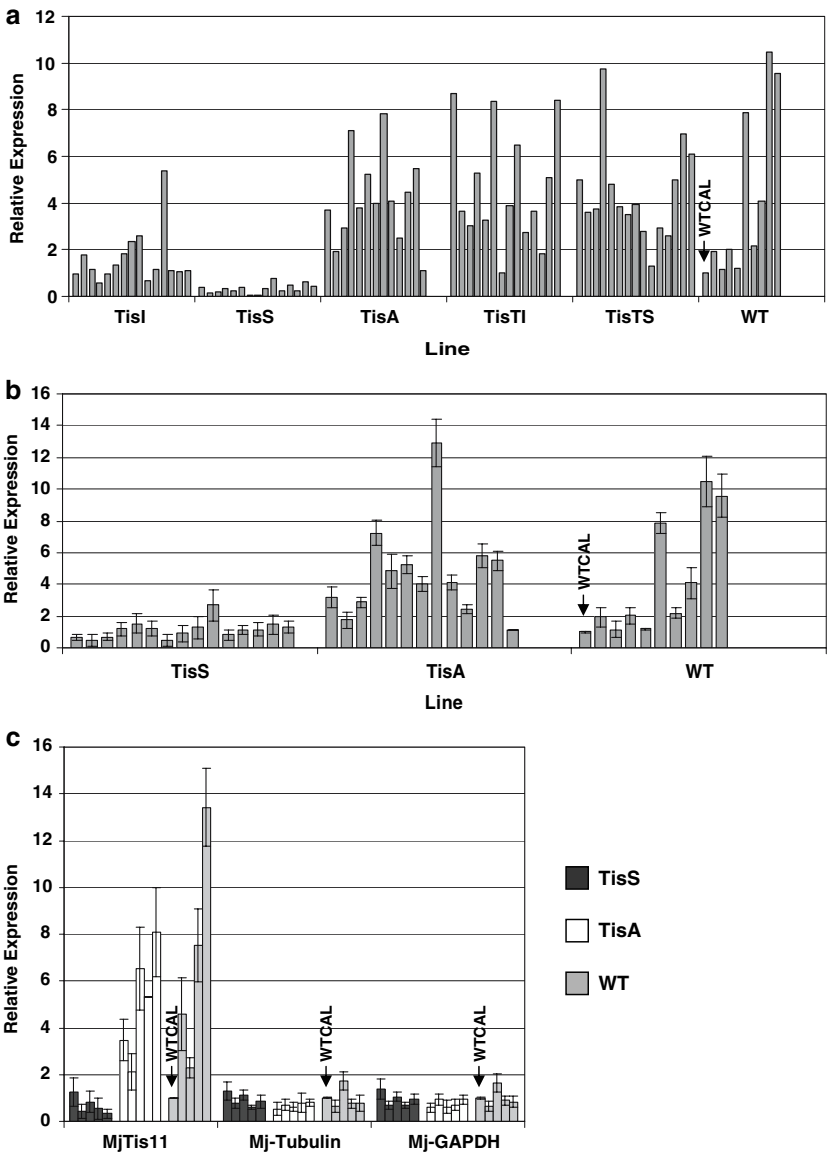


**Fig. 4** Silencing of the *MjTis11* gene in nematodes feeding on transgenic plants is reproducible and dependent on the structure of the introduced construct. Real-time qRT-PCR was used to determine mRNA abundance of *M. javanica* genes from nematodes living in wild-type and transgenic plants from independent transgenic lines. mRNA abundance was normalized to actin levels and shown relative to mRNA levels in wild-type sample WTCAL. **a** Each *bar* represents the mean of duplicate assays for an individual WT plant or transgenic line. **b** Each *bar* represents the mean of duplicate assays repeated twice for three clonal replicates of the TisS lines and individual WT and TisA plants. **c** Expression levels of *MjTis11*, *Mjα-tubulin* (GenBank #BG736650) and *Mj-GAPDH* (GenBank #BM881590) in nematodes living in roots of selected TisS (*black*), TisA (*white*) and WT (*grey*) plants. Each *bar* represents the mean of duplicate assays repeated three times. *Error bars* representing standard error of the mean are shown

To verify the results from the preliminary screening, additional experiments were carried out focusing on the best performing construct (TisS) and using two different controls (TisA and WT). In this occasion, duplicate qRT-PCR determinations were performed on three biological replicates for each TisS line analysed. The results shown in Fig. 4b confirmed that nematodes feeding on transgenic plants carrying the TisS construct had significantly lower levels of *MjTis11* than nematodes feeding on either control TisA lines or WT plants (Student's *t* test, alpha value = 0.05). Relative expression levels for all the 15 TisS lines analysed were very consistent and similar to those obtained in the preliminary screen (Fig. 3a).

