Technical Advance

Application of RNA Interference to Root-Knot Nematode Genes Encoding Esophageal Gland Proteins

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Plant parasitic nematodes have been, so far, refractory to transformation or mutagenesis. The functional analysis of nematode genes relies on the development of reverse genetic tools adapted to these obligate parasites. Here, we describe the application of RNA interference (RNAi) to the root-knot nematode *Meloidogyne incognita* for the knock-down of two genes expressed in the subventral esophageal glands of the nematode and potentially involved in parasitism, the calreticulin (*Mi-crt*) and the polygalacturonase (*Mi-pg-1*) genes. Incubation in 1% resorcinol for 4 h induced double-stranded RNA uptake through the alimentary track of the nematodes and led to up to 92% depletion of *Mi-crt* transcripts. Timecourse analysis of the silencing showed different temporal patterns for *Mi-crt* and *Mi-pg-1*. The silencing of *Mi-crt* was optimal 20 h after soaking, whereas the silencing of *Mi-pg-1* was optimal 44 h after soaking. For the two genes, the silencing effect was highly time-limited, since no transcript depletion was detectable 68 h after soaking.

Additional keywords: gene silencing, secretions.

Root-knot nematodes, genus *Meloidogyne*, are root endoparasites that can develop on a wide range of plant species. The infective second stage juvenile (J2) penetrates the root tip and migrates intercellularly until it reaches the differentiating vascular cylinder. During invasion, the nematode secretes, via its stylet, cell-wall-degrading enzymes that are produced by two subventral esophageal gland cells (Davis et al. 2000; Doyle and Lambert 2002). These enzymes are thought to be involved in the softening of the plant cell walls in order to facilitate the migration of the parasite. The nematode then perforates the wall of five to seven selected parenchyma cells with its stylet. In response to nematode stimuli, the selected cells differentiate into multinucleate hypertrophied giant cells that form a permanent feeding site. The identification of nematode-secreted proteins involved in giant-cell induction is important in understanding the molecular events controlling the plant-nematode interaction. Esophageal gland secretions, including secretions produced by the dorsal esophageal gland cell, are thought to be responsible for the formation of the feeding site. Many genes en-

coding nematode proteins potentially secreted from esophageal gland cells during parasitism have been identified (Vanholme et al. 2004). Plant parasitic nematodes have been so far refractory to transformation or mutagenesis, and no reverse genetic tool is available to analyze the roles of these genes in parasitism.

RNA-mediated genetic interference (RNAi) offers an opportunity to address the function of putative nematode pathogenicity factors. RNAi regulates gene expression at the post-transcriptional level (Mello and Conte 2004). Exogenous double-stranded (ds)RNA triggers the depletion of the homologous endogenous mRNA, which leads to the depletion of the encoded protein. In *Caenorhabditis elegans*, targeting the dsRNA into the nematode intestine by microinjection or ingestion allows the silencing of genes expressed in distal tissues (Guo and Kempheus 1995; Tabara et al. 1998; Timmons and Fire 1998). Recently, RNAi has been applied successfully to animal parasitic nematodes. In the adult stage of *Nippostrongylus brasiliensis*, dsRNA uptake into the nematode body was induced by the cationic lipid polymer lipofectin (Hussein et al. 2002). In the case of *Brugia malayi*, a low-volume culture system was developed to expose females to dsRNA (Aboobaker and Blaxter 2003). No in vitro culture system is yet available for plant parasitic nematodes, and the only free-living stage is the infective J2. A major challenge in applying RNAi to plant parasitic nematodes is getting the dsRNA ingested by the nonfeeding J2. Uptake of dsRNA was induced in plant parasitic cyst nematode J2 by addition of the neurotransmitter octopamine (Urwin et al. 2002). Obtaining a gene knockdown phenotype is dependent on other factors as well as dsRNA uptake efficiency. For example, the phenotype will be influenced by the duration of the silencing effect and by the turnover of the targeted protein.

Here, we present the application of RNAi to the plant parasitic root-knot nematode *M. incognita*. We tested RNAi efficiency on two single-copy genes whose active transcription in J2 subventral esophageal glands has previously been demonstrated. The first gene, *Mi-crt*, encodes a calreticulin protein that was first identified in stylet secretions purified from freshly hatched J2 (Jaubert et al. 2002a) and is secreted in planta by sedentary J3, J4, and females (M.-N. Rosso, unpublished data). The second gene, *Mi-pg-1*, encodes a polygalacturonase (E.C. 2.1.15) specifically expressed in the subventral gland cells of J2 (Jaubert et al. 2002b). The efficiency of silencing of these genes and the duration of the silencing effect are described.

