RESEARCH PAPER

Tobacco rattle virus mediates gene silencing in a plant parasitic root-knot nematode

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Abstract

Root-knot nematodes (RKNs) are sedentary biotrophic parasites that induce the differentiation of root cells into feeding cells that provide the nematodes with the nutrients necessary for their development. The development of new control methods against RKNs relies greatly on the functional analysis of genes that are crucial for the development of the pathogen or the success of parasitism. In the absence of genetic transformation, RNA interference (RNAi) allows for phenotype analysis of nematode development and nematode establishment in its host after sequence-specific knock-down of the targeted genes. Strategies used to induce RNAi in RKNs are so far restricted to small-scale analyses. In the search for a new RNAi strategy amenable to large-scale screenings the possibility of using RNA viruses to produce the RNAi triggers in plants was tested. Tobacco rattle virus (TRV) was tested as a means to introduce double-stranded RNA (dsRNA) triggers into the feeding cells and to mediate RKN gene silencing. It was demonstrated that virus-inoculated plants can produce dsRNA and siRNA silencing triggers for delivery to the feeding nematodes. Interestingly, the knock-down of the targeted genes was observed in the progeny of the feeding nematodes, suggesting that continuous ingestion of dsRNA triggers could be used for the functional analysis of genes involved in early development. However, the heterogeneity in RNAi efficiency between TRV-inoculated plants appears as a limitation to the use of TRV-mediated silencing for the high-throughput functional analysis of the targeted nematode genes.

Key words: Reverse genetics, RNA interference, virus-induced gene silencing.

Introduction

Plants, animals, fungi, and protists have developed similar defence mechanisms against invading genetic elements, based on the sequence-specific degradation of double-stranded RNA (dsRNA). These processes—known as post-transcriptional gene silencing (PTGS) in plants, RNA interference (RNAi) in animals, and quelling in fungi—involve common degradation processes. In all cases the gene silencing is activated by a dsRNA recognized by the cell as aberrant RNA and processed by a Dicer RNase into 21–26 bp duplexes. These short interfering RNAs (siRNA) are incorporated into a dsRNA-induced silencing complex (RISC) and guide the degradation of homologous transcripts (Meister and Tuschl, 2004). In addition, siRNA can guide gene silencing through inhibition of RNA translation or chromatin rearrangement. PTGS is used as a functional genomics tool for knocking out gene expression in plants (Brodersen and Voinnet, 2006; Vaucheret, 2006). In the case of virus-induced gene silencing (VIGS) the silencing
is triggered by viral vectors that deliver dsRNA fragments homologous to the targeted plant genes, and leads to the degradation of both the viral genome and the targeted transcripts (Ratcliff et al., 1997; Baulcombe, 1999; Ratcliff et al., 2001; Lacomme et al., 2003; Hileman et al., 2005). VIGS has been successfully used on leaves to decipher plant responses to pathogens (Kandoth et al., 2007; An et al., 2008; Brueggeman et al., 2008). In addition, functional genomics on plant parasitoids is required to decipher the molecular events that govern plant–parasite interactions.

Root-knot nematodes (RKNs), Meloidogyne sp., are obligate sedentary root parasites of >2000 plant species (reviewed by Caillaud et al., 2007). The only free-living stage is the second-stage juvenile (J2), which hatches from eggs in the soil. J2s attracted by root exudates penetrate the root tip and migrate towards the differentiation zone. The nematodes become sedentary and perforate the cell wall of selected parenchyma cells with their stylet to induce the differentiation of root cells into hypertrophied multinucleate feeding cells—giant cells—formed by repeated cycles of mitosis without cytokinesis (Jones, 1981). Expanding multinucleate giant cells with a dense cytoplasm are formed 7 d post-inoculation (Jammes et al., 2005). The giant cells are the sole source of nutrients for the parasite during the 6–8 weeks required for completion of its life cycle. Each nematode feeding site consists of 5–7 giant cells embedded in a root gall formed by the hyperplasia and hypertrophy of the surrounding cortex cells. Silencing of plant parasitic nematode genes has been obtained by soaking free-living J2s in dsRNA solutions (reviewed by Rosso et al., 2009). The ingestion of dsRNA by the non-feeding J2s was stimulated artificially by adding neurostimulant chemicals. This strategy is limited to the functional analysis of genes involved in the early steps of parasitism, as production of the targeted transcripts is often restored a few days after soaking (Fanelli et al., 2005; Rosso et al., 2005; Dubreuil et al., 2007; Bakhetia et al., 2008). Alternatively, RKN genes were silenced by growing the nematodes on Arabidopsis and tobacco plants that constitutively express hairpin RNA homologous to a nematode gene (Huang et al., 2006; Yadav et al., 2006; Fairbairn et al., 2007). The targeted mRNAs were found to be depleted in the nematodes feeding on the transgenic plants, suggesting that the nematodes efficiently ingested RNAi triggers produced by the plant. However, the need for large-scale functional analyses is emerging as more RKN genes potentially involved in parasitism and development are being identified (Abad et al., 2008; Bellafiore et al., 2008).

