



Post-transcriptional gene silencing of root-knot nematode in transformed soybean roots

Heba M.M. Ibrahim^{a,c}, Nadim W. Alkharouf^b, Susan L.F. Meyer^a, Mohammed A.M. Aly^c,
Abd El Kader Y. Gamal El-Din^c, Ebtissam H.A. Hussein^c, Benjamin F. Matthews^{a,*}

^a United States Department of Agriculture, Plant Sciences Institute, Beltsville, MD 20705, USA

^b Department of Computer and Information Sciences, Towson University, Towson, MD 21252, USA

^c Genetics Department, Faculty of Agriculture, Cairo University, Giza, Egypt

ARTICLE INFO

Article history:

Received 29 January 2010

Received in revised form 15 June 2010

Accepted 29 June 2010

Available online 3 July 2010

Keywords:

Gene silencing

RNA interference

RNAi

Resistance

In planta RNAi

Plant-parasitic nematode

Root-knot nematode (RKN)

Meloidogyne incognita

Soybean

Glycine max

Root transformation

ABSTRACT

RNAi constructs targeted to four different genes were examined to determine their efficacy to reduce galls formed by *Meloidogyne incognita* in soybean roots. These genes have high similarity with essential soybean cyst nematode (*Heterodera glycines*) and *Caenorhabditis elegans* genes. Transformed roots were challenged with *M. incognita*. Two constructs, targeted to genes encoding tyrosine phosphatase (TP) and mitochondrial stress-70 protein precursor (MSP), respectively, strongly interfered with *M. incognita* gall formation. The number of galls formed on roots transformed with constructs targeting the *M. incognita* TP and MSP genes was reduced by 92% and 94.7%, respectively. The diameter of *M. incognita* inside these transformed roots was 5.4 and 6.5 times less than the diameter of *M. incognita* found inside control plants transformed with the empty vector. These results indicate that silencing the genes encoding TP and MSP can greatly decrease gall formation and shows a promising solution for broadening resistance of plants against this plant-parasitic nematode.

Published by Elsevier Inc.

1. Introduction

Plant-parasitic nematodes cause about \$100 billion in crop losses annually (Koenning et al., 1999; Sasser and Freckman, 1987). Root-knot nematodes (RKN; *Meloidogyne* spp.) are sedentary endoparasites and *Meloidogyne incognita* in particular is widespread and considered economically as a very important species. The host range of *M. incognita* is very wide and it attacks almost all plant species. These nematodes cause dramatic morphological and physiological changes in plant cells. Recently, scientists have

identified several gene products secreted by the nematode during infection (Davis et al., 2004; Davis and Mitchum, 2005). Some of the genes encoding these proteins are similar to microbial genes or genes of animal-parasitic nematodes. Knowledge about these secreted proteins from the nematode and interaction of these proteins with targets within the plant cell during infection provides a better understanding of the interaction between the host cells and the parasite (Williamson and Gleason, 2003).

The nematode life cycle starts with the eggs, which are found in soil and in plant tissues. Second-stage juveniles (J2) hatch from eggs, search for plant hosts and infect the root close to the root tip. Within the root, J2 root-knot nematodes establish a feeding site and induce the formation of giant cells. These nematodes become sedentary and molt three times to reach maturity. The mature female deposits its eggs in a gelatinous mass. The RKN can be easily recognized by the “knots” or “galls” where they feed and develop (Caillaud et al., 2008). The focus of this study is to disrupt the development of the nematode and the host plant cell.

When RKN infects plant roots, there is a sophisticated interactive relationship with the host cell. Within the nematode's esophageal gland cells, there are different gene products that are

Abbreviations: EST, expressed sequence tag; hai, hours after inoculation; dai, days after inoculation; RKN, root-knot nematode; J2, second-stage juvenile; eGFP, enhanced green fluorescent protein; FMV-sgt, Figwort Mosaic Virus promoter sub-genomic transcript; qRT-PCR, Quantitative Real Time-PCR; LDH, lactate dehydrogenase; MSP, mitochondrial stress-70 protein precursor; ATP, ATP synthase beta-chain mitochondrial precursor; TP, tyrosine phosphatase; F, forward primer; R, reverse primer.

