Eur J Plant Pathol (2012) 134:131–144
DOI 10.1007/s10658-012-9971-y

RNAi silencing of the *Meloidogyne incognita Rpn7* gene reduces nematode parasitic success

Junhai Niu · Heng Jian · Jianmei Xu · Changlong Chen · Quanxin Guo · Qian Liu · Yangdong Guo

Accepted: 22 February 2012 / Published online: 3 March 2012

Abstract RNA interference (RNAi) techniques provide a major breakthrough in functional analysis for plant parasitic nematodes (PPNs). It offers the possibility of identifying new essential targets and consequently developing new resistance transgenes. To validate the potential of *Mi-Rpn7* as a target for controlling root knot nematode *Meloidogyne incognita* and to evaluate the feasibility of our modified platform for assessing silencing phenotypes, we knocked down the *Rpn7* gene of *M. incognita* using RNAi in vitro and in vivo. After soaking with 408-bp *Rpn7* dsRNA, pre-parasitic second-stage juvenile (J2) nematodes showed specific transcript knockdown, resulting in an interrupted locomotion in an attraction assay with Pluronic gel medium, and consequently in a reduction of nematode infection ranging from 55.2% to 66.5%. With in vivo expression of *Rpn7* dsRNA in transformed composite plants, the amount of egg mass per gram root tissue was reduced by 34% ($P<0.05$) and the number of eggs per gram root tissue was reduced by 50.8% ($P<0.05$). Our results demonstrated that the silencing of the *Rpn7* gene in *M. incognita* J2s significantly reduced motility and infectivity. Although it does not confer complete resistance, *Mi-Rpn7* RNAi in hairy roots produced significant negative impacts on reproduction and motility of *M. incognita*. In addition, the presented modified procedure provides technique reference for PPN genes functional analysis or target screening.

Keywords *Meloidogyne incognita* · *Rpn7* · RNAi · Composite plant · Reproduction · Motility

J. Niu · H. Jian (✉) · J. Xu · C. Chen · Q. Guo · Q. Liu
Key Laboratory of Plant Pathology, Ministry of Agriculture of China, Department of Plant Pathology,
China Agricultural University,
Beijing 100193, China
e-mail: hengjian@cau.edu.cn

J. Niu
Tropical Crops Genetic Resources Institute, CATAS,
Danzhou, Hainan 571737, China

J. Niu · Y. Guo
Department of Vegetable Science,
China Agricultural University,
Beijing 100193, China

Introduction

Plant parasitic nematodes (PPN) represent one of the major biotic constraints in world agriculture, which caused crop losses estimated at US$125 billion in 2003 (Chitwood 2003). Even though no recent and comprehensive surveys have been carried out, without a doubt, the economic value of nematode damage per annum may be higher currently. Nematodes of the order Tylenchida, especially the sedentary endoparasitic nematodes (root knot nematodes and cyst nematodes) are responsible for the majority of this damage.

Although various options for nematode control such as chemicals, cultural practices and use of

resistant varieties are currently available, none is perfect with respect to cost, environmental safety or efficacy against the harmful nematodes. During the last 5 years, RNA interference (RNAi) based plant biotechnology has been considered to meet these requirements and consequently has attracted wide attention.

RNAi has been adopted as a powerful tool for gene functional analysis and also has been employed for engineering plant resistance to various pathogens (Lindbo and Dougherty 2005; Chi-Ham et al. 2010; Niu et al. 2010). However, the same RNAi protocols developed on *C. elegans* by injection or soaking cannot be employed in research on PPNs, because they are too small to be microinjected with dsRNA and do not normally ingest fluid until they have infected a host plant (Bakhetia et al. 2005). In 2002 in a landmark study, Urwin et al. broke a technological bottleneck by using a chemical neuroactive stimulant (octopamine) to stimulate uptake by pre-parasitic second stage juveniles of soybean cyst nematodes (*Heterodera glycines*) and potato cyst nematodes (*Globodera pallida*). In that study, RNAi of the targeted genes of cysteine proteinases caused a shift from the normal female/male ratio of 3:1 to 1:1 by 14 days post-infection (dpi), and exposure of *H. glycines* to *hgctl* dsRNA corresponding to a protein with homology to C-type lectins (RT-PCR and Northern analysis confirmed the inhibition of target transcripts) resulted in a reduction of established nematode infection (Urwin et al. 2002). Soon after, the in vitro RNAi assays have also been established for sedentary forms including root knot nematodes (e.g. *Meloidogyne*), cyst nematodes (e.g. *Heterodera* and *Globodera*), migratory banana-root nematode (*Radopholus similis*) (Haegeman et al. 2009) and pine nematodes (*Bursaphelenchus xylophilus*) (Park et al. 2008). A number of genes of PPNs in different species, expressed in a range of different tissues and cell types, have been successfully targeted for silencing (Lilley et al. 2007; Rosso et al. 2009).

However, inducing RNAi by soaking (usually termed as in vitro RNAi) is potentially limited in that the effects are temporary, and it cannot be carried out on the parasitic stage of the PPNs. An alternative strategy termed in vivo RNAi is to engineer host plants with a hairpin struture of the target nematode gene, which would provide continuous delivery of the silencing triggers and should ultimately help identify gene targets for nematode control. In 2006, Yadav et al. (2006) developed transgenic tobacco lines expressing dsRNA for the *M. incognita* splicing factor and integrase gene, which induced down-regulation of the target genes and reduced parasitic infection (Yadav et al. 2006). Huang et al. (2006) transformed *Arabidopsis* with a hairpin structure of the essential parasitism gene *16D10* from root knot nematodes, resulting in significant reduction of gall formation by the common *Meloidogyne* species, ranging from 63% to 90%. Meanwhile, Steeves et al. (2006) demonstrated soybean host-induced silencing of the major sperm protein gene from *H. glycines*, and the bioassay data showed that up to a 68% reduction in eggs g^{-1} root tissue and a 75% reduction in eggs g^{-1} root tissue for progeny nematodes. Recently, Charlton et al. (2010) crossed two transgenic *Arabidopsis thaliana* lines expressing dsRNAs for *Mispc3* and *Miduox*, which were similar in a ≥50% reduction in nematode numbers in the roots and retarded female development. The crossing of transgenic lines that combined expression of both dsRNAs resulted in an additive effect that further reduced nematode numbers and developmental capacity (Charlton et al. 2010). Combining the above studies and other related publications, shows convincingly that *in planta* delivery of dsRNA is a powerful technique for investigating the function of nematode genes and identification of potential targets for parasite control.

Although there are about 30 successful applications that have been published on in vitro and in vivo RNAi in PPNs, this technology is still not routinely used as an investigative tool in molecular plant nematology. It cannot yet be employed in high-throughput functional analysis to play the same role as it does in *C. elegans*. One main restriction lies in technical discrepancies in the literature. In the documented studies, there are different experimental parameters with regard to soaking duration, nematode density, dsRNA length, stimulator and methods for evaluation of phenotypes. Associated with the methodological disparities, potential false positive or negative results make it difficult to directly compare results between and even within different laboratories (Dalzell et al. 2009; Rosso et al. 2009). Therefore, only an extensive study of different approaches will be able to identify the most effective and practical means of investigation for plant-parasitic nematodes using this technology.

To this end, in the current study, we analyzed the phenotype in *M. incognita* resulting from silencing of *Mi-Rpn7* using modified methods. We selected the

target gene based on the fact that it is essential for the integrity of the 26S proteasome, not only for eliminating damaged or misfolded proteins but also for degrading short-lived regulatory proteins involved in cell cycle regulation, DNA repair, signal transduction, apoptosis, and metabolic regulation (Hershko and Ciechanover 1998; Schwartz and Ciechanover 1999). In addition, knockdown of its *C. elegans* ortholog resulted in a variety of abnormal damaging phenotypes in multiple independent reports (Takahashi et al. 2002; Kamath et al. 2003; Rual et al. 2004; Sonnichsen et al. 2005). Therefore, we hypothesized that silencing the *Rpn7* gene could induce the corresponding phenotype and may provide a strong target for control of *M. incognita*. In this study, we focused on in vitro and in vivo RNAi screens using, respectively, a Pluronic F-127 gel and transgenic hairy roots of soybean which were adapted to evaluate the silencing phenotypes. We believe this attempt may provide a reference technique in PPNs RNAi research and target gene selection.