RESULTS

Induction of dsRNA uptake.

Six compounds were tested for their ability to induce solute uptake by freshly hatched *M. incognita* J2. Fluorescein isothiocyanate (FITC) was used as a tracer for uptake efficiency. Freshly hatched J2, assessed as healthy by their high mobility, were soaked in FITC in M9 buffer with resorcinol (0.1 to 3%), serotonin (50 mM), octopamine (10 to 70 mM), tomato root intercellular fluid, lipofectin (0.1 to 14%), or collagenase (1 to 100 units/ μ l). J2 were washed in M9 buffer prior to observation. As a control, nematodes were soaked in FITC in M9 buffer. As shown in Table 1, FITC uptake was most effectively induced by resorcinol and serotonin. In 1% resorcinol, FITC uptake was clearly observed in the pharyngeal lumen of the juveniles and in the intestine posterior to the median bulb (Fig. 1A and A'). After incubation for 4 h in 1% resorcinol, the nematodes did not show any defect in mobility and infectivity (data not shown). In contrast, longer incubation times in resorcinol showed a detrimental effect on the nematodes, since about 10% of the nematodes were dead after 6 h of incubation in 1, 2, or 3% resorcinol. Incubations in lower resorcinol concentrations (0.5 to 0.8%) led to a marked reduction in FITC uptake, and no FITC uptake was visible after incubations in 0.1 to 0.4% resorcinol (data not shown). In the presence of 50 mM serotonin, FITC uptake was observed in the pharyngeal lumen (Fig. 1B and B'). Although a faint FITC signal was observed after 2 h of incubation, a strong signal was obtained from 4 h of incubation (Table 1). Intercellular fluids from healthy and infested tomato roots induced a weak FITC uptake through the pharyngeal lumen, although this uptake was observed in 80% of the nematodes after 16 h of incubation (Table 1). Octopamine treatment resulted in a very weak FITC signal in the pharyngeal lumen. However, 10% of the nematodes showed a strong signal in the amphids from 4 h of incubation on (Fig. 1C and C'). This signal in the amphids was stronger than the signal occasionally observed after incubating the nematodes in FITC in water or M9 buffer (Fig. 1D and D'). Octopamine concentrations ranging from 10 to 70 mM gave similar results. Lipofectin (0.1%) did not induce FITC uptake and showed a detrimental effect on the nematodes, since 2% of the nematodes were dead after 6 h of incubation and 80% were rod-shaped from 16 h of incubation on. Increasing the lipofectin concentration did not improve FITC uptake (data not shown). Nematodes incubated in FITC in the presence of 1 to 100 units of collagenase or M9 buffer per microliter showed autofluorescence in intestine lipid vesicles and occasional uptake in the amphids (Fig. 1D and D').

In order to check whether dsRNA would be ingested similarly to FITC, dsRNA from the *Mi-crt* gene (AF402771) was

labeled with fluorescent dye Cy3. The *Mi-crt* cDNA was used as a template to synthesize a 300-bp long dsRNA spanning the 390 to 690-nt region of the coding sequence. The dsRNA was labeled with Cy3 and dsRNA uptake was induced by incubating freshly hatched juveniles for 4 h in 2 mg of Cy3-dsRNA per 1 ml of 1% resorcinol. The nematodes were washed in water before observation. As controls, nematodes were soaked in Cy3-dsRNA in water or M9 buffer. Cy3-dsRNA uptake was observed in the pharyngeal lumen of the juveniles after soaking in 1% resorcinol (Fig. 1E and E').

Silencing efficiency assessment.

We examined whether dsRNA could direct RNAi in *M. incognita* J2 by in situ hybridization. Silencing of *Mi-crt* was analyzed by soaking 10,000 freshly hatched J2 in 40 μ l of a *Mi-crt* dsRNA solution (4 mg/ml) spanning the 390 to 690-nt region of the coding sequence in of 1% resorcinol for 4 h. After soaking, the nematodes were washed in water. Digoxigenin-labeled sense and antisense probes were synthesized from the 770 to 1,380-nt region of the *Mi-crt* cDNA. For controls, J2 were soaked in green fluorescent protein (GFP) *gfp5* dsRNA in 1% resorcinol or in 1% resorcinol without dsRNA. The coding sequence of *gfp5* (Siemering et al. 1996; accession number U87974) was used as a template for the synthesis of the 270-bp long dsRNA. As shown in Figure 2, a major decrease in *Mi-crt* transcript abundance in the esophageal gland cells was observed after soaking the nematodes in *Mi-crt* dsRNA. No *Mi-crt* transcript was visible in the majority of the silenced nematodes. Incomplete silencing, revealed by a reduction of transcript abundance, was observed in less than 10% of the silenced nematodes. The silencing effect observed by in situ hybridizations was further confirmed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (Fig. 3).