* Corresponding author. Address: USDA-ARS PSI, Soybean Genomics and Improvement Laboratory, 10300 Baltimore Ave., Bldg. 006, Beltsville, MD 20705, USA. Fax: +1 (301) 504 5728.

E-mail address: ben.mathews@ars.usda.gov (B.F. Matthews).

expressed to help the nematode establish a feeding site from the host's cells. Some of these proteins secreted by the nematode are injected into the host cell and cause modification of the cell wall (Davis et al., 2004). According to that investigation, some of the proteins appear to influence the progression of the host's cell cycle, cell defense response, protein degradation and regulatory system in the nucleus.

RNA silencing is a genetic mechanism that triggers degradation of mRNA to regulate gene expression at the post-transcriptional level and to degrade foreign RNA during virus infection as a defense response (Voinnet, 2001; Rosso et al., 2009). RNAi or post-transcriptional gene silencing has been used successfully in the free-living nematode, *Caenorhabditis elegans*, to silence the *unc-22* gene, which is responsible for the non-essential myofibrillar protein in muscle cells (Fire et al., 1998). Also, it has been used to silence specific genes in *C. elegans* to study gene function in loss-of-function mutants (Kamath et al., 2003). Recently, Dalzell et al. (2010) have shown that 21 bp siRNAs, specific to the gene encoding FMRFa-mide-like peptide (flp), are sufficient to silence the gene in infective (J2) stage juveniles of potato cyst nematode, *Globodera pallida*, and root-knot nematode, *M. incognita*. Charlton et al. (2010) showed that suppression of two *M. incognita* genes (dual oxidase and a subunit of a signal peptidase required for the processing of nematode secreted proteins) using RNAi resulted in the reduction in the number of nematodes by 50%. In that study, they showed an additive effect on nematode development by silencing two genes at the same time. Moreover, RNAi has been used to target a number of specific genes of two plant-parasitic cyst nematodes i.e., *Heterodera glycines* (Alkharouf et al., 2007) and *G. pallida* (Urwin et al., 2002) by oral ingestion using octopamine to enhance the oral uptake of the double-stranded RNA. *In planta* silencing of the RKN parasitism gene *16D10* has been examined by Huang et al. (2006b) using transformed *Arabidopsis*. The gene *16D10* encodes a 13-amino-acid peptide secreted from the subventral esophageal gland of *M. incognita* and is highly conserved among four *Meloidogyne* species (*M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*). This gene is responsible for signal transduction that is required for the formation and establishment of the feeding site for the RKN (Huang et al., 2006a). The *in planta* expression of *16D10* dsRNA in *Arabidopsis* resulted in 63–90% reduction in the number of galls and in reduced gall size compared with galls on roots transformed with empty vector. This study proved that *16D10* is needed in the early stage of parasitism and silencing it can increase resistance against the four major RKN species. Also, *in planta* RNAi has been used successfully to silence three sugar beet cyst nematode (*Heterodera schachtii*) genes individually with four different RNAi constructs. *Arabidopsis* plants were transformed with RNAi fragments that targeted the *3B05* gene, which codes for a cellulose-binding protein, the *4G06* gene responsible for a predicted protein with similarity to plant ubiquitins, and two different RNAi constructs targeted the *8H07* gene, which codes for a protein that has similarity to the SKP1 protein of plants. Transcript levels of the targeted genes were reduced, which resulted in reduction of the number of mature females by about 23–64% (Sindhu et al., 2009). Thus, silencing of some nematode genes appears to have a more profound effect on nematode development than does the silencing of other nematode genes. Moreover, Steeves et al. (2006) successfully reduced the reproductive potential of *H. glycines* in soybean roots by targeting the major sperm protein transcript with RNAi.