Materials and methods

Biological materials

The *M. incognita* isolate TSH1 was maintained on susceptible tomato (*Solanum lycopersium* cv. Zhongshu6) under greenhouse conditions. Roots were harvested from infected plants, and egg masses were collected by brief dissolution in sodium hypochlorite (2.5% v/v) for 2 min. The free eggs were washed thoroughly with sterile water and placed in water under complete darkness at 25°C for obtaining hatched juveniles (J2s).

The *Agrobacterium rhizogenes* strain K599 was used in the transformation experiments (a gift from Dr. Peter M Gresshoff, The University of Queensland, Australia). The binary vector pCAMBIA3301 contains the β-glucuronidase (GUS) reporter gene and the phosphinothricin acetyl transferase (Bar) gene and was optimized with DL-phosphinothricin (PPT) as a selectable marker in plant transformation. The pSAT6 RNAi vector with an RNAi cassette under the control of a 35S cauliflower mosaic virus (CaMV35S) promoter was a kind gift from Dr. Tzfira (Michigan University, USA) (Yelin et al. 2007).

Tomato (Zhongshu6 and Rutgers) seeds were sterilized and allowed to germinate as previously described (Wang et al. 2009b). Susceptible soybean (*Glycine max* L.) variety Kenfeng16 seeds were kept by the laboratory. The seeds were sterilized using chlorine gas (3.3 ml concentrated HCl added into 100 ml chlorine bleach) in a closed container overnight, then placed in the laminar flow hood for 30 min to remove residue chlorine gas and finally stored in sealed petri plates.

Initial cloning of *Mi-Rpn7* cDNA sequence

RNA was extracted from eggs, preparasitic second-stage juvenile (pre-J2), parasitic J2 (par-J2) and adult females using Trizol reagent (Invitrogen, UK) and contaminated DNA was digested by DNase I. cDNA was synthesized using SuperScript III (Invitrogen) according to the manufacturer's instructions. For preparing *Mi-Rpn7* template for dsRNA synthesis, a region corresponding to the 5′ end of the *Rpn7* gene was PCR-amplified from the cDNA isolated from preparasitic J2 of *M. incognita*, using $R7F_1/R7R_1$ primer pairs which were designed from the *Mi-Rpn7* sequence (AW570829) (Fig. 1a). The PCR product was cloned into the pGEM-T Easy vector (Promega, USA), transformed into DH5α competent cells and validated by sequencing. For semi-quantitative RT-PCR analysis, the synthesized cDNAs then were diluted to a concentration equivalent to 10 ng/μl, *M. incognita* β-actin was employed as an endogenous control reference for constitutively expressed housekeeping gene for normalization, and the replicated RT-PCR reactions were performed using gene specific primers R7 F_2/R7 R_2 (Table 1, Fig. 1b).

DsRNA synthesis and in vitro RNAi

The *Mi-Rpn7* cDNA was then used as a template for dsRNA synthesis using MEGA script RNAi kit (Ambion, USA) according to the manufacturer's instructions. T7-labeled gene-specific primers (see Table 1, *Rpn7* T7F and *Rpn7* T7R) were designed to amplify a 408 bp region of the *Rpn7* gene transcript. To avoid non-targeting effects, sequences homologous to the *Mi-Rpn7* target region were searched using the BlastN program against the *M. incognita* genome and EST database generated by the nematode whole genome sequencing project (Abad et al. 2008; http://meloidogyne.toulouse.inra.fr/). The gel-purified PCR products were used as templates for synthesis of sense and antisense *Rpn7* RNAs in a single tube.

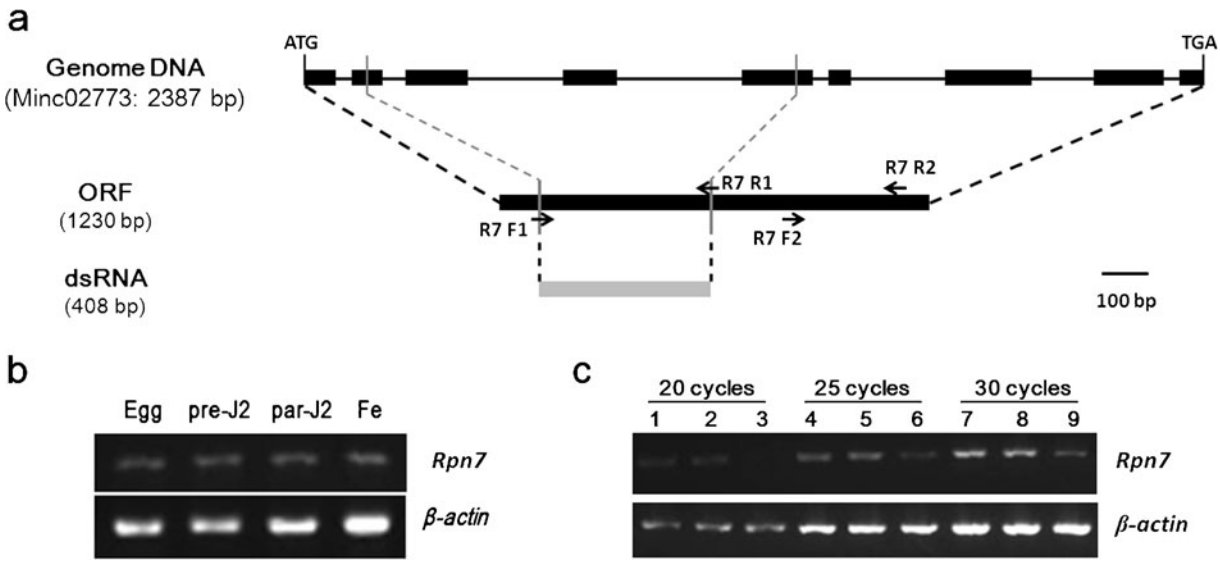

Fig. 1 Schematic of the target region for the *Mi-Rpn7* gene and transcript abundance after soaking. **a** Illustration of the *Mi-Rpn7* open reading frames (ORFs) and the regions with which the double-stranded RNAs (*gray*) used in this study have homology. Solid black boxes represent the relative positions of the encoded peptides. Minc02773: the retrieved *Rpn7* gene from the *M. incognita* genome annotation (MiV1ctg54: 66694..69160). **b** *Rpn7* expressing pattern on various developmental stages. pre-J2: preparasitic second-stage juvenile, par-J2: parasitic J2, Fe: adult females. **c** Semi-quantitative RT-PCR analysis of *Rpn7* transcript abundance after dsRNA soaking. Amplified products were visualized at 25, 30 and 35 cycles. Lanes 1, 4 and 7 represent J2 controls soaked in M9 buffer only; lane 2, 5, and 8 represent J2 controls soaked in M9 + octopamine buffer; lanes 3, 6 and 9 represent nematodes soaked in M9 + octopamine + dsRNA buffer

Freshly hatched J2s (≈10,000) of *M. incognita* were soaked in 0.25× M9 buffer (10.9 mM Na_2HPO4, 5.5 mM KH_2PO4, 2.1 mM NaCl and 4.7 mM NH_4Cl) containing 2 mg/ml dsRNA, 50 mM octopamine, 0.1 mg/ml FITC isomer I, 0.05% gelatin, and 3 mM spermidine and incubated for 12 h in the dark at room temperature on a rotator. Control samples were incubated in the same solution but without octopamine or dsRNA.