In order to score the silencing efficiency, the nematodes were incubated in *Mi-crt* dsRNA in 1% resorcinol for 4 h and the number of silenced nematodes was quantified by in situ hybridization. After soaking, the nematodes were washed in water and were hybridized with a *Mi-crt* antisense probe. The nematodes showing *Mi-crt* transcripts were counted. As a control, the same experiment was performed using *gfp5* dsRNA in resorcinol. The silencing efficiency was quantified by comparing the number of nematodes showing *Mi-crt* transcripts under these two conditions. The experiment was conducted in triplicate. Silencing efficiencies ranging from 75 to 92% were obtained (Table 2). Moreover, the silencing effect started to be visible 12 h after soaking.

Since octopamine was successfully applied to cyst nematode RNAi (Urwin et al. 2002), we tested the silencing of *Mi-crt*

Table 1. Percentage of *Meloidogyne incognita* second stage juveniles showing fluorescein isothiocyanate (FITC) uptake after exposure to various compounds^a

Incubations	1 h	2 h	4 h	6 h	16 h	24 h
Strong FITC uptake in the esophagus						
1% resorcinol	—	—	+++	++++	++++	++++
2% resorcinol	—	+	++++	++++	++++	++++
3% resorcinol	—	++	++++	++++	++++	++++
50 mM serotonin	—	+	+	++	++	+++
Weak FITC uptake in the esophagus						
Root intercellular fluid	—	+/-	++	++	+++	+++
50 mM octopamine	—	—	+/-	+/-	+/-	+/-
No FITC uptake						
0.1% lipofectin	—	—	—	—	—	—
10 units/ μ l collagenase	—	—	—	—	—	—
M9	—	—	—	—	—	—

^a FITC uptake was analyzed by fluorescence microscopy after incubation in the appropriate solution for 1, 2, 4, 6, 16 or 24 h and washing in M9 buffer. — = 0%; +/- = $\leq$ 10%; + = $\leq$ 25%; ++ = $\leq$ 60%; +++ = $\leq$ 80%; ++++ = $\leq$ 100%.

Fig. 1. Fluorescence microscopy showing fluorescein isothiocyanate (FITC) and double-stranded (ds)RNA uptake by *Meloidogyne incognita* second stage juveniles (J2). **A** and **A'**, After soaking for 4 h in FITC (1 mg/ml) and 1% resorcinol and washing in water, FITC was apparent in the lumen of the stylet, the pharyngeal lumen, the median bulb and the lumen of the intestine posterior to the median bulb. The lipid vesicles from the intestine showed auto-fluorescence. **B** and **B'**, After soaking in 50 mM serotonin, FITC in the lumen of the stylet, the pharyngeal lumen, and the median bulb. **C** and **C'**, After soaking in 50 mM octopamine, a strong FITC signal was visible in the amphids in 10% of the nematodes. **D** and **D'**, J2 soaked in FITC (1 mg/ml) and M9 buffer occasionally showed weak FITC uptake in the amphids. **E** and **E'**, After soaking in Cy3-dsRNA and 1% resorcinol, fluorescence was apparent in the pharyngeal lumen and the median bulb. a = amphids; i = intestine; il = intestine lumen; m = median bulb; pl = pharyngeal lumen; s = stylet. A,B,C,D,E show nematodes observed with differential interference contrast. A', B', C', D', and E' show nematodes observed using FITC filter setup. Bar = 10 μ m

after incubation in *Mi-crt* dsRNA in the presence of octopamine. The nematodes were incubated for 24 h in *Mi-crt* dsRNA in the presence of 50 mM octopamine or M9 buffer. After soaking, the nematodes were washed and directly analyzed by in situ hybridization or were incubated in water for 6 or 20 h prior to analysis. No reduction in *Mi-crt* transcripts was observed as compared with control nematodes incubated in dsRNA in M9 buffer (data not shown). Similarly, root intercellular fluid from healthy tomato plants did not induce any significant silencing effect (data not shown).

Duration of the silencing effect.