Alkharouf et al. (2007) identified over 1500 *H. glycines* genes that are orthologs of essential *C. elegans* genes that have lethal phenotypes or impede *C. elegans* development, if they are silenced. They demonstrated that silencing the ribosomal gene *Hg-rps-23* from *H. glycines* was lethal to the nematode. Urwin et al. (2002) found that silencing of genes encoding cysteine proteinase, C-type

lectins and major sperm protein affected the male: female ratio, inhibited the development of the nematode inside the roots, and reduced the number of sperm formed by the mature male. In addition, silencing the gene encoding amino peptidase with RNAi in *H. glycines* J2s resulted in a significant reduction in the number of J2 nematodes that were able to infect plant roots (Lilley et al., 2005). Furthermore, RNAi has been used to target transcripts of some genes of *Meloidogyne* spp., including the gene encoding dual oxidase (Bakheta et al., 2005) and chitin synthase (Fanelli et al., 2005). In *M. incognita*, the function of the L-cysteine proteinase was identified using RNAi. Silencing this gene resulted in a 60% reduction in the number of females that were able to reach the adult stage and produce eggs. This enzyme is synthesized in the intestine and is predicted to have a digestive function (Shingles et al., 2007).

In this investigation, we attempted to broaden resistance of soybean against the root-knot nematode *M. incognita* by silencing the genes encoding L-lactate dehydrogenase, mitochondrial stress-70 protein precursor, ATP synthase beta-chain mitochondrial precursor, and tyrosine phosphatase using RNAi gene silencing. These four *M. incognita* genes were silenced individually, which resulted in a substantial reduction in the number of galls formed on the roots after 1 month of infection. Some of the genes targeted by RNAi were chosen for their high degree of similarity to *C. elegans* genes found to have lethal phenotypes if mutated or silenced. Selection of other targets was based on similarity to *H. glycines* genes that were over expressed during feeding (Alkharouf et al., 2007; Klink et al., 2009b). *Agrobacterium rhizogenes* was used to transform soybean roots with the RNAi gene constructs. The transformed roots were challenged with *M. incognita* and nematode development was examined in the transformed roots from early infection to gall formation at 30 days after infection.

2. Materials and methods

2.1. Nematode

Meloidogyne incognita was grown at the United State Department of Agriculture Soybean Genomics and Improvement Laboratory Beltsville, MD, USA. RKN eggs were harvested from roots of *Glycine max* 2–4 months after inoculation using a method modified from those previously described in Meyer et al. (2000) and Nitao et al. (2002). Eggs were used to inoculate roots of soybean seedlings (*Glycine max*, cv. Williams 82) grown in Promix in 20 × 20 × 10 cm flats for a week. Thirty days after inoculation, plant roots were stained with acid fuchsin to monitor nematode invasion and development inside the roots according to Byrd et al. (1983).

2.2. First strand cDNA synthesis

Total RNA was extracted from *M. incognita* J2 using the Ultra Clean Plant RNA Isolation Kit (MOBIO, Carlsbad, CA). The RNA was used to synthesize the first strand cDNA using SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA) and using oligo d(T) as primer according to the manufacturer's instructions.

2.3. Amplification and extraction of the four *M. incognita* genes

Two target genes, L-lactate dehydrogenase (LDH; Accession No. AW828669) and tyrosine phosphatase (TP; Accession No. AW570920) were selected because of their high similarity to two *C. elegans* genes that were predicted to be lethal if silenced, according to the method described by Alkharouf et al. (2007) to identify

lethal genes. Briefly, RKN ESTs were downloaded from GenBank. The CAP3 assembly program (Huang and Madan, 1999) was used to cluster the *M. incognita* ESTs into contigs (data not shown). The contigs were then subjected to *BlastX* and *BlastN* comparisons against the *C. elegans* genome (Altschul et al., 1997). *M. incognita* genes that were highly similar (*e*-value of 1E-40 or lower) to a *C. elegans* gene were chosen and subjected to *BlastX* and *BlastN* in WormBase (www.wormbase.org) using the WormMart tool to find those that were lethal to *C. elegans* if mutated or silenced. The other two genes, mitochondrial stress-70 protein precursor (MSP; Accession No. BI773411) and ATP synthase beta-chain mitochondrial precursor (ATP; Accession Nos. BI773402 and BI773383), are highly similar to genes in *H. glycines* that are important during feeding (CK350603 and CB281634, respectively). We identified the *M. incognita* targeted genes by performing a BLAST sequence alignment using *BlastN*, *BlastX*, and *tBlastX* of the *M. incognita* EST sequences versus the *C. elegans* and *H. glycine* EST databases. We performed the same alignment search with the human and soybean genomes and we found no similarity with any human or soybean genomes. This would suggest that our constructs should not silence any gene in soybean or human.