Examination of the effect of RNAi treatment in vitro

After soaking, the nematodes were collected by centrifugation at 7000×*g* for 2 min and washed three times with RNase-free water. About 200 nematodes from each of the treatments were observed with a fluorescence microscope (Olympus, Melville, NY) to monitor uptake of FITC, and the remaining nematodes were then recovered for 24 h. For examining the transcriptional silencing effect of *Rpn7* gene, 500 treated individuals were removed and RNA extracted using the Dynabeads mRNA Direct™ Kit (Invitrogen). After treatment with DNase I, the RNA was used to synthesize first strand cDNA and perform semi-quantitative RT-PCR as in "Initial cloning of Mi-Rpn7 cDNA sequence". The reactions were destructively sampled at 20, 25 and 30 cycles of PCR, to ensure comparisons were made during the exponential phase of the reactions.

To analyze the phenotypic and motor impact of target knockdown, we performed an attraction and migration assay modified from a previously described method (Wang et al. 2009b). Six thousand J2s were added into 30 ml 23% Pluronic gel F-127 and mixed uniformly at 15°C. The mixture was pipetted into a 24-well culture plate with 1 ml volume in each well. Subsequently, sterile 5-day-old tomato root tips (Zhongshu6 and Rutgers varieties) were placed in the centre of the gel, and the plates were incubated at room temperature for the gel to solidify. Beginning 8 h after the start of the assay, the distributions of J2s were monitored and photographed every hour. The photographs were taken with a Nikon SMZ-U dissecting microscope (Nikon, Melville, NY, USA). Infected roots were stained at 36 h with acid fuchsin to detect J2 as described (Byrd et al. 1983). The infected nematodes in tomato roots were counted, and the data was analyzed using standard statistical software (SPSS,

Table 1 A list of primers used in this study

Primers	Sequence (5′→3′)	Product size	Tests	Reference
R7 F_1	ATGATTCTTCTACTTGGCTGGCTTTACT	434 bp	RT-PCR	This study
R7 R_1	TTTCTGTCCCAATCACCTCCCTTT			
R7 F_2	CAGTTAAACGCAGAAATTGGCC	365 bp	RT-PCR	This study
R7 R_2	TGGTCAGTTTGGGCTGTTTCA			
Rpn7-T7 F	***TAATACGACTCACTATAGGG***[a]TTGGCTGGCTTTACTTGAA	408 bp	dsRNA synthesis	This study
Rpn7-T7 R	***TAATACGACTCACTATAGGG***[a]AATCACCTCCCTTTTCCAT			
Rpn7BglII	CC***AGATCT***TTGGCTGGCTTTACTTGAA [b]	408 bp	RNAi vector	This study
Rpn7EcoRI	CC***GAATTC***AATCACCTCCCTTTTCCAT [b]			
Rpn7SalI	CC***GTCGAC***AATCACCTCCCTTTTCCAT [b]	408 bp	RNAi vector	This study
Rpn7BamHI-BstE II	CC***GGATCCGGTTACC***TTGGCTGGCTTTACTTGAA [b]			
β- actin	TGATGATGCTCCTCGT	283 bp	RT-PCR	This study
β- actin	GACCCATTCCAA CCATAACT			
BarF25	GCCAGTTTCCCGTGCTTGAAGCCGGC	1000 bp	PCR	This study
BarR25	GGCGGTCTGCACCATCGTCAACCAC			
GUS-P1	GATGATAGTTACAGAACCGACG	750 bp	Probe amplification	Jian et al. 2009
GUS-P2	CATTCGGAATCTCCACGTTAC			
rolB-P1	GCTCTTGCAGTGCTAGATTT	450 bp	PCR	Furner et al. 1986
rolB-P2	GAAGGTGCAAGCTACCTCTC			
virG-P1	TTATCTGAGTGAAGTCGTCTCAGG	1000 bp	PCR	Lee et al. 2004
virG-P2	CGTCGCCTGAGCTTAAGTGTC			

[a] Italics and underlined letters refer to T7 promoter sequences.

[b] Italics and underlined letters refer to *Bgl* II, *EcoR* I, *Sal* I, *BamH* I and *BstE* II restriction sites, respectively.

version 16) to determine statistically significant differences by one-way analysis of variance tests.

Plasmid constructs

The binary vector pCAMBIA3301 contains a GUS reporter and the selectable bar gene encoding the enzyme phosphinothricin acetyl transferase (PAT) that confers resistance to the herbicide phosphinothricin. Both of these genes were driven by the CaMV 35S promoter. To assess the *Mi-Rpn7* silencing effect at the lateral stages after infection and validate the potential for *M. incognita* suppression, we cloned the *Mi-Rpn7* hairpin RNA expression cassette into the pCAMBIA3301 vector. The sense and anti-sense cDNA sequences *Rpn7*gene used in the in vitro RNAi experiments were amplified from the R7 F_1/R_1 clone with the gene-specific primers (Table 1) that introduced *Bgl* II, *EcoR* I, *Sal* I, or *BamH* I-*BstI* I restriction sites. The fragments were cloned into the *Bgl* II-*EcoR* I sites or the *Sal* I-*BamH* I sites of pSAT6 RNAi vector (Dafny-Yelin et al. 2007) to generate pSAT6 *Rpn7* RNAi (Fig. 4a) and then confirmed by sequencing. The constructs made in the pSAT6 RNAi vector were subcloned as *Nco* I-*BstI* I fragments into the binary vector pCAMBIA3301 to produce highly effective intron-containing "hairpin" RNA silencing constructs (pCAMBIA3301: *Rpn7* RNAi) (Fig. 4a). The pCAMBIA3301-derived RNAi constructs including the empty vector pCAMBIA3301 as a control were introduced into the *A. rhizogenes* strain K599 by the freeze-thaw method (Chen et al. 1994), and the transformations were confirmed by PCR.

Induction of hairy roots

A modified transformation system (Collier et al. 2005; Klink et al. 2009) was used to produce and screen transformed soybean roots. Transformed *A. rhizogenes* K599 clones (pCAMBIA3301 control and *Rpn7*-

RNAi construct) were grown individually in 5 ml YEB medium containing 100 mg/l kanamycin for 24 h at 28°C on a rotary shaker at 250 rpm until the *A. rhizogenes* concentrations achieved OD_{600nm}=0.6. The cultures were centrifuged at 5000×g for 10 min, and then the pellets were resuspended in distilled water. Five-day-old soybean seedlings were inoculated with *A. rhizogenes* by an injection three times parallel to the vascular bundle of hypocotyls as previously described (Li et al. 2010a, b). Later, seedlings were transferred to a tray covered with a plastic lid to keep high humidity. Over 5 to 8 days, a globular tumour developed at the wound site and visible hairy roots emerged from the tumour 2 weeks after infection. At 3 weeks post-infection, the transformed hairy roots (about 1–2 cm in length) with herbicide resistance were selected by smearing 2 mg/l PPT (determined based on selection from a series of PPT concentrations in a preliminary assay) on the root tips for two times continuously. After 3–5 days, the selected plant carrying abundant hairy roots for putative transformation were transplanted into soil and grown in a greenhouse (28°C, 16 h, light/24°C, 8 h, dark), with removal of primary roots by cutting the hypocotyl under the wounded site.

Histochemical GUS assay

To confirm the *A. rhizogenes*-mediated transformation and selection system, composite plants transformed with K599 harbouring vector pCAMBIA3301 were analyzed for GUS activity when the hairy roots were about 2–3 cm long. The activity of GUS was measured histochemically as described earlier (Jefferson et al. 1987). Tissues were incubated for 8 h at 37°C in staining solution containing 0.1 mM X-Gluc, 100 mM sodium phosphate buffer (pH7), 5 mM EDTA, 1 mM $K_4Fe(CN)_6$, 1 mM $K_3Fe(CN)_6$ and 0.1% (v/v) Triton X-100. The pigments were then removed by extraction with 95% ethanol prior to observation.

Southern blot analysis

To further confirm the transformation and selection system, both empty pCAMBIA3301 control and *Rpn7* RNAi caste composite plants were analyzed by PCR and Southern blot 4 weeks after transplantation. The same protocol for isolation of DNA from hairy roots was used as described earlier (Murray and Thompson 1980). PCR amplification was carried out to check the integration of the rolB rooting gene and the bar selection marker gene using specific primers (rolBF/rolBR and BarF25/BarF25, Table 1). In addition, the *A. rhizogenes* virG gene was detected to confirm the absence of bacterial contamination.