In other systems, the duration of the gene-silencing effect induced by RNAi partly depends on the transcription level and the turnover of the targeted transcripts. We assessed the duration of the silencing effect on *Mi-crt* and on the polygalacturonase gene *Mi-pg-1* (AY098646). Like *Mi-crt*, *Mi-pg-1* is highly expressed in the subventral esophageal glands of freshly hatched J2 (Jaubert et al. 2002b). *Mi-pg-1* dsRNA was synthesized from the 1,240 to 1,730-nt region of the *Mi-pg-1* coding sequence. The sense and antisense probes used for in situ hybridizations were synthesized from the 160 to 610-nt region of the *Mi-pg-1* coding sequence. In situ hybridizations were carried out after soaking the nematodes for 4 h in dsRNA in 1% resorcinol, followed by washing and incubation in water for 20, 44, or 68 h. The silencing efficiency in these different conditions was assessed as described above. In the case of *Mi-crt*, the highest silencing effect was obtained 20 h after soaking. This effect was reduced 44 h after soaking and was no longer detectable 68 h after soaking (Table 3). For *Mi-pg-1*, no silencing was observed 20 h after soaking and the highest silencing effect was observed 44 h after soaking. As observed for *Mi-crt*, no silencing of *Mi-pg-1* was detectable 68 h after soaking (Table 3). Three repetitions of the experiment showed the same results.

DISCUSSION

The silencing of root-knot nematode genes by soaking in dsRNA highly depends on dsRNA uptake by nonfeeding J2. Resorcinol (0.4%) was previously used to induce the secretion of esophageal proteins via the stylet by freshly hatched juveniles (Jaubert et al. 2002a). Here, we show that incubating J2 in 1% resorcinol for 4 h resulted in active pharyngeal uptake. Although long incubation times showed a deleterious effect on the nematodes, 4-h incubations did not show detrimental effects on nematode infectivity and development.

The biogenic amines serotonin and octopamine elicit specific and opposite behavioral responses in *C. elegans*. The rate

of pharyngeal contraction is increased by serotonin and suppressed by octopamine (Komuniecki et al. 2004). Rhythmic contraction and relaxation of the pharyngeal muscle transports bacteria from the mouth of the pharynx posteriorly to the intestine. Consistent with its activity on *C. elegans*, we observed

Fig. 3. *Mi-crt* transcript depletion in double-stranded (ds)RNA treated nematodes. A semiquantitative reverse transcriptase-polymerase chain reaction was realized on 50,000 *Meloidogyne incognita* second stage juveniles treated with resorcinol alone (1), *gfp5* double-stranded (ds)RNA (4 mg/ml) and 1% resorcinol (2), or *Mi-crt* dsRNA (4 mg/ml) and 1% resorcinol (3). The 390 to 690-nt region of the *Mi-crt* coding sequence was used for amplification. Transcripts from *troponin C* were used as a control. A specific decrease in *Mi-crt* transcript abundance was observed in nematodes treated with *Mi-crt* dsRNA (3). M = molecular weight marker.

Table 2. Quantification of the silencing efficiency^a

Stained nematodes	Experiment 1 ^b	Experiment 2	Experiment 3
<i>gfp5</i> dsRNA	100	100	100
<i>Mi-crt</i> dsRNA	10	8	25
Deduced silencing efficiency	90	92	75

^a Comparison of the number of nematodes showing *Mi-crt* transcripts in their esophageal glands after soaking in *gfp5* dsRNA or *Mi-crt* dsRNA. For each experiment, a single batch of nematodes was split into two samples for incubation in *gfp5* dsRNA or *Mi-crt* dsRNA. The percentage of nematodes showing *Mi-crt* transcripts after soaking in *gfp5* dsRNA was used to score the in situ hybridization efficiency.

^b Results shown as percentages.

Table 3. Comparison of the duration of the silencing effect for *Mi-crt* and *Mi-pg-1*^a

Incubation condition	% <i>Mi-crt</i> transcripts	% <i>Mi-pg-1</i> transcripts
4 h in target gene dsRNA + 20 h in H ₂ O	35	70
4 h in target gene dsRNA + 44 h in H ₂ O	65	45
4 h in target gene dsRNA + 68 h in H ₂ O	80	65
4 h in <i>gfp5</i> dsRNA + 20 h in H ₂ O	80	75

^a Assessed by in situ hybridization.