The four *M. incognita* genes were amplified from cDNA prepared from *M. incognita* using gene specific primers (Table 1) to yield 400 bp products. A CACC sequence was added to the 5' end of each forward primer to allow the insertion of the amplified sequence in the pENTR vector (Invitrogen) as described below. The PCR products were separated on 1% agarose gel and extracted using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA).

2.4. Gene cloning and RNAi construct design

The purified PCR fragments were cloned into the pENTR cloning vector using a pENTR™ Directional TOPO® Cloning Kit (Invitrogen, Carlsbad, CA). Vectors containing genes of interest were transformed into competent *Escherichia coli* cells using One Shot® Mach1™-T1^R chemically competent *E. coli* (Invitrogen, Carlsbad, CA). Transformed *E. coli* were grown and harvested using QIAprep® Miniprep kit (QIAGEN, Valencia, CA) and the plasmid contents were confirmed by DNA sequencing. The genes of interest were moved from the pENTR vector to pRAP17 (Klink et al., 2009b) using Invitrogen's Gateway technology: pRAP17 is designed to express dsRNA using a double pair of Gateway components; one pair is in the opposite orientation of the other pair for easy cloning of DNA fragments in opposite directions. When the cloned DNA fragments are expressed, dsRNA is produced with a hairpin sequence in between (Fig. 1). The cloning reaction was mediated by Gateway® LR Clonase™ II Enzyme Mix (Invitrogen, Carlsbad, CA). The RNAi DNA fragments replace the *ccdB* genes and are inserted into the vector by the crossing over between the *attR* sites (on Prap17) and *attL* sites (on Pentr) Transformation into the pRAP17 vector was confirmed by PCR using three different primer sets. The first primer set was used to confirm the presence of the

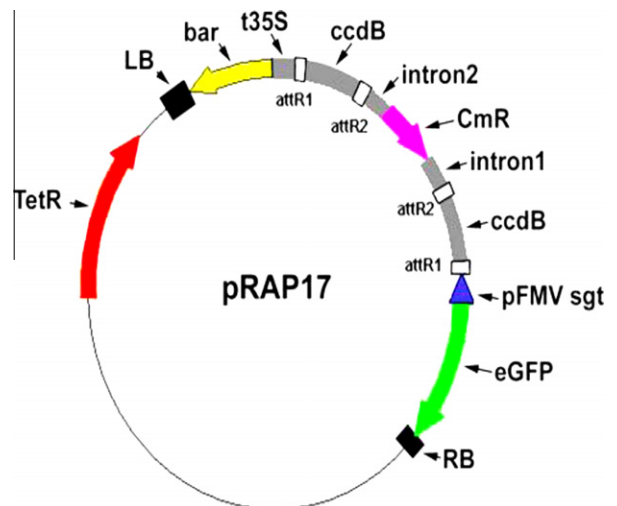


Fig. 1. pRAP17 vector. Tetracycline resistance gene TetR (red), left border LB, Basta₂ resistance gene bar (yellow), 35S terminator t35S, lethality gene *ccdB*, where the RNAi construct is inserted. It replaces *ccdB* (Bernard and Couturier, 1991; Salmon et al., 1994); intron 2, chloramphenicol resistance gene CmR (pink); intron 1, figwort mosaic virus sub-genomic transcript promoter FMV-sgt (blue), enhanced green fluorescent protein cassette containing the *roID* promoter and the 35S terminator eGFP (green), right border RB, bacteriophage k-derived recombination site #1attR1 LR, bacteriophage k-derived recombination site #2 attR2 LR.