For Southern blot assays, approximately 30 μg of DNA from each sample was digested overnight with *Sac* I. The DNA fragments were separated by electrophoresis in a 0.8% agarose gel and transferred to a Hybond-N^+ (Amersham Biosciences, USA) nylon membrane. The DIG-dUTP (Roche, Germany) labelled GUS (750 bp) and *Rpn7* (408 bp) gene were used as probes for control and *Rpn7* silenced lines, respectively. Hybridization was carried out according to the manufacturer's instruction of the DIG high prime DNA labeling and detection starter kit II (Roche, Germany).

Challenge tests with *M. incognita*

To explore the potential of *Rpn7* gene silencing for nematode suppression, composite plants expressing empty vector control or the RNAi constructs were transplanted into sterilized soil (compost: sand=1:4) and maintained under greenhouse conditions as described in "Induction of hairy roots". After 1 week, in both groups, 30 plant root systems were challenged with 5000 freshly hatched *M. incognita* J2s per plant. The numbers of roots, egg masses and eggs g^{-1} root were counted 40 days post-inoculation, and the collected data were analyzed using a standard statistical package (SPSS version 16).

Results

The efficacy of silencing *Mi-Rpn7* by dsRNA soaking

To confirm *Mi-Rpn7* silencing, RT-PCR was performed on cDNA obtained from the dsRNA treated and control J2s. It was evident that the *Mi-Rpn7* transcript was reduced in dsRNA soaked J2s compared with untreated controls, and only a very faint residual amplification could be detected after 25 cycles (Fig. 1c). Meanwhile, the β-actin control gene showed equivalent expression levels in the nematodes soaked with dsRNA and the controls (M9 with octopamine or not) (Fig. 1c). The expression pattern was confirmed

by triplicate amplification reactions. Thus, the RNAi silencing effect was specific to *Mi-Rpn7* and not due to global RNA degradation in nematodes.

In the migration assay, once the Pluronic gel solidified, the dispersing nematodes from the two control groups were strongly attracted and aggregated to the root tips rapidly. By 4 h, most of the nematodes had arrived at the zone of the root tips, and the nematode clusters were observed clearly. After 8 h, the attached nematodes were invading, and none were moving away from the roots, although the few J2s which perhaps were set out at a distance were still swimming towards the roots (Fig. 2). However, significant differences were seen in the dsRNA-soaked nematodes, many of which lost the typical body wave shape and displayed abnormal locomotor behaviour (not shown). Some moved in situ continually, while others aggregated at the root tips but were significantly slower compared with the controls. After 8 h, there were still many nematodes distributed away from the root tips (Fig. 2). Because the number of nematodes near the root tips was too great to count with confidence, the juveniles that had invaded into the roots were stained with acid fuchsin after 36 h and counted under a microscope (Fig. 3a). The results indicated that the number of invading dsRNA soaked J2 was significantly fewer than those from control groups ($P<0.01$), with a reduction ranging from 55.2% to 66.5%, and no significant difference ($P<0.05$) between the two control groups (Fig. 3b). These experiments were repeated on two tomato cultivars, Zhongshu6 and Rutgers, and similar results were found.

Co-transformation and selection

The chimeric root systems formed by *A. rhizogenes* K599 transformation are composed of both transformed and non-transformed roots. Although high co-transformation efficiencies were proven, some roots were not considered in further quantitative characterization. In the in vivo RNAi assay, 3–5 days after screening with PPT, the control plants inoculated with *A. rhizogenes* K599 containing no binary vectors showed very poor root development and turned brown. However, clones carrying either the pCAMBIA3301 or pCAMBIA3301:*Rpn7* RNAi binary vector induced composite plants that still had numerous roots with normal growth.

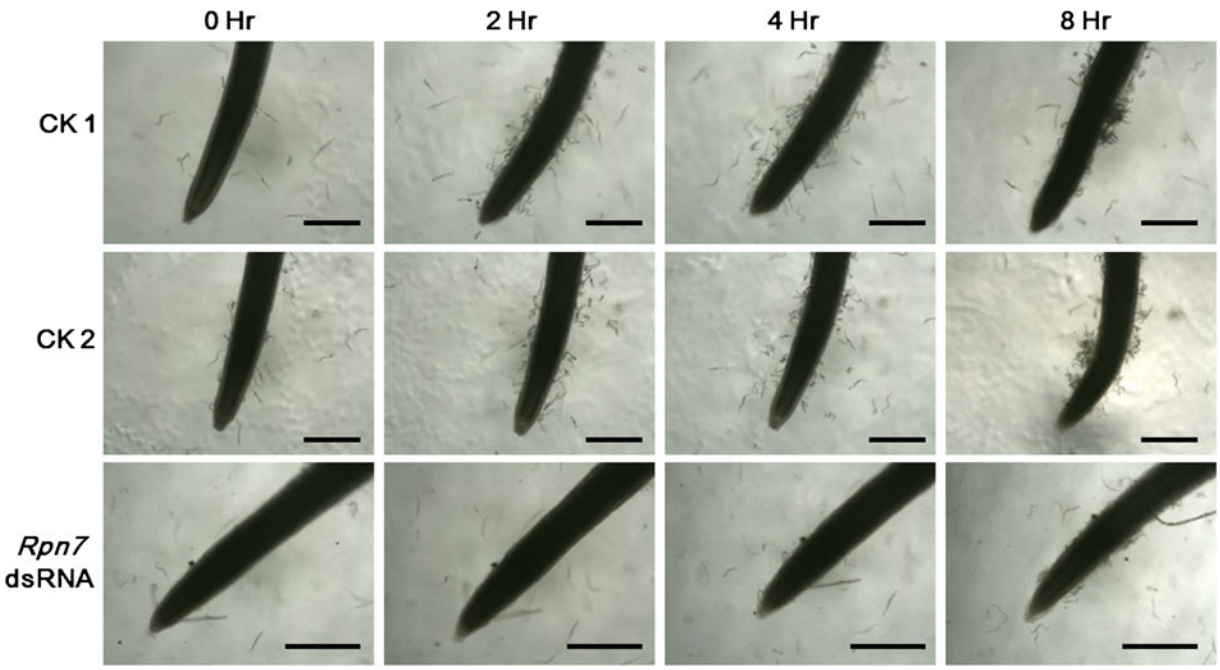

Fig. 2 Movement and attraction of *M. incognita* J2 in attraction assays with Pluronic gel medium. Different nematodes after soaking were attracted to tomato root tips and were monitored at 0, 2, 4 and 8 h. CK1: control nematodes soaked in M9 buffer only; CK2: control nematodes soaked in M9 buffer containing 50 mm octopamine; *Rpn7* RNAi: nematodes soaked in M9 containing 50 mm octopamine and dsRNA. Scale bar=1 mm