Viral vectors can be used to mediate dsRNA production in plants more rapidly than stable transgenesis and are amenable to high-throughput screenings. Vectors constructed from tobacco rattle virus (TRV) are the most efficient vectors for triggering dsRNA-mediated silencing in root tissues, including root meristems, to date (Matthews, 1991; MacFarlane and Popovich, 2000; Valentine et al., 2004; Jablonska et al., 2007). TRV is a positive-strand RNA virus with a bipartite genome. RNA1 encodes an RNA-dependent RNA polymerase and a movement protein (Mp). RNA2 encodes a coat protein (Cp), protein 2b (required for natural vector transmission), and protein 2c, of unknown function (MacFarlane, 1999). The non-essential proteins 2b and 2c can be deleted from TRV vectors and replaced by nucleotide fragments homologous to targeted genes. Virus invasion in the plant generates the dsRNA triggering the sequence-specific degradation of the targeted transcripts (Ratcliff et al., 2001; Dong et al., 2007). TRV vectors were used by Valentine et al. (2007) to deliver proteins and dsRNA to cyst nematodes feeding on Arabidopsis thaliana. Cyst nematodes feed on specialized syncytia formed by breakdown of plant cell walls and fusion of adjacent protoplasts. Despite the demonstration that TRV can deliver macromolecules to the feeding nematodes, the authors noticed a low proportion of syncytia producing dsRNA and consequently observed a moderate penetrance of RNAi in nematodes grown on TRV-inoculated A. thaliana plants.

Here the use of TRV to target RKN genes in Nicotiana benthamiana is described. Nicotiana benthamiana was chosen as it is a good host for Meloidogyne incognita, it allows uniform root invasion by TRV (MacFarlane and Popovich, 2000), and it is more susceptible to virus infection than many other host plants (Liu and Page, 2008). It is demonstrated that TRV propagates in RKN-infected roots and mediates the production of RNAi triggers in the giant cells that are the source of nutrients for the feeding nematode. Virus-derived RNAi triggers induced the silencing of the targeted genes in the progeny of the feeding nematodes. Troponin C was used as a target for gene silencing because the knock-out of a troponin C gene in the model nematode Caenorhabditis elegans caused severe embryo and larval arrest phenotypes (Kagawa et al., 1997; Terami et al., 1999; Ono and Ono, 2004). It was shown that TRV-mediated silencing of the orthologous gene Mi-tnc in M. incognita led to a defect in hatching of the juveniles. The calreticulin gene Mi-crt encodes an effector of parasitism secreted in the plant tissue throughout parasitism (Jaubert et al., 2005). TRV-mediated silencing of Mi-crt was obtained and a correlation was observed between Mi-crt transcript level and the ability of the nematode to establish successfully in the root tissue and complete its life cycle.

Materials and methods

Biological material

All work involving virus-infected material was carried out in containment chambers under licence no. 4061 from the Ministry for Youth, Education and Research. Nicotiana benthamiana plants were grown in compost, in 50 ml containers, under controlled conditions (25 °C, 16 h photo-period). Seeds from 35S:GFP transgenic N. benthamiana were kindly provided by Dr O Voinnet (Sainsbury Laboratory, Norwich, UK, present address IBPM, Strasbourg, France). Meloidogyne incognita nematodes from the
Calissane population were grown on tomato plants (*Solanum lycopersicum* L., cv. St. Pierre) in a greenhouse. Eggs and infective J2s were extracted as described by Rosso *et al.* (1999).

**Viral constructs and infections**

A 381 bp fragment of the nematode troponin C gene (*Mi-tnc*, AY861686) and a 304 bp fragment of the nematode calreticulin gene (*Mi-crt*, AF402771) were amplified by PCR from the *M. incognita* cDNA, using *Taq* DNA polymerase and the primers TNC-F-13 (5′-CGGGATCCCGATGAGATGTTTGCTG-3′) and TNC-R-303 (5′-CGACACCTCGTCAACAGGA-3′) for *Mi-tnc*-1, and CRT-F-318 (5′-AATGGATCTGACTTGTTGGCCTTGC-3′) and CRT-R-621 (5′-AATAAAGCTTGGCAAACTCCTCCAATCAGC-3′) for *Mi-crt*. The TNC and CRT PCR products were digested with *Hin*dIII and *Bam*HI, respectively, and inserted into PTV00 (Ratcliff *et al.*, 2001) digested with the same enzymes, to generate TRV::TNC and TRV::CRT (Fig. 1). The pTV.PDS and pTV.P vectors (Ratcliff *et al.*, 2001) were used to silence the endogenous phytotoxic desaturase gene (*PDS*) in wild-type *N. benthamiana*, and *GFP* in 35S::*GFP* *N. benthamiana* plants, respectively. The pBINTRA6, pTV00, pTV.PDS, and pTV.P vectors were kindly provided by Dr O Voinnet (Sainsbury Laboratory, Norwich, UK, present address IBPM, Strasbourg, France).