RNAi gene in the forward direction using the gene specific forward primer and intron 1 reverse primer. The second one was used to confirm the presence of the Figwort Mosaic Virus promoter sub-genomic transcript (FMV-sgt) and the RNAi gene in the reverse direction using the forward primer for FMV and the gene specific reverse primer. The vector pRAP17 also contains the gene encoding enhanced green fluorescent protein (eGFP) under control of the *roID* promoter. Finally, the presence of the eGFP gene was confirmed using the forward and reverse primers specific to the enhanced green fluorescent protein gene (eGFP). The transformed pRAP17 clones were moved individually to competent *A. rhizogenes* and the transformations were confirmed by PCR using the same three primer sets. Transformed roots were identified by fluorescence of eGFP as visualized under blue light using the Dark Reader Spot Lamp that emits 400–500 nm (Clare Chemical Research; Dolores, CO).

2.5. Preparation of the RNAi transformed *A. rhizogenes* co-cultivated media

Five transformed *A. rhizogenes* clones (pRAP17 control, LDH-RNAi, MSP-RNAi, ATP-RNAi, and TP-RNAi constructs) were grown individually in 5 ml Terrific Broth (Research Products International Corp., Mt. Prospect, IL) medium containing 5 µg/ml tetracycline overnight at room temperature on a rotary shaker at 250 rpm. The 5 ml cultures were used to inoculate five 1-l flasks of TB + 5 µg/ml tetracycline medium and were incubated on a rotary shaker at 250 rpm overnight at room temperature. The cultures were centrifuged at 5000 rpm at 4 °C for 30 min. The pellets were resuspended in Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) with 3% sucrose and 500 µM acetosyringone (pH 5.7) according to Klink et al. (2009b). A mock control MS medium (MS medium inoculated with the same amount of water instead of the transformed *A. rhizogenes* culture) was prepared for the non-transformed control plants.

2.6. Plant transformation and challenging with *M. incognita*

A modified version of the soybean transformation system (Collier et al., 2005; Klink et al., 2009b) was used to produce trans-

Table 1
Primers used in PCR amplification and sequencing.

Name	Sequences (5'–3')
LDH-F	CACCGCACCGATTATGCCAAAAC
LDH-R	GCAGAGAGATTGACCCCTTGC
MSP-F	CACCTCAACCCGTGATGAGGCTGTT
MSP-R	TGCTGCTCTTCCAGTACC
ATP-F	CACCCAACCTGGAAGGGCTGAGAA
ATP-R	ACATGGGGAGTATGCAGGAA
TP-F	CACCACTCGCCGTTTCTGCAAT
TP-R	TACATTGGGCAACCTCTTCC
Intron-R	ATATGGACACGTTAACTGG
FMV-F	GGAGCCCTCCAGCTTCAAAG
eGFP-F	ATCGATGAATTTGTCGTGAACTATTAGTTGCGG
eGFP-R	ATCGATGCATGCTGCAGGTCACTGGATTTTG

formed soybean roots. Fifty soybean plants (cv. William 82) were grown for each experiment in Promix for 9 days before transformation. The plants were cut at the soil line with a fresh sharp razor and the stem ends were placed into small beakers containing the transformed *A. rhizogenes* co-cultivation solution. The beakers were placed into a vacuum chamber for 30 min to infiltrate the co-cultivation solution into the plants. The vacuum was released and the plants were co-cultivated overnight in a covered plastic tub on a rotary shaker at 65 rpm at room temperature. The plant stems were washed with tap water to remove excess *A. rhizogenes* and placed in a small beaker containing distilled water and stored inside an uncovered plastic tub overnight. The next day the soybean plants were planted in 50-cell flats filled with pre-wetted vermiculite. Five weeks after planting, the plants were screened to identify transformed roots. Transformed roots expressed GFP and were recognized by their green color when viewed under blue light; while the non-transformed roots did not express GFP. Non-GFP roots were trimmed and the plants were replanted in vermiculite to encourage growth of transformed roots. Control plants were trimmed in a similar manner. After 2 weeks, non-GFP roots were removed a second time and control plant were trimmed in a similar manner. By then, 60% of the transformed plants had large enough roots to be challenged with *M. incognita*. Twenty plant roots were washed with distilled water to remove the vermiculite from