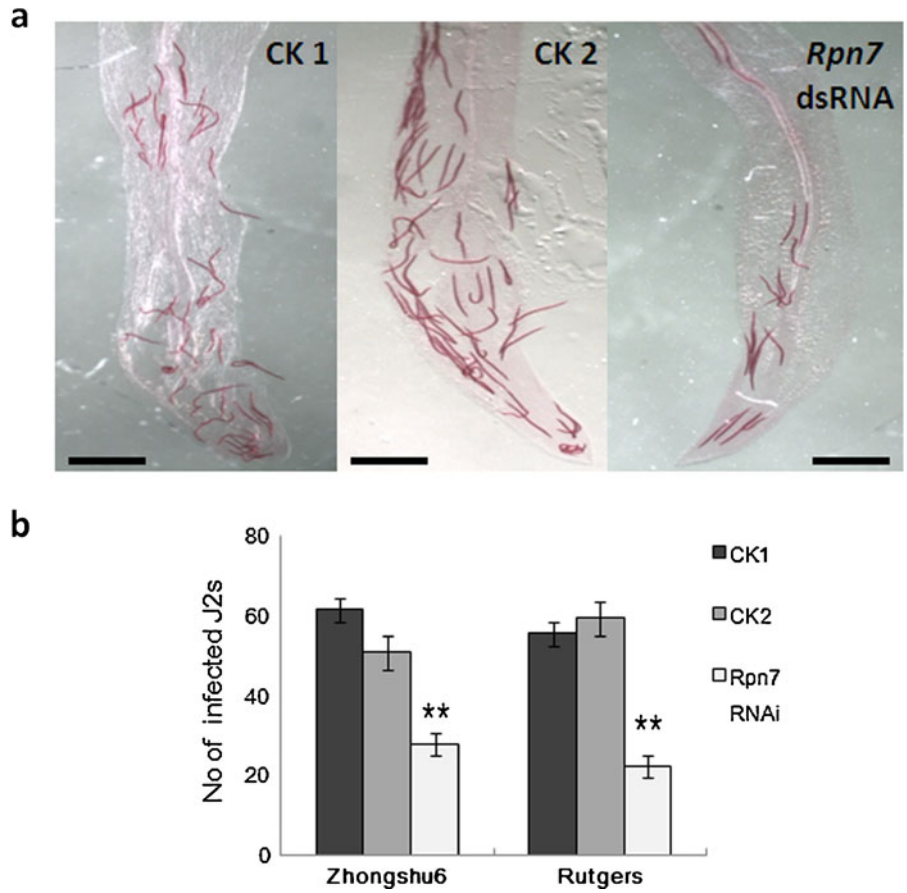

Fig. 3 Primary invading nematodes in tomato roots. **a** Nematodes stained with acid fuchsin at 36 h after inoculation on tomato. Scale bar=1 mm. **b** The number of J2s attracted to two genotypes of tomato and penetrated roots at 36 h after inoculation. *Error bars* indicate standard error. ** $P<0.01$

GUS staining, PCR and Southern blotting

In the sampled composite plants inoculated with K599:pCAMBIA3301, some roots showed negative expression of GUS in the plants without PPT treatment (Fig. 4b). Moreover, almost all of the surviving roots exhibited GUS activity in the plants through PPT screening (Fig. 4b). The co-transformation and selection was further confirmed by PCR analysis with GUS-P1/GUS-P2 primers. For the plants transformed with the RNAi construct of *Mi-Rpn7*, genomic DNA were isolated from the roots of separate composite plants to initially screen for plant transformation, and PCR was performed for testing the presence of target fragments. As expected, the *Mi-Rpn7* fragment was amplified from all putative transgenic roots that grew rapidly under selection. No amplification was observed from control root samples transformed with empty pCAMBIA3301.

To further verify the co-transformation and selection in soybean hairy roots, Southern blot analysis were carried out on both sampled plants transformed with pCAMBIA3301 and pCAMBIA3301: *Rpn7* RNAi constructs. The hybridization results with the GUS probe indicated that integration was detected in the root samples of control plants P1–P4, and in the sampled plant P1, co-transformation events were confirmed with four hairy roots (lane 2–5, Fig. 5a). Meanwhile, no hybridization signals were detected in root samples induced by K599 (no binary vector) only (lane 1, Fig. 5a). With the *Rpn7* probe, at least four hybridized bands were detected in the *Rpn7* RNAi hairy roots samples (lanes 4–6, Fig. 5b). As expected, no signals were found in root samples induced by empty *A. rhizogenes* K599 (lane1, Fig. 5b) or containing pCAMBIA3301 (lane 2–3, Fig. 5b). Overall, the hybridization results correlated well with the results obtained from the PCR analysis.

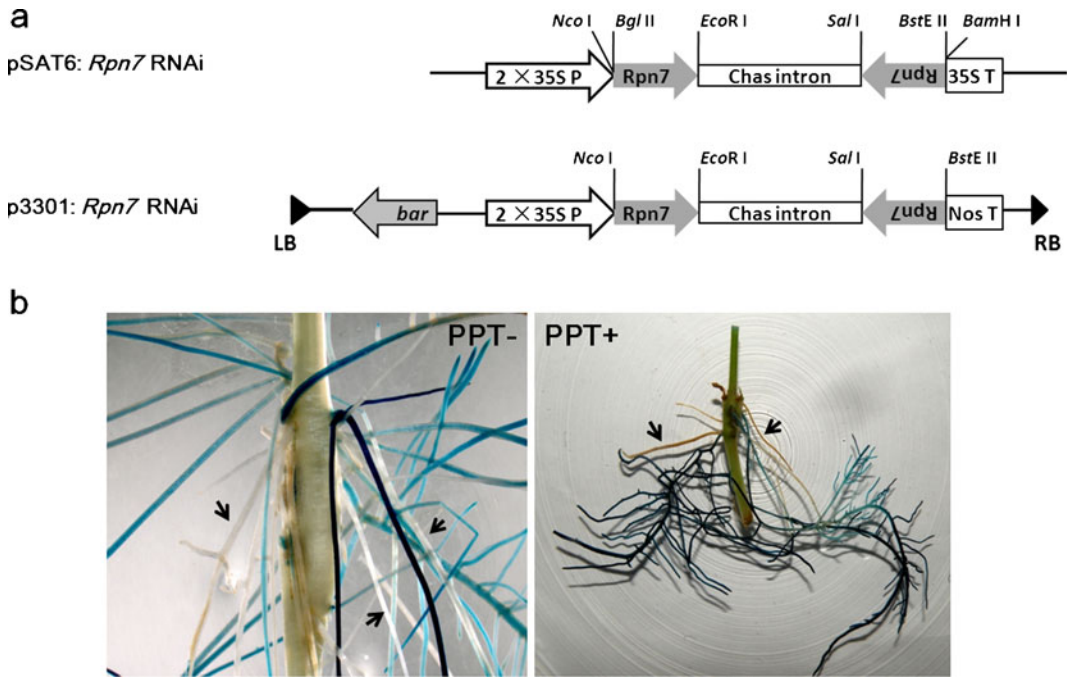

Fig. 4 Construction of *Rpn7* RNAi vector and hairy root transformation. **a** *Rpn7* RNAi construct map of the expression cassette. pSAT6 *Rpn7* RNAi: *Rpn7* RNAi construct was cloned into the multiple cloning site, without the T-border region. p3301 *Rpn7* RNAi: schematic diagram of the binary vector pCAMBIA3301 harboring the *Rpn7* RNAi construct, which was used in the production of *Rpn7* dsRNA in transgenic plants. Nopaline synthase terminator (Nos T), promoter 35S of CaMV (35S), phosphinothricin acetyltransferase (*bar*), left border (LB), right border (RB). **b** β-glucuronidase (GUS) histochemical assay of transgenic roots transformed with pCAMBIA3301. Hairy root systems without (*left*) or with (*right*) a PPT screening procedure are shown. The *arrows* indicate non-transformed hairy roots

Bioassays

Nematode demographic assays were performed to detect *M. incognita* suppression by inoculated fresh hatched J2s on composite plants. The plants were harvested 40 days after inoculation. Although the primary root numbers per chimeric plant were variable, ranging from 4 to 14, the branch roots develop more extensively, and all of the soybean plants developed advanced root systems. By monitoring the development of the nematode, we found that nematodes in both pCAMBIA3301 and pCAMBIA3301: *Rpn7* RNAi harbouring roots had reached the final stage in their life cycle, and silencing the *Rpn7* gene did not have a strong suppressive effect on the nematode development. In addition, comparisons of the numbers of root galls, egg masses and eggs g^{-1} root tissue, indicated the level of resistance against inoculated J2s. The results of the hairy root bioassays, as summarized in Table 2, indicated that although a very small reduction was found in the number of galls between pCAMBIA3301:*Rpn7* RNAi and the empty pCAMBIA3301 lines, this difference was not statistically significant (Table 2). However, the numbers of egg masses and eggs g^{-1} root tissue of *Rpn7* RNAi plants were significantly different from the control lines according to the Tukey test ($P<0.05$). For the pCAMBIA3301: *Rpn7* RNAi and the empty pCAMBIA3301 lines, the mean values of egg masses g^{-1} root tissue were 24.7±1.83 and 37.4±3.04, respectively, (34% reduction); and mean values of eggs g^{-1} root tissue were 3510±157.93 and 7130±136.12, respectively (50.8% reduction) (Table 2). Together, these results indicated that silencing the *Rpn7* genes significantly interrupted the reproduction of the nematode.