Fig. 2. Detection of *Mi-crt* transcripts in the subventral esophageal gland cells of *Meloidogyne incognita* second stage juveniles by in situ hybridization. **A** and **B**, After soaking in *gfp5* double-stranded (ds)RNA (4 mg/ml) and 1% resorcinol. **C** and **D**, After soaking in *Mi-crt* dsRNA (4 mg/ml) and 1% resorcinol.

that 50 mM serotonin induced FITC uptake through the pharyngeal lumen of *M. incognita* J2. In plant parasitic cyst nematodes, octopamine was shown to induce FITC uptake via the pharyngeal lumen and the excretory/secretory system (Urwin et al. 2002). In our hands, incubating *M. incognita* J2 in presence of 10 to 70 mM octopamine led to a very weak FITC uptake through the pharynx, and no visible uptake through the excretory/secretory pore was observed. However, FITC was more abundant in the amphids after octopamine treatment than in control conditions. It is tempting to suggest that neurotransmitters act differently on the pharyngeal and amphidial nerve synapses and on the excretory/secretory pore activity of cyst and root-knot nematodes.

Root intercellular fluids extracted from healthy or nematode-infested tomato plants induced FITC uptake via the pharyngeal lumen in 80% of the nematodes after 16 h of incubation. Yet the observed FITC uptake was weaker than after resorcinol or serotonin treatment. Although 7% lipofectin was successfully applied to the animal parasitic nematode *Nippostrongylus brasiliensis* (Hussein et al. 2002), incubating *M. incognita* juveniles in 0.1% lipofectin showed a strong deleterious effect on the nematodes and no induction of FITC uptake. This absence of FITC uptake is probably due to the difference of cuticle structure in plant and animal parasitic nematodes. Similarly, collagenase was used in an attempt to improve the permeability of the nematode cuticle (Sela et al. 1998) but did not induce solute uptake through the body wall.

The data presented here demonstrate that root-knot nematode genes expressed in the subventral esophageal glands can efficiently be silenced by RNA interference. Effective transcript depletion could be observed using two subventral gland genes, *Mi-crt* and *Mi-pg-1*. Consistent with the FITC and dsRNA uptake assays, gene silencing was optimal after incubating the nematodes in resorcinol, and up to 92% of the nematodes were silenced. In contrast with previous studies on cyst nematodes (Urwin et al. 2002), no silencing was observed after incubation in octopamine. Incubating the nematodes in root intercellular fluids led to a very weak silencing effect. This result is in accordance with the weak FITC uptake and the RNase activity we observed in the intercellular fluids (data not shown).

The delay in transcript depletion is dependent on the transcription level and the turnover of the transcripts. We show here that the timecourse of the silencing of two genes expressed in the same tissue can differ. The silencing of *Mi-crt* was visible 16 h after soaking and was optimal 20 h after soaking, whereas the silencing of *Mi-pg-1* was visible 44 h after soaking. The observed silencing effect is highly time-limited. The duration of the silencing effect observed for *Mi-crt* and *Mi-pg-1*—less than 72 h—is very short compared with the 14 days observed after silencing of the major sperm protein gene in cyst nematode J2 (Urwin et al. 2002) and the 6 days observed in the case of the acetylcholinesterase gene of *N. brasiliensis* (Hussein et al. 2002). Such a short duration of the RNAi effect could be due to the very high transcriptional activity of the subventral esophageal glands of root-knot nematode infective juveniles (Hussey and Mims 1990).

Depletion of the protein is necessary to observe a phenotypic effect of the silencing. In our hands, attempts to score the depletion of the Mi-CRT protein in nematodes by whole-mount immunofluorescence were unsuccessful, due to the low number of labeled nematodes in all conditions tested. Such an investigation would require the optimization of the fixation and permeabilization of the nematode tissues. The reduction in Mi-CRT protein was also tested by Western blot. Immunodetection by enhanced chemiluminescence did not allow the observation of a quantitative difference between silenced nematodes and controls (data not shown). Consequently, the depletion of

the Mi-CRT protein may be too low to be detected and could impair further pathogenicity tests.

Our results show that the silencing of two genes expressed in the subventral esophageal glands is highly time-limited. If this was a common feature of parasitism genes expressed in esophageal glands, the application of RNAi as a functional analysis tool would be limited to genes involved in the early stages of parasitism, up to 2 days postinoculation, i.e., during the invasion of the root tissue and the induction of the feeding site. Testing the silencing efficiency and duration for genes expressed in other tissues, in particular the dorsal esophageal gland cell, will be important for the functional analysis of genes involved in later stages, such as feeding site maintenance. Alternatively, feeding the nematodes with planta-produced dsRNA via plant RNA viruses could allow the knockout of functions involved in the late phase of parasitism. The recent development of efficient systems for dsRNA replication in plant roots opens up their application to the study of plant-nematode interactions (Ryu et al. 2004; Valentine et al. 2004).