*Nicotiana benthamiana* was infected with the virus by co-agroinfiltration with *Agrobacterium tumefaciens* strain GV3101 carrying the TRV constructs and the pBINTRA6 vector required for viral replication and movement in the plant. Transformed *A. tumefaciens* were grown separately and centrifuged for 15 min at 3300 g. The pellet was washed in water, resuspended in 10 mM MgCl2, 10 mM MES, and 150 μM acetosyringone, kept at room temperature for 3 h, and diluted to an OD600 of 0.8. Separate cultures containing TRV constructs were mixed in a 1:1 ratio with the pBINTRA6 culture. Tobacco plants at the four-leaf stage were infiltrated with 200–300 μl of the mixture of *Agrobacterium* cultures, applied to the underside of the leaves with a 2 ml syringe with no needle. No increase of the silencing efficiency in the roots by use of the agrodrench method was observed (Ryu *et al.*, 2004). As a control of virus invasion, wild-type *N. benthamiana* were co-agroinfiltrated with a TRV::PDS construct targeted to the *PDS* gene (Ratcliff *et al.*, 2001). The virus propagation was visualized 1 week after co-agroinfiltration onwards by photobleaching typical to *PDS* inhibition in leaves, stems, axillary shoots, and sepals, as previously described (Ratcliff *et al.*, 2001; data not shown).

**Microscopy**

For observation of *GFP* silencing in galls, roots were embedded in 5% agar and cut into 150–200 μm sections with a Microm HM650V vibroslice. *GFP* expression in whole roots was observed with a MZCL III stereomicroscope (Leica Microsystems, Rueil-Malmaison, France) equipped with a E995 Coolpix camera (Nikon, Champigny sur Marne, France). Gall sections were observed with an Axioplan 2 microscope (Carl Zeiss, Le Pecq, France) equipped for epifluorescence microscopy, and images were collected with a digital Axiocam (Carl Zeiss).

**Transcript analyses**

Total RNA was extracted from eggs or freshly hatched juveniles by sonication in Trizol (Invitrogen, Paisley, UK) according to the manufacturer’s recommendations. Plant tissues were collected in liquid nitrogen and ground with a mortar and pestle for RNA extraction in Trizol. Total RNA was reverse transcribed using random hexamer primers and the Superscript III reverse transcriptase (Invitrogen). The presence of TRV RNA in galls was detected by reverse transcription-PCR (RT-PCR), using the coat protein-specific primers TRV-CP-F (5′-ACTCACGGGCTAAACAGTGCT-3′) and TRV-CP-R (5′-GACGATATGCGGACCTCACT-3′). The production of *tnc::GFP* fusion RNA in galls was detected using the primers TNC-142-F (5′-CGGGATCCCGTTTTGTCGGAGAGGTCGGGG-3′) and TNC-R (5′-GGATCCCTGACTGGACGAAATGGCTA-3′) digested with the *Hind*III and *Bam*HI–*Bam*HI–*Bam*HI mixture of *Agrobacterium* cultures, applied to the un-

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**Fig. 1.** Constructs used in TRV-mediated gene silencing. (A) Map of the *M. incognita* troponin C and calreticulin genes. The *Bam*HI–*Hind*III fragments were amplified by PCR with the primers TNC-F-13 and TNC-R-303 for *Mi-tnc* and CRT-F-318 and CRT-R-621 for *Mi-crt*, and inserted into pTV.P to produce the TRV::TNC and TRV::CRT constructs. The silencing of *Mi-tnc* and *Mi-crt* in nematodes was quantified by quantitative RT-PCR using the primer pairs TNC-F-368+TNC-R-491 and CRT-F-703+CRT-R-848, respectively. (B) Map of TRV vectors used for the in planta production of *Mi-tnc*, *Mi-crt*, and GFP dsRNA. The cDNA fragments were cloned between the left and right borders of the T-DNA (Lb and Rb), and their transcription was controlled by the cauliflower mosaic virus 35S promoter (35S) and terminator (T). Cp, coat protein; MCS, multiple cloning site. The TRV::GFP construct is from the pTV.P vector described by Ratcliff *et al.* (2001).
specific fragment. The ACT-F (5'-AGGGTTTGTCTGGA-
GATGATG-3') and ACT-R (5'-CGGGTTAAGGTTG-
CTTCAG-3') primers were used as a control for the
detection of plant actin transcripts.

Quantitative PCR was performed on a DNA Engine 2
(MJ Research, Minnesota, MN, USA) with Opticon 3.1
software (run: 95 °C for 10 min, followed by 40 cycles of
95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s) and
qPCR MasterMix (Eurogentec, Seraing, Belgium). Stan-
dard curves were established with four serial dilutions—
from 1/10 to 1/10 000—of Mi. incognita cDNA extracted
from 30 000 juveniles. For reference, the Mi. incognita 18S
ribosomal cDNA (GenBank accession no. U81578) was
amplified using the primers 18S-F-852 (5'-ACCGTGG-
CCAGACAAACTAC-3') and 18S-R-966 (5'-GATCGC-
TAGTTGGGATCGTT-3'). Transcripts from the Mi-tnc
gene were quantified using the primers TNC-F-368 (5'-
TTGACAAAGGGCAATGGT-3') and TNC-R-491 (5'-CTTCCGTCTTGTCGATTTCC-3') (Fig. 1) in 20 egg
masses collected randomly over the root system of each
individual plant. Transcripts from Mi-crt were quantified
using the primers CRT-F-703 (5'-GCTGAGGATTGG-
GATGAG-3') and CRT-R-848 (5'-TTTTGTGGCTCC-
CATTCTC-3') (Fig. 1) in J2s hatched from 20 egg masses
randomly collected on each individual plant. Relative
abundance was calculated with the relative standard curve
method (Applied Biosystems, Foster City, CA, USA) for
three technical replicates.

For northern blot analyses, 250 mg of leaf or gall tissue
was ground in liquid nitrogen and mixed with 1 ml of
Trizol. The suspension was centrifuged at 12 000 g for
10 min. The supernatant was collected, mixed with 200 μl
of chloroform, and centrifuged at 12 000 g for 15 min.
RNAs present in the aqueous phase were precipitated by
the addition of 500 μl of isopropanol and washed with 75%
ethanol. Isolated RNA was dissolved in water. As a marker
on RNA gels, 21–23 base siRNAs were obtained in vitro
from Dicer-digested dsRNA. For this purpose, Mi-tnc
dsRNA that covered the 289 bp fragment of Mi-tnc was
synthesized using the T7 RiboMax kit (Promega) using the
primers TNC-T7F-13 (5'-TAATACGACTCACTATA-
GGCCGAATAGAAATCC-3') and TNC-T7R-303 (5'-TAATACGACTCACTATAGGGCAGATTTTG-
CCGC-3'). After titration by spectrophotometry, 40 ng of
dsRNA were Dicer digested with 1 U of human Dicer
enzyme (Ambion, Huntingdon, UK) according to the
manufacturer’s recommendations. Small RNAs were sepa-
rated by electrophoresis on 15% polyacrylamide gels
containing 8 M urea and 1× TBE, stained with ethidium
bromide and electropherotransferred on BrightStar-Plus Po-
ositively Charged Nylon Membranes (Ambion). The mem-
brane were hybridized overnight at 42 °C in RNA
hybridization buffer (ULTRAhyb-Oligo, Ambion). The
riboprobe was synthesized from the 289 bp Mi-tnc fragment
with the kit MAXIscript T7 (Ambion) in the presence of
[32P]UTP (GE Healthcare, Buckinghamshire, UK) and
purified on a Sephadex G50 column. The membranes were
washed twice for 5 min and twice for 20 min in 2× SSC,
0.1% SDS at 42 °C, and exposed to X-ray films for 10 d
at −80 °C.

Nematode inoculations and phenotype analyses
To test the impact of Mi-tnc silencing on egg and larva
development, tobacco plants were virus inoculated and
infested with 500 J2s per plant, 3 weeks after agroinfil-
tration. Six weeks after nematode inoculation, the roots were
collected and egg masses were stained with 0.45% eosin B
(Sigma, Saint-Quentin Fallavier, France). Galls and egg
masses from 10 plants were counted for each treatment. To
analyse egg development and hatching of the juveniles, 10
gall masses per plant were randomly collected from four
plants for each treatment and set for hatching. The egg
masses were treated with 9.6% sodium hypochlorite for
2 min to dissolve the gelatinous matrix. The eggs were
placed on 10 μm pore sieves in water. Hatched J2s were
scored periodically from 3 d to 21 d after egg extraction. An
independent replicate of the whole experiment was done on
a second batch of agroinfiltrated plants.

To test the impact of Mi-crt silencing on nematode
virulence, eight tobacco plants were agroinfiltrated with
TRV::CRT and four plants were agroinfiltrated with
TRV::GFP as a control. The number of galls and egg
masses per plant was counted for each treatment. Twenty
egg masses per plant were randomly collected as mentioned
above and eggs were set for hatching. Freshly hatched J2s
issued from each individual tobacco plants were inoculated
on four tomato plants. Two hundred J2s were inoculated
per plant. The rest of the J2s were stored at −80°C for real-
time quantitative PCR. Six weeks after inoculation, the
tomato roots were washed, egg masses were stained with
0.45% eosin B, and the number of galls and egg masses was
counted.

Results

TRV-mediated GFP silencing in nematode feeding cells
The ability of TRV to propagate and mediate gene silencing
in roots and RKN feeding sites in N. benthamiana was first
investigated. Plants that constitutively express the GFP gene
under the control of a 35S promoter were used to monitor
TRV-mediated gene silencing in the root system. GFP
silencing was induced by co-infiltration of transgenic
35S::GFP plants with two clones of A. tumefaciens. One
A. tumefaciens clone contained the plasmid pBINTRA6, which
encodes the TRV RNA polymerase and Mp for virus replication and propagation (Ratcliff et al., 2001). For
virion formation and gene silencing targeting, leaves were
co-agroinfiltrated with a A. tumefaciens clone that contained
the TRV::GFP construct encoding the virus Cp fused to the 3’ terminus of GFP (Fig. 1). As a control of
agroinfiltration and virus invasion, wild-type N. benthamiana were agroinfiltrated with a pTV::PDS con-
struct targeted to the endogenous PDS gene (Ratcliff et al.,
2001). The virus propagation was visualized in leaves from
1 week after agroinfiltration onwards by photobleaching typical of PDS inhibition in leaves, stems, axillary shoots, and sepals as previously described (Ratcliff et al., 2001; data not shown). One week after agroinfiltration of 35S::GFP plants with the TRV::GFP construct, GFP silencing was observed in the lateral roots (Fig. 2A); this silencing extended to the entire root system, including the apex, 3 weeks after agroinfiltration. The intensity of residual GFP fluorescence differed between plants, possibly due to disparity in TRV propagation in the root systems.

To evaluate the ability of TRV to mediate dsRNA or siRNA production in the RKN feeding cells, 35S::GFP plants were agroinfiltrated with the TRV::GFP construct and they were inoculated with nematodes 3 weeks after agroinfiltration. GFP silencing was monitored periodically in the galls induced by RKNs and in the feeding cells. Agroinfiltration with TRV::GFP did not affect gall formation, and all galls displayed GFP silencing (Fig. 2B, C). One week after inoculation with the nematodes, gall sections showed uniform GFP silencing in all cell layers, including the inner tissues of the gall containing the giant cells (Fig. 2D–I). Gene silencing in the galls remained effective up to 5 weeks after nematode infection. As expected, GFP expression was later reduced in galls, due to 35S promoter downregulation (Goddijn et al., 1993), but the silencing was still visible in plants agroinfiltrated with the TRV::GFP construct. The presence of viral RNA in galls was confirmed by the detection of the Cp transcripts (Fig. 2J). Using RT-PCR the expected decline in Cp transcripts with time was not observed, possibly due to lack of quantitative relevance for this method. The observed silencing of GFP in the feeding cells showed that TRV can be used as a dsRNA delivery system for the feeding nematodes.

TRV-mediated silencing of nematode genes

The efficiency of TRV in silencing nematode genes critical for two different biological processes was assessed. The troponin C gene, important for embryo development and larval mobility in C. elegans, was targeted using a TRV::TNC construct where the GFP gene portion is fused to an additional 381 bp fragment of the RKN Mi-tnc cDNA. The calreticulin parasitism gene involved in the interaction with the plant was targeted using a TRV::CRT construct where the GFP gene portion is fused to a 304 bp fragment of the nematode Mi-crt cDNA (Fig. 1). The Mi-tnc and Mi-crt gene fragments were selected on the basis of a lack of local similarity with plant genes from the Viridiplantae databanks, to prevent off-target effects on plant genes. The presence of both genes as single copies in the nematode genome and the absence of detected local similarity with other genes in M. incognita reduced the risk of an off-target effect of the silencing in the nematode. Plants agroinfiltrated with TRV::GFP were used as a control. Agroinfiltrated plants were inoculated with RKNs and grown until the completion of the nematode life cycle. After giant cell induction, RKN juveniles developed into feeding stages and females extruded their eggs onto the root surface. The depletion of Mi-tnc and Mi-crt transcripts in eggs was analysed by quantitative RT-PCR (RT-qPCR) and compared with transcript levels in eggs grown on plants agroinfiltrated with the TRV::GFP construct. For both Mi-tnc and Mi-crt, a high disparity in the extent to which transcript levels were reduced was observed between plants. From six plants agroinfiltrated with TRV::TNC, two displayed <50% reduction in Mi-tnc transcripts in eggs, two displayed ~50% reduction, and two displayed 80–90% reduction in Mi-tnc transcripts (Fig. 3A). An analysis was carried out to determine if the silencing of Mi-tnc in the nematode progeny could be triggered by siRNA produced by the plant after TRV invasion. Tobacco plants were agroinfiltrated with TRV::TNC, inoculated with RKNs, and leaves and galls were collected 6 weeks after inoculation. As controls, leaves and galls were collected from non-agroinfiltrated plants and from plants agroinfiltrated with the TRV::GFP construct. In RNA blot analysis, Mi-tnc siRNAs were detected in the leaves of plants infiltrated with TRV::TNC and were absent from leaves of control plants. Because the nematodes are exclusively present in the root tissues, this result indicates that the plants are able to Dicer digest the Mi-tnc dsRNA produced during TRV invasion. Short siRNAs were also detected in the galls. Because galls contain both plant cells and the feeding nematodes, these siRNAs could originate from either the plant or the parasite. The results suggest that the feeding nematodes can ingest both Mi-tnc dsRNA and siRNA produced by the plant silencing pathway (Fig. 3B, C).

The silencing of the calreticulin gene was assessed in the progeny of RKNs feeding on tobacco plants agroinfiltrated with TRV::CRT. Eggs produced by the feeding nematodes were set for hatching and Mi-crt transcript depletion was analysed in the freshly hatched juveniles. Similar to what was observed after troponin C silencing, high disparity was observed in the reduction of the targeted nematode transcripts between plants. A 55% and 75% reduction in Mi-crt transcripts was observed in the progeny collected from two plants (Fig. 3D, plants D and H).

Phenotype analysis of the silenced progeny

An investigation was carried out to determine whether TRV-mediated silencing of the troponin C gene in M. incognita resulted in a phenotype similar to the embryo and larval arrest phenotypes observed after gene knock-out in the free-living nematode C. elegans. Nicotiana benthamiana plants agroinfiltrated with the TRV::TNC construct were inoculated with nematodes, and the numbers of galls and egg masses were scored 5 weeks after inoculation. Tobacco plants agroinfiltrated with TRV::TNC had no fewer galls or egg masses than non-infiltrated plants or plants agroinfiltrated with TRV::GFP, suggesting that the ingestion of Mi-tnc dsRNA/siRNA had no effect on the establishment of the nematode in the root tissue. The effect of Mi-tnc silencing on egg and juvenile development was further analysed. Direct counting and microscopy showed no difference between treatments in terms of the number of...
eggs per egg mass or in the development of the juveniles coiled in the eggs. The eggs were set for hatching, and freshly hatched J2s were counted periodically. Eggs collected from plants agroinfiltrated with TRV::TNC displayed a strong hatching defect during the first 13 d (Fig. 4). The total number of hatched J2s was 59% lower for eggs collected from TRV::TNC-infiltrated plants than for those from control plants, 21 d after hatching (Fisher’s LSD test, $P=0.0028$). This RNAi phenotype suggested that depletion of troponin C in juveniles did not affect J2 development but reduced the ability of juveniles to extrude from the egg shell, possibly due to altered muscle contraction.

The calreticulin gene Mi-crt is thought to be important for parasitism because its product is secreted into the feeding site by the nematode throughout parasitism (Jaubert et al., 2005). To investigate the potential value of TRV-mediated silencing for studying nematode parasitism genes, N. benthamiana plants were agroinfiltrated with TRV::CRT and inoculated with nematodes. No decrease was observed in the number of galls or egg masses in agroinfiltrated tobacco plants, suggesting that ingestion of Mi-crt dsRNA/siRNA by the feeding nematode had no effect on the nematode after the feeding site was formed and the nematode started feeding. To test the virulence of the silenced progeny, eggs produced by the feeding nematodes were set for hatching, and freshly hatched J2s issued from these eggs were used to inoculate tomato plants. The number of galls on tomato plants was scored 5 weeks after inoculation. As a control, juveniles hatched from eggs collected on tobacco plants agroinfiltrated with TRV::GFP were also inoculated on tomato plants (Fig. 5). Although juveniles collected from one control plant induced a surprisingly low number of galls on tomato plants, a correlation was observed between the abundance of Mi-crt transcripts and the ability of the nematodes to induce gall symptoms on tomato. The juveniles recovered from silenced progeny (plants D and H, Fig. 3D) induced 63.5% and 84% fewer galls on tomato than the controls.

![Fig. 2. TRV-mediated silencing of GFP in the roots and galls of transgenic 35S::GFP N. benthamiana. (A) GFP expression in a lateral root of a 35S::GFP plant (a) and a lateral root of a TRV::GFP-agroinfiltrated plant (b), 3 weeks after agroinfiltration. The displayed lateral roots have been sectioned from the root system. (B and B’). GFP expression in the root and in galls of a 35S::GFP plant, 5 weeks after nematode inoculation (wai). Galls are indicated by arrows. Nematode egg masses extruded on the root surface are visible as brown dots. (C, C’, and C’’) GFP silencing in the root and in galls of a 35S::GFP plant agroinfiltrated with TRV::GFP, 5 wai. (D–I) GFP silencing observed on 200 μm sections of galls collected on 35S::GFP plants agroinfiltrated with TRV::GFP, 1 wai (D), 2 wai (E), and 5 wai (F). Galls from non-agroinfiltrated plants were used as a control for each time point (G, H, I). (J) Viral RNAs detected by RT-PCR in tobacco roots 6 h after inoculation (6 h) and in galls dissected from plants agroinfiltrated with TRV::GFP at 1, 2, and 4 wai. The TRV invasion in galls was detected by the amplification of a coat protein-specific fragment. Actin was used as a positive control, and a PCR mixture with no template was used as a negative control (−). Bar=50 μm.](image-url)
Discussion

RNAi is a powerful tool for reverse genetics in many organisms and its application to high-throughput functional analyses in obligate biotrophic parasites such as RKNs could allow efficient exploitation of expanding genomic data. Here tests were conducted to determine whether virus-mediated silencing could be an effective strategy for the delivery of RNAi triggers to the feeding nematodes and silencing of RKN genes. TRV vectors were used because TRV is able to propagate efficiently in roots and because TRV vectors are amenable to high-throughput screenings (Dong et al., 2007; Jablonska et al., 2007). The ability of TRV to produce RNAi triggers in the feeding cells induced on tobacco roots by the nematode was tested. TRV-mediated silencing of a GFP transgene in the feeding cells suggested that TRV can be used to deliver RNAi triggers to the feeding nematodes. These results were in agreement with the report that TRV vectors can deliver small proteins and dsRNA to cyst nematodes Heterodera schachtii feeding on A. thaliana (Valentine et al., 2007). However, using TRV to produce fluorescent proteins in the nematode feeding sites, the authors observed a low proportion of syncytia effectively producing the recombinant proteins. In contrast, using N. benthamiana as a host, homogeneous silencing of

Fig. 3. Silencing of Mi-tnc and Mi-crt in the progeny of nematodes grown on TRV-agroinfiltrated tobacco plants and detection of Mi-tnc siRNA. (A) Relative abundance of Mi-tnc transcripts in eggs collected on TRV::GFP- or TRV::TNC-agroinfiltrated plants. For each treatment, the results obtained for egg masses collected from six individual plants are shown. The relative abundance of Mi-tnc transcripts was determined with respect to the ribosomal 18S internal standard, and is presented as the ratio related to the abundance of these transcripts in the control plant A agroinfiltrated with the TRV::GFP construct. Each bar represents the mean±SD of technical triplicate experiments. The results obtained from two independent experiments are shown. (B and C) RNA-blot analysis of Mi-tnc siRNA. (B) RNAs extracted from leaves and galls were separated on polyacrylamide gels and stained with ethidium bromide. (C) After blotting on a membrane, small RNAs were hybridized with a Mi-tnc-specific probe. As a size marker, Mi-tnc dsRNA was Dicer digested in vitro. (D) Relative abundance of Mi-crt transcripts in juveniles issued from TRV::GFP or TRV::CRT co-agroinfiltrated plants. For each treatment, 20 egg masses collected randomly on individual plants were set for hatching. The relative abundance of Mi-crt transcripts in freshly hatched juveniles is presented as the ratio related to the abundance of these transcripts in the control plant A agroinfiltrated with the TRV::GFP construct. Each bar represents the mean±SD of technical triplicate experiments.

Fig. 4. Analysis of nematode hatching after Mi-tnc TRV-mediated silencing. Eggs were collected from N. benthamiana plants agroinfiltrated with TRV::TNC and from non-agroinfiltrated plants or TRV::GFP-infiltrated plants as controls. A juvenile coiled in the egg (A) and a freshly hatched juvenile (B) are shown. The head region of the nematode is indicated by its stylet(s). Hatched J2s were counted periodically (C). Each bar represents the mean±SD of five values. The data presented are from one of two independent experiments, the results of which were comparable. Bar=15 μm.
the GFP transgene was observed throughout the root system and similar silencing efficiency was seen in all galls induced by the feeding RKNs. It was shown that TRV invasion triggered the RNAi machinery of the plant, leading to the production in galls of siRNAs homologous to the targeted gene. These siRNAs could be ingested by the feeding nematode. In addition, the nematode could possibly ingest the dsRNA molecules produced during virus replication. By targeting two RKN genes, silencing in the progeny of the feeding nematodes was observed. However, a high variability in gene silencing efficiency was observed between progeny collected from individual plants. Noticeably, variability in silencing efficiency was also observed between root systems of GFP transgenic plants inoculated with a TRV construct aimed at GFP silencing. Variability in the efficiency of virus propagation or in the trafficking of virus-derived RNAi triggers (Voinnet, 2005) could result in variations in dsRNA or siRNA accumulation between the root systems of individual plants. A threshold level of ingested RNAi triggers together with variability in efficiency of RNAi inheritance may determine the efficiency of silencing in the nematode progeny. Recently, the 16 kDa protein encoded by the TRV RNAi was shown to act as a suppressor of local silencing in N. benthamiana (Ghazala et al., 2008). As a consequence, TRV vectors that do not encode the 16 kDa protein could possibly provide better silencing efficiency, although the suppressor activity of the protein was only observed with low dosages of dsRNA inducer (Martínez-Priego et al., 2008).

The mechanisms of RNAi inheritance in nematodes are not yet understood and could involve chromatin remodelling as well as transcriptional silencing (Vastenhouw et al., 2006). In C. elegans, transmission of RNAi to the first generation has been observed for several genes, and in most cases RNAi is lost in later generations, except for some genes expressed in the maternal germline (Grishok et al., 2000). Analysis of RNAi spreading in C. elegans mutants has shown the presence of specific transcytosis pathways that transport silencing signals in the germline (reviewed by Whangbo and Hunter, 2008). Surprisingly, no orthologue has been found so far in the genome of parasitic nematodes for C. elegans genes involved in RNAi spreading and inheritance (Ghedin et al., 2007; Abad et al., 2008; Opperman et al., 2008), suggesting that these genes might not be conserved or may be rapidly evolving within the phylum.

Troponin C is a component of thin filaments that regulates body wall muscle contraction in response to changes in intracellular calcium. RNAi silencing of the troponin C gene tnc-1(pat-10) in C. elegans led to sterility by inhibiting ovarian contraction (Ono and Ono, 2004). The phenotype of M. incognita after TRV-mediated silencing of the orthologous gene Mi-tnc was analysed. Although efficient silencing was detected in the progeny of the feeding nematodes, no defect in egg production or embryo development was observed. However, Mi-tnc silencing led to a defect in hatching of the juveniles possibly due to a defect in muscle contraction. The absence of a lethal phenotype after Mi-tnc silencing could be explained by the observed incomplete silencing of the gene in the progeny, by subtle differences in troponin C functions in C. elegans and M. incognita, or by the presence in M. incognita of genes able to compensate for Mi-tnc knock-down.

Calreticulins from the endoplasmic reticulum act as chaperones whereas calreticulins located at the nuclear envelope, in the cytoplasm, or at the cell surface regulate numerous cell functions via calcium binding or direct interaction with signalling proteins. The calreticulin gene crt-1 from C. elegans is expressed in numerous tissues including intestine, pharynx, hypodermis, body wall muscle, uterus, and sperm. Silencing of crt-1 in genome-wide RNAi screening led to an abnormal locomotion phenotype (Kamath and Ahringer, 2003), and deletion mutants are affected in sperm and oocyte development (Park et al., 2001). It was previously observed that in M. incognita, the gene Mi-crt is expressed in the gonads of females, suggesting a role in oocyte development (Jaubert et al., 2005). In addition, Mi-crt is expressed in the secretory oesophageal glands of the nematode. The protein is secreted into the plant tissue and accumulates in the galls at the cell wall of the feeding cells, suggesting that Mi-CRT may play a role in the establishment of the nematode in the host (Jaubert et al. 2005). Here a correlation is shown between Mi-crt transcript abundance and the ability of the nematodes to

Fig. 5. Graphical representation of the correlation between Mi-crt transcript abundance in the nematode progeny and pathogenicity defects on tomato plants. Freshly hatched J2s issued from eight tobacco plants infiltrated with TRV::CRT (filled circles) and four tobacco plants infiltrated with TRV::GFP (open circles) were separated into two aliquots. One aliquot was used for quantification of Mi-crt transcript abundance (x-axis ± SD). The second aliquot was used to inoculate four tomato plants (200 J2s per tomato plant). The mean number of galls induced by each J2 sample is shown (y-axis ± SD). The diagonal line corresponds to the linear regression between the mean points. The linear correlation coefficient between the mean points is 0.61 (n=12, P=0.036).
induce galls, and additional evidence is provided for the importance of Mi-crt in parasitism. Because freshly hatched juveniles from the silenced progeny did not seem affected in terms of mobility, the reduction in gall induction may be related to a defect in feeding cell formation. Detailed analyses of root penetration, root invasion, and feeding cell induction after TRV-mediated silencing will help in defining the role of Mi-crt in parasitism.

Taken together the present results showed that although TRV is able to mediate the delivery of RNAi triggers to the feeding nematodes, its use for large-scale functional analysis of RKN genes is limited by heterogeneity in RNAi efficiency between individual plants, and that incomplete knock-out of the targeted genes in treated nematodes makes the interpretation of RNAi phenotypes difficult.

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