Discussion

In the present study, we combined a modified protocol to evaluate the knockdown phenotype of *Mi-Rpn7* by both in vitro and in vivo RNAi methods. By a simple soaking procedure, a 408-bp length dsRNA was

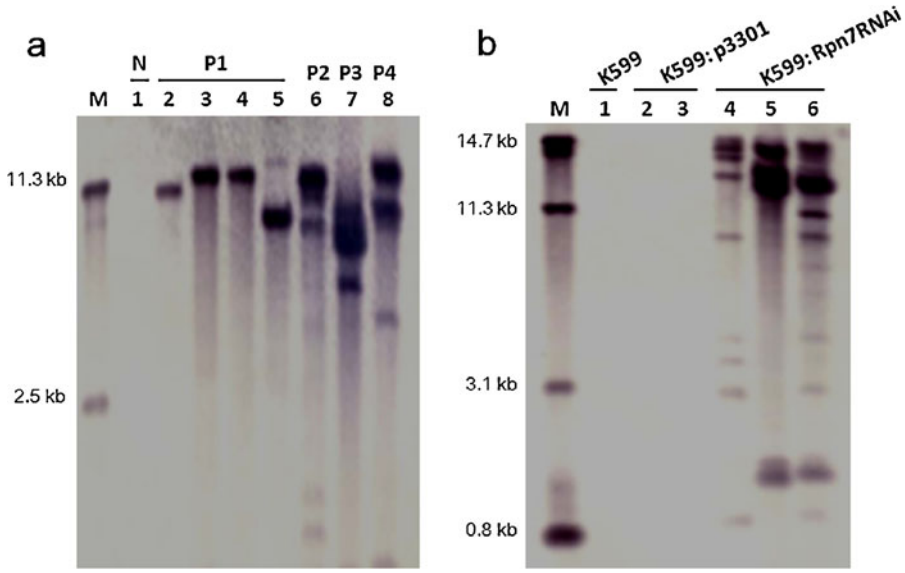

Fig. 5 Southern blot analysis of the transgenic hairy roots. **a** Southern blot confirm the integration of pCAMBIA3301 empty vector in control lines using the GUS fragment as a probe. M: Marker; 1: negative control line in which roots were induced by *A. rhizogenes* (K599) without vector. 2–5: four independent hairy roots from a composite transgenic plant (P1). 6–8: another three individual chimeric plants (P2-P4) were pooled. **b** Southern blot confirming the *Rpn7* RNAi lines using the *Rpn7* fragment as a probe. M: marker; 1: pooled DNA of hairy roots induced by *A. rhizogenes* K599 (no binary vector); 2–3: pooled DNA of hairy roots induced by K599 harboring p3301 empty vector; 4–6: three individual chimeric plants (P2–P4) transformed with the *Rpn7* RNAi construct were pooled

transmitted into *M. incognita* J2s. In attraction assays with Pluronic gel medium, the treated J2s moved to roots much more slowly and fewer numbers (reduction ranging from 55.2% to 66.5%) reached and infected the host than did the control. In addition, we transformed the soybean hairy roots with DNA constructs encoding hairpin shaped dsRNA of the same fragment in *Mi-Rpn7*, and the transformed roots were screened and challenged with *M. incognita*. Compared to non-transgenic plants, the chimeric plants had significantly fewer numbers of egg masses g^{-1} root (34% reduction) and eggs g^{-1} root (50.8% reduction). These results indicated that silencing the *Rpn7* genes interrupted the motility, invasiveness, fitness or fertility of the nematode significantly, which are partly consistent with previous findings in *C. elegans* (Takahashi et al. 2002; Kamath et al. 2003; Rual et al. 2004).

Pluronic F-127 is a clear, non-toxic co-polymer that forms a gel at room temperature and a liquid under 15°C when the concentration is 20–30%. Furthermore, unlike agarose medium (juveniles of root knot nematodes only move on the surface of agar) (Wallace 1968), the Pluronic gel has similar features to that of soil, and second-stage juveniles can move freely within the medium. Because of these advantages, the gel has been applied in chemotaxis studies (Wang et al. 2009a, 2010; Reynolds et al. 2011). In our preliminary experiment, Pluronic F-127 gels containing a series of J2s were added onto tomato roots, and then the infected nematodes were counted after staining with acid fuschin. The

Table 2 Comparison reproduction from the bioassay with negative control and *Rpn7* dsRNA transgenic roots

Treatment	No.of Plants	No.of roots/plant	No.of Egg galls g^{-1} root	No.of Egg mass g^{-1} root	Eggs g^{-1} root
Control[a]	30	8±4	56.2	37.4±17.4	7130±1345
Rpn7 RNAi[b]	30	8.6±5.4	51.9	24.7±15.3**	3510±897**

[a] Hairy roots inoculated with K599 : p3301 as negative control.

[b] Hairy roots inoculated with K599 : p3301: rpn7 RNAi.

"**" represents significant differences between treatments ($P<0.01$).

result showed that the numbers increased proportionally with of the inoculation dose until more than 300 J2s were added (unpublished observation). The possible reason for this saturation effect is that the pre-parasitic juveniles prefer invading the elongating portion of the root tip which can attract many nematodes but has limited volume and is not capable of accommodating them. Furthermore, more inoculated nematodes would seriously damage the roots through penetrating the epidermal cell and shuttling inside the root. Therefore, in the present study, we employed the Pluronic F-127 gel in the nematode attraction assays. When the gel at 15°C was mixed with 200 J2s and pipetted into a 1-cm diameter well containing a sterile 5-day-old tomato plant, the stained roots were shown to keep an integrated shape, and the invading nematodes could be counted clearly under a microscope (Fig. 3a).

Successful in vitro RNAi tests have been observed in both cyst and root-knot nematodes treated with dsRNA ranging in size from 42 bp to 1300 bp, with incubation periods ranging from 4 h to 7 days (Rosso et al. 2009). Thus, it was reasonable that we chose to soak nematodes in a solution containing 408-bp dsRNA for 12 h in an RNAi treatment procedure. In most cases, depletion of transcripts after soaking is transient, and the transcript level of the targeted genes would return to normal once nematodes are removed from exposure to dsRNA. In addition, different nematode species and/or different genes may have varying time limits of effective RNAi-mediated knockdown. For example, a glutathione S-transferase gene (*Mi-gsts-1*) of *M. incognita*, was effectively silenced beginning at 1 h after a 1-h soaking period in dsRNA solution with 4% resorcinol and 25 m serotonin, and the silencing was no longer detectable 48 h after the treatment (Dubreuil et al. 2007). After a 4-h incubation of dsRNA, the calreticulin (*Mi-crt*) and polygalacturonase (*Mi-pg-1*) genes of *M. incognita*, displayed maximum transcript repression after a further 20 h and 44 h respectively, and for both genes, no transcript depletion was detectable at 68 h post-soaking (Rosso et al. 2005). In *H. glycines*, transcript repression of a *β-1, 4*-endoglucancase gene was observed immediately following a 16-h dsRNA treatment, and transcript recovery was observed at 6 to 10 days after soaking (Bakhetia et al. 2007). In this study, after a 12-h soaking in *Mi-Rpn7* dsRNA, *M. incognita* J2s were incubated in nuclease-free water for 24 h at room temperature to allow the RNAi effects to be proceed before being assayed by semi-quantitative RT-PCR. The *Mi-Rpn7* transcript was reduced in the treated J2s compared with the control groups (Fig. 1c), demonstrating that the silencing effect was achieved. However, the transcript was still faintly detectable, similar to the observations in previous studies (Rosso et al. 2005; Lilley et al. 2005), which is possibly due to the testing period of the treated nematodes not corresponding to the peak of transcript repression.