The down-regulation observed for *MjTis11* does not affect other nematode genes

Selected TisS lines were further examined to determine whether the decrease in transcript levels observed for *MjTis11* was specific for this gene or whether other sequence-unrelated genes were also affected in the nematodes. For this purpose, the expression levels of *MjTis11*, *Mjα-tubulin* and *MjGAPDH* in nematodes feeding on the roots of TisS lines, control TisA or WT plants were measured by qRT-PCR and normalized to nematode actin levels. As observed before (Fig. 4a, b), there was a significant (Student's *t* test, alpha value = 0.05) and reproducible reduction in *MjTis11* transcript levels in *M. javanica* feeding on the roots of TisS lines, when compared to nematodes feeding on control TisA and WT plants (Fig. 4c). In contrast, the levels of *Mjα-tubulin* and *MjGAPDH* were not significantly different among the three groups of plants studied, suggesting that the down-regulation observed for *MjTis11* is sequence-specific. It is important to note that in Fig. 4c, *MjTis11* levels are only comparable among the three populations of plants (TisS, TisA and WT), but are not comparable with the levels observed for *Mjα-tubulin* and *MjGAPDH*.

Down-regulation of *MjTisII* is associated with the existence of small interfering RNAs in the transgenic tobacco line

In order to determine whether the transgenic lines causing down-regulation of *MjTis11* in nematodes were producing small interfering RNAs (siRNAs) with homology to the *MjTis11* dsRNA, we analysed samples from TisS, TisI, TisA and WT plants. Leaves of TisS and TisI plants, but not TisA and non-transgenic controls, showed the presence of siRNAs (Fig. 5). To provide positive and negative controls for siRNA detection, transgenic tobacco plants expressing the *β*-glucuronidase (*GUS*) gene were retransformed with a *GUS* hairpin construct. A number of lines showed silencing of the *GUS* gene, while in others no silencing was observed. The presence of *GUS* siRNAs was detected in *GUS* silenced, but not in non-silenced plants (Fig. 5). Although not directly comparable, it appears that the levels of siRNAs were lower in the *MjTis11* plants than those observed in the leaves of *GUS* silenced plants.

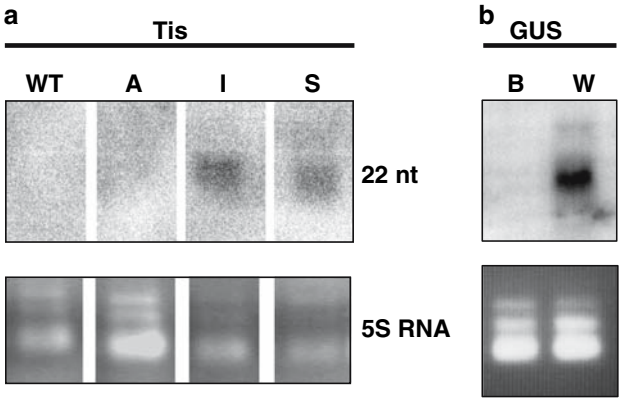


**Fig. 5** Silencing of the *MjTis11* gene is specific and associated with the presence of siRNAs. **a** RNA gel-blot showing the accumulation of *MjTis11* siRNAs in TisS (*lane S*) and TisI (*lane I*) plants, but not in TisA (*lane A*) and WT controls. **b** RNA gel-blot showing the accumulation of *GUS* siRNAs in silenced (*lane W*) and non-silenced (*lane B*) GUS transgenic tobacco plants expressing a GUS hairpin construct. Ethidium bromide stained gels are shown as loading controls

## Discussion

Previous studies have shown that it is possible to achieve an RNAi-like response in cyst and RKN nematodes (Urwin et al. 2002; Bakhetia et al. 2005a; Chen et al. 2005; Rosso et al. 2005). Nevertheless, these nematodes do not feed until they are established inside their host plants; therefore, the above-mentioned studies induced the nematodes to ingest dsRNA by octopamine or resorcinol treatment. The silencing achieved was transient with normal transcript levels returning 68 h after soaking (Rosso et al. 2005). A key question for the potential application of HD-RNAi was whether nematodes can ingest dsRNA when feeding on plant cells expressing hairpin constructs. Root-knot and cyst nematodes produce specialized structures inside the cells on which they feed called feeding tubes (Hussey and Mims 1991). These act as molecular sieves and may help to protect the nematode from ingesting plant defence macromolecules. The size exclusion limit is unknown, but experiments using GFP (28 kDa) and different dyes indicate a size exclusion between 20 and 40 kDa (Bockenhoff and Grundler 1994; Urwin et al. 1997). It has been suggested that linear dsRNA as well as double-stranded small interfering RNAs (siRNAs) of 21–23 nucleotides could be taken up lengthwise by *M. incognita* (Bakhetia et al. 2005b). The siRNAs are produced from long dsRNA molecules by an

RNase III-like ribonuclease before being incorporated into an RNA-induced silencing complex (RISC) that identifies and degrades mRNAs showing homology to the dsRNA. However, the feeding tube would be expected to exclude both non-linear large dsRNA molecules and siRNAs incorporated into RISC (360 kDa; Nykanen et al. 2001).

The ultimate aim of our research was to devise methods to control nematode infestation in agriculturally important crops. The selection of the targeted gene (*MjTis11*) was based on the fact that RNAi-driven silencing of some homologous *C. elegans* genes resulted in an embryo lethal phenotype. Down-regulation of *MjTis11* transcript levels in *M. javanica* nematodes did not result in a significant decrease in fecundity or egg hatching rate in any of the lines analysed (results not shown), indicating that either (1) this gene is not a good candidate to achieve an embryo lethal phenotype in *M. javanica* and/or (2) the levels of down-regulation achieved for *MjTis11* are not sufficient to compromise its biological role. In fact, even though silencing of the *C. elegans* genes coding for the MjTIS11 homologous proteins MEX-1, POS-1 or PIE-1 resulted in an embryo lethal phenotype, silencing of ten other *C. elegans* TIS11 transcription factors, also homologous to MjTIS11 and clustering together in the phylogenetic tree (Fig. 2), produced a wild-type phenotype (http://www.wormbase.org). Therefore, for this particular family of transcription factors, identifying an *M. javanica* orthologue of an essential *C. elegans* gene in order to achieve an embryo lethal phenotype could be difficult based solely on *in-silico* analyses, as the protein similarity is confined to the zinc finger motifs. Regardless, the fact that partial silencing of *MjTis11* was obtained demonstrates that plants can be used as delivery systems to induce down-regulation of targeted genes in parasitic nematodes. High-throughput strategies can now be devised to find appropriate gene targets in *M. javanica*. During the production of this manuscript, a report by Yadav et al. (2006) has been published describing the use of hairpin constructs to control infestation of a different plant parasitic nematode, *Meloidogyne incognita* in tobacco. Down-regulation of the target genes selected by Yadav et al. (2006), an integrase and a splicing factor, protected the plant against infection by *M. incognita*. Even more recently, Huang et al. (2006) found that expression in *Arabidopsis thaliana* of a conserved RKN gene essential for plant parasitism resulted in broad range resistance against four major RKN species.

The large differences observed in *MjTis11* expression levels (Fig. 3a, b and MjTis11 in c) within each specific genotype, but most apparent in WT and non-silenced lines (TisA, TisTI and TisTA), could reflect the developmental stage of the nematode population present in the sample. We have shown that *MjTis11* expression levels can increase up to 12 times from juveniles to adult females; therefore, a particular sample enriched in adult females could result in increased levels of *MjTis11* message. In fact, this variation was not observed when more "developmentally constant genes" such as *Mjα-tubulin* and *MjGAPDH* were measured, proving that the difference in *MjTis11* were not due to experimental artifacts (Fig. 4c). It is important to note that *MjTis11* expression levels did not show such large variation in silenced TisS lines.

Our data indicated that the decrease in transcript levels observed for *MjTis11* was specific for this gene and did not affect two other unrelated nematode genes *Mjα-tubulin* and *MjGAPDH* (Fig. 4c). This suggests that (1) the silencing is caused by the transgene introduced in the host plants and (2) that the silencing is caused by an RNAi-like mechanism. Nevertheless, introduction of a hairpin-containing transgene is not sufficient to induce silencing unless it is linked to a strong promoter, as is shown by the absence of silencing observed in nematodes feeding on lines driven by the weak TobRB7 promoter (Fig. 3a). Another characteristic of RNAi is the production of siRNAs with homology to the dsRNA trigger, which was detected in transgenic lines. In plants, fungi and nematodes siRNAs can serve as primers for the synthesis of dsRNA by RNA-dependent RNA polymerases, producing secondary siRNAs by "transitive RNAi" and resulting in strong amplification of RNAi silencing (Lipardi et al. 2001; Sijen et al. 2001; Vaistij et al. 2002). However, no secondary siRNAs are expected to be produced in the TisS and TisI lines, since the targeted sequence is not present in the tobacco genome. Our data suggested that transgenic lines expressed relatively low levels of *MjTis11* siRNAs compared to those detected in GUS-silenced plants (Fig. 5). These observations provide a potential explanation why complete silencing of the nematode *MjTis11* gene was not achieved since it is believed that siRNAs must accumulate above a certain threshold for complete silencing to occur. In this respect, the simultaneous expression of the *MjTis11* gene and the *MjTis11* hairpin structure in root galls may boost the cellular levels of *MjTis11* siRNAs, resulting in more effective silencing of the nematode gene.

In summary, this paper describes the production of transgenic tobacco plants expressing dsRNAs targeting a root-knot nematode (*Meloidogyne javanica*) putative zinc finger transcription factor, *MjTis11*. We provide evidence that *MjTis11* was silenced in nematodes feeding on the roots of these transgenic plants and that the silencing probably resulted from an RNAi-like mechanism. Our results demonstrate that plants can be used as delivery systems to induce RNAi-mediated gene silencing in parasites such as root-knot nematodes.Host-delivered RNA interference (HD-RNAi) will allow the development of novel methods to control nematode infestation in plants. However, further research is needed to increase both the level of silencing

and to identify nematode genes whose silencing results in a lethal phenotype. The basic principle behind HD-RNAi is not restricted to nematode pests and, in theory, could also be applied to all those agricultural pests that feed on plant tissues and are susceptible to an RNAi-type gene silencing.

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