MATERIALS AND METHODS

FITC and dsRNA uptake.

M. incognita population Calissane were grown in a greenhouse on *Lycopersicon esculentum* plants, and freshly hatched J2 were collected as described by Rosso and associates (1999). Fluorescein isothiocyanate (FITC) uptake was analyzed by soaking approximately 2,000 freshly hatched J2 in 200 µl of 1 mg/ml FITC isomer I (Sigma-Aldrich, Lyon, France) in M9 buffer (43.6 mM Na₂HPO₄, 22 mM KH₂PO₄, 2.1 mM NaCl, 4.7 mM NH₄Cl) with resorcinol (0.1% to 3%), serotonin (50 mM), octopamine (10 to 70 mM), tomato-root intercellular fluid, lipofectin (7 to 14%), or collagenase (1 to 100 units/µl). Tomato-root intercellular fluids were extracted as described by Hammond-Kosack (1992) from soil-grown healthy plants and infested plants six weeks after inoculation with *M. incognita*. J2 were slightly shaken in a rotary incubator for 1, 2, 4, 6, 16, or 24 h at room temperature. After soaking, the nematodes were centrifuged for 2 min at 13,000 rpm and were washed three times with M9 buffer. FITC uptake was observed by fluorescence, using a Zeiss microscope with suitable filters. Each experiment was repeated six times. As a control, nematodes were soaked in 1 mg of FITC per milliliter of water or M9 buffer.

dsRNA uptake was assessed after labeling *Mi-crt* dsRNA with the Silencer siRNA labeling kit (Ambion, Huntingdon, U.K.) according to the manufacturer's recommendations.

dsRNA synthesis.

In vitro transcriptions were performed using the MEGAscript T7 kit (Ambion) according to the manufacturer's recommendations. The *Mi-crt* cDNA was used as a template to synthesize dsRNA using primers CRTFo (5'-T7 adapter-TGACTTG GTGGCGGTTATC-3') and CRTBa (5'-T7 adapter-CGGCAA CAACTCCCAATCAG-3'). *Mi-pg-1* dsRNA was synthesized from *Mi-pg-1* cDNA using primers PGFo (5'-T7 adapter-GGC AAGGGGCTCCCGTCCA-3') and PGBa (5'-T7 adapter-ATA TTTCTCCATCATGTCCTACTG-3'). The coding sequence of *gfp5* (Siemering et al. 1996) was used as a template for the synthesis of *gfp5* dsRNA, using primers GFP5Fo (5'-T7 adapter-AGAGGGTGAAGGTGATGC-3') and GFP5Ba (5'-T7 adapter-TCCTGTTGACGAGGGTGT-3').

Transcript analyses.

For each silencing assay, 10,000 nematodes were collected after soaking in a dsRNA solution (4 mg/ml) or control buffer. Sense and antisense probes were synthesized from *Mi-crt* and

Mi-pg-1 cDNA using the forward and backward primers CRTFo2 (5'-GATAAGAAACCCGAAGATTG-3'), CRTBa2 (5'-ATTACGACGAAAAGTGAAC-3') and PGFo2 (5'-ATTATGTGTTCTCCGTTAG-3'), PGBa2 (5'-AAGGCTGAAGTTGAGGAAGA-3'), respectively. In situ hybridizations were conducted as described previously (Jaubert et al. 2002b). Sense probes were systematically used to control the specificity of the hybridizations.

Semiquantitative RT-PCR was performed on 50,000 *M. incognita* J2 incubated with a dsRNA solution (4 mg/ml) or control buffer. PolyA mRNA were purified using the Quick prep micro mRNA purification kit (Amersham Biosciences, Freiburg, Germany). First-strand cDNA were synthesized using a poly(dT) (5'-TGAAGGTTCCAGAATCGATAGGAATTC T₁₆G-3') primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). The 324-bp fragment from *M. incognita* troponin C (AY861686) coding sequence was amplified using primers TROPOFo (5'-TTCTTCGATATGTTTGACCGTGG-3') and TROPOBa (5'-TTTGTCTGGAGAGGTCGGGGCGATTTCGTG-3'). Amplification products were visualized on 1% agarose gels stained with 0.5 µg of ethidium bromide per milliliter.

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