Although it is possible that neuro-stimulants may not be absolutely required to stimulate solute ingestion by J2 of root knot nematodes (Rosso et al. 2009), to facilitate the uptake of dsRNA, we incubated *M. incognita* infective (J2) juveniles in 50 mM octopamine (as previously used for this purpose). Taking into account the observation that octopamine can inhibit the motility of *G. pallida* juveniles and therefore prevent analysis of RNAi-induced locomotor defects, we carried out the experiment with octopamine as a control in parallel. Similar to most previous reports (Urwin et al. 2002; Chen et al. 2005; Lilley et al. 2005; Alkharouf et al. 2007; Shingles et al. 2007), no impaired motility phenotype was observed in our migration assays. A possible reason is that *M. incognita* has a different sensitivity level to octopamine than *G. pallida*, but even in comparing different batches of nematodes of the same species, they may not all respond in the same way to neuro-stimulants (Rosso et al. 2009). Therefore, we concluded from the migration assay that the impaired motility of *M. incognita* was derived from effects of the *Mi-Rpn7* silencing. It is noteworthy that both *M. incognita* and *G. pallida* J2s present abnormal phenotypes and behaviour on exposure to non-nematode dsRNAs, so it is possible that the toxic RNA motifs, enzymatic cleavage, translational suppression or other unknown confounding issues induced inhibitory phenomenon (Dalzell et al. 2009), so non-nematode dsRNAs (such as GFP dsRNA) should be employed and facilitated performing a more rigorous analysis of RNAi phenotype in future research.

We employed tomato and soybean in in vitro and in vivo RNAi analysis separately. Both species are hosts of *M. incognita* and could be infected successfully, but tomato attracted J2s much more quickly than soybean. Moreover, tomato roots with a smaller-size are facilitated to be placed in culture plate wells in migration assay. In recent publications, a rapid and non-axenic

procedure for obtaining soybean transgenic hairy roots was developed using *A. rhizogenes*. This led to the production of so-called "Ex vitro composite plants" comprising of a transgenic hairy root system attached to non-transformed shoots and leaves (Collier et al. 2005; Kereszt et al. 2007; Cao et al. 2009). This procedure has been considered an attractive alternative for the production of plant-mediated RNAi to suppress *H. glycines* (Klink et al. 2009; Li et al. 2010a, b) and *M. incognita* (Ibrahim et al. 2011). There are two main challenges that would limit the application of chimeric plants for in vivo RNAi analysis on PPNs. First, it is currently not feasible to obtain a transgenic soybean plant from the hairy root, so the material cannot be maintained or introduced into other lines by crossing. Notably, the transgenic hairy root from *Lotus corniculatus*, which is a legume crop, has been regenerated into plants, so it is promising that transgenic soybean plants would be obtained from *A. rhizogenes*-induced hairy roots by tissue culture aided by plant growth regulators. Second, the hairy root system of 'composite' plants, consisting of co-transformed and non-transformed roots, makes quantitative analyses complicated. Li et al. (2010a, b) has induced hairy roots via *A. rhizogenes* carrying pIG121Hm and pANDA35HK vectors, both of which harboured the hygromycin resistance gene (HPT) and neomycin phosphotransferase gene (NPTII) so that inoculated hypocotyls could be screened on an MS-based medium containing either 200 mg/l kanamycin or 20 mg/l hygromycin to suppress untransformed roots. Ibrahim et al. (2011) transformed hairy roots with binary vectors carrying the GFP marker, and transformed roots were recognized by their green colour when viewed under blue light. Therefore, non-transformed roots without GFP expression could be trimmed before replanting.

In this investigation, we demonstrated a modified version of the soybean hairy root transformation procedure with the pAMBIA3301 vector, which carries the bar gene and the facility to use PPT as a selection agent to eliminate non-transformed roots. Based on the GUS assay after screening, the frequency of positive roots per chimeric plant was 92.3%. Southern blot analysis was performed to further confirm the integration of transgenic DNA in soybean hairy roots. However, a minority of non-transgenic roots still survived; because the escapes were shorter and unbranched, they may have developed after the PPT screening. In addition, based on the Southern blot results, an individual chimeric plant could be produced through multiple independent transformation events, with different copies and integrating loci in the genome, giving rise to variable expression levels of the transgene. However, the present modified procedure was developed to identify the promising genes for PPNs control by engineered host-delivered RNAi. The genes of the highest interest always have significant roles in reproduction or fitness, and their silencing would not be misevaluated. For example, targeting *M. incognita* TP and MSP genes in composite plants resulted in reduction of galls by 92% and 94.7%, respectively (Ibrahim et al. 2011). Expressing dsRNA of *H. glycines Cpn-1* gene in transgenic hairy roots led to a 95% reduction of eggs in roots (Li et al. 2010b). Therefore, the use of chimeric plants was shown to be a fast and reliable *in planta* method for testing transgene efficacy against cyst and root-knot nematodes.

Compared with the control lines, the *Mi-Rpn7* RNAi plants had no statistically significant difference in the number of galls, even though a very small reduction was found. However, the transformed composite plants showed a 34% reduction in egg masses g^{-1} root tissue and a 50.8% reduction in eggs g^{-1} root tissue (Table 2), so we suspected that *Rpn7* plays a more important role in propagation than during early development. Although the dsRNA transcripts and effect of siRNA in roots were not confirmed by Northern blotting, these results still indicated that silencing the *Rpn7* genes significantly interrupted the reproduction of the nematode. However, *Mi-Rpn7* RNAi did not result in an equivalent resistance to *M. incognita* as with other targets, such as 16D10 (Huang et al. 2006), splicing factor and integrase genes (Yadav et al. 2006), TP and MSP (Ibrahim et al. 2011), all of which produced almost complete resistance.

In conclusion, our results demonstrate that silencing of the *Rpn7* gene had a substantial detrimental effect on *M. incognita* motility and invasiveness. Plant-delivered *Rpn7* RNAi resulted in a significant although not complete level of resistance in transgenic hairy roots. This gene may not be an ideal control target by itself, but can potentially be employed as a candidate in combination with other targets to silence in order to enhance the efficacy of RNAi for nematode control. In addition, the presented modified attraction assays and transformation procedure may provide a

reference for functional genomic analysis of PPNs or screening of promising control genes.

Acknowledgments The authors would like to thank Professors Tevi Tzfira and Dr. Peter M Gresshoff, the University of Queensland, Australia, for providing pSAT6 RNAi vector, pCAMBIA3301 vector and *A. rhizogenes* strain K599 kindly. This study was financially supported by Program for Changjiang Scholars and Innovative Research Team in University (IRT1042), and the Special Fund for Agro-scientific Research in the Public Interest, China (No. 20113018) and the National Foundation of Natural Sciences, China (No. 30971901), and project 948 from the Ministry of Agriculture of China (No. 2011-G4)

References

Abad, P., Gouzy, J., Aury, J. M., et al. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nature Biotechnology, 26*, 909–915.

Alkharouf, N. W., Klink, V. P., & Matthews, B. F. (2007). Identification of *Heterodera glycines* (soybean cyst nematode [SCN]) cDNA sequences with high identity to those of *Caenorhabditis elegans* having lethal mutant or RNAi phenotypes. *Experimental Parasitology, 115*, 247–258.

Bakhetia, M., Charlton, W. L., Urwin, P. E., et al. (2005). RNA interference and plant parasitic nematodes. *Trends in Plant Science, 10*, 362–367.

Bakhetia, M., Urwin, P. E., & Atkinson, H. J. (2007). qPCR analysis and RNAi define pharyngeal gland cell-expressed genes of *Heterodera glycines* required for initial interactions with the host. *Molecular Plant-Microbe Interactions, 20*, 306–312.

Byrd, D. W., Kirkpatrick, T., & Barker, K. R. (1983). An improved technique for clearing and staining plant tissue for detection of nematodes. *Journal of Nematology, 15*, 142–143.

Cao, D., Hou, W. S., Song, S. K., et al. (2009). Assessment of conditions affecting *Agrobacterium rhizogenes*-mediated transformation of soybean. *Plant Cell, Tissue and Organ Culture, 96*, 45–52.

Charlton, W. L., Harel, H. Y., Bakhetia, M., et al. (2010). Additive effects of plant expressed double-stranded RNAs on root-knot nematode development. *International Journal for Parasitology, 40*, 855–864.

Chen, H., Nelson, R. S., & Sherwood, J. L. (1994). Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. *Biotechniques, 16*, 664–670.

Chen, Q., Rehman, S., Smant, G., et al. (2005). Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi. *Molecular Plant-Microbe Interactions, 18*, 621–625.

Chi-Ham, C. L., Clark, K. L., & Bennett, A. B. (2010). The intellectual property landscape for gene suppression technologies in plants. *Nature Biotechnology, 28*, 32–36.

Chitwood, D. J. (2003). Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture-Agricultural Research Service. *Pest Management Science, 59*, 748–753.

Collier, R., Fuchs, B., Walter, N., et al. (2005). Ex vitro chimeric plants: an inexpensive, rapid method for root biology. *The Plant Journal, 43*, 449–457.

Dafny-Yelin, M., Chung, S. M., Frankman, E. L., et al. (2007). pSAT RNA interference vectors: a modular series for multiple gene down-regulation in plants. *Plant Physiology, 45*, 1272–1281.

Dalzell, J. J., McMaster, S., Johnston, M. J., et al. (2009). Non-nematode-derived double-stranded RNAs induce profound phenotypic changes in *Meloidogyne incognita* and *Globodera pallida* infective juveniles. *International Journal for Parasitology, 39*, 1503–1516.

Dubreuil, G., Magliano, M., Deleury, E., et al. (2007). Transcriptome analysis of root-knot nematode functions induced in the early stages of parasitism. *New Phytologist, 176*, 426–436.

Furner, I. J., Huffman, G. A., Amasino, R. M., et al. (1986). An Agrobacterium transformation in the evolution of the genus Nicotiana. *Nature, 319*, 422–427.

Haegeman, A., Vanholme, B., & Gheysen, G. (2009). Characterization of a putative endoxylanase in the migratory plant-parasitic nematode *Radopholus similis*. *Molecular Plant Pathology, 10*, 389–401.

Hershko, A., & Ciechanover, A. (1998). The ubiquitin system. *Annual Review of Biochemistry, 67*, 425–479.

Huang, G., Allen, R., Davis, E. L., et al. (2006). Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 14302–14306.

Ibrahim, H. M., Alkharouf, N. W., Meyer, S. L., et al. (2011). Post-transcriptional gene silencing of root-knot nematode in transformed soybean roots. *Experimental Parasitology, 127*, 90–99.

Jefferson, R. A., Kavanagh, T. A., & Bevan, M. W. (1987). GUS-fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal, 6*, 3901–3907.

Jian, B., Hou, W., Wu, C., Liu, B., Liu, W., Song, S., et al. (2009). *Agrobacterium rhizogenes*-mediated transformation of Superroot-derived Lotus corniculatus plants: a valuable tool for functional genomics. *BMC Plant Biology, 9*, 1–14.

Kamath, R. S., Fraser, A. G., Dong, Y., et al. (2003). Systemic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature, 421*, 231–237.

Kereszt, A., Li, D. X., Indrasumunar, A., et al. (2007). Testing gene function in transgenic soybean roots. *Nature Protocols, 2*, 948–952.

Klink, V. P., Kim, K. H., Martins, V., et al. (2009). A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of development of female *Heterodera glycines* cyst formation during infection of *Glycine max*. *Planta, 230*, 53–71.

Lee, M. H., Yoon, E. S., Jeong, J. H., et al. (2004). *Agrobacterium rhizogenes*-mediated transformation of Taraxacum platy-carpum and changes of morphological characters. *Plant Cell Reports, 22*, 822–827.

Li, J., Todd, T. C., Oakley, T. R., et al. (2010a). Host-derived suppression of nematode reproductive and fitness genes decreases fecundity of *Heterodera glycines* Ichinohe. *Planta, 232*, 775–785.

Li, J., Todd, T. C., & Trick, H. N. (2010b). Rapid in planta evaluation of root expressed transgenes in chimeric soybean plants. *Plant Cell Reports, 29*, 113–123.

Lilley, C. J., Goodchild, S. A., Atkinson, H. J., et al. (2005). Cloning and characterization of a *Heterodera glycines* aminopeptidase cDNA. *International Journal for Parasitology, 35*, 1577–1585.

Lilley, C. J., Bakhetia, M., Charlton, W. L., et al. (2007). Recent progress in the development of RNA interference for plant parasitic nematodes. *Molecular Plant Pathology, 8*, 701–711.

Lindbo, J. A., & Dougherty, W. G. (2005). Plant pathology and RNAi: a brief history. *Annual Review of Phytopathology, 43*, 191–204.

Murray, M. G., & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research, 8*, 4321–4326.

Niu, J. H., Jian, H., Xu, J. M., et al. (2010). RNAi technology extends its reach: engineering plant resistance against harmful eukaryotes. *African Journal of Biotechnology, 9*, 7573–7582.

Park, J., Lee, K., Lee, S. J., et al. (2008). The efficiency of RNA interference in *Bursaphelenchus xylophilus*. *Molecules and Cells, 26*, 81–86.

Reynolds, A. M., Dutta, T. K., Curtis, R. H., et al. (2011). Chemotaxis can take plant-parasitic nematodes to the source of a chemo-attractant via the shortest possible routes. *Journal of the Royal Society, Interface, 8*, 568–577.

Rosso, M. N., Dubrana, M. P., Cimbolini, N., et al. (2005). Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. *Molecular Plant-Microbe Interactions, 18*, 615–620.

Rosso, M. N., Jones, J. T., & Abad, P. (2009). RNAi and functional genomics in plant parasitic nematodes. *Annual Review of Phytopathology, 47*, 207–232.

Rual, J. F., Ceron, J., Koreth, J., et al. (2004). Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Research, 14*, 2162–2168.

Schwartz, A. L., & Ciechanover, A. (1999). The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annual Review of Medicine, 50*, 57–74.

Shingles, J., Lilley, C. J., Atkinson, H. J., et al. (2007). *Meloidogyne incognita*: molecular and biochemical characterization of a cathepsin L cysteine proteinase and the effect on parasitism following RNAi. *Experimental Parasitology, 115*, 114–120.

Sonnichsen, B., Koski, L. B., Walsh, A., et al. (2005). Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. *Nature, 434*, 462–469.

Steeves, R. M., Todd, T. C., Essig, J. S., et al. (2006). Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. *Functional Plant Biology, 33*, 991–999.

Takahashi, M., Iwasaki, H., Inoue, H., et al. (2002). Reverse genetic analysis of the *Caenorhabditis elegans* 26S proteasome subunits by RNA interference. *Biological Chemistry, 383*, 1263–1266.

Urwin, P. E., Lilley, C. J., & Atkinson, H. J. (2002). Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. *Molecular Plant-Microbe Interactions, 15*, 747–752.

Wallace, H. R. (1968). The dynamics of nematode movement. *Annual Review of Phytopathology, 6*, 91–114.

Wang, C., Bruening, G., & Williamson, V. M. (2009a). Determination of preferred pH for root-knot nematode aggregation using pluronic F-127 gel. *Journal of Chemical Ecology, 35*, 1242–1251.

Wang, C., Lower, S., & Williamson, V. M. (2009b). Application of pluronic gel to the study of root-knot nematode behaviour. *Nematology, 11*, 453–464.

Wang, C., Lower, S., Thomas, V. P., et al. (2010). Root-knot nematodes exhibit strain-specific clumping behavior that is inherited as a simple genetic trait. *PLoS ONE, 5*, e15148.

Yadav, B. C., Veluthambi, K., & Subramaniam, K. (2006). Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. *Molecular and Biochemical Parasitology, 148*, 219–222.

Yelin, M. D., Chung, S. M., Frankman, E. L., & Tzfira, T. (2007). pSAT RNA interference vectors: a modular series for multiple gene down-regulation in plants. *Plant Physiology, 145*, 1272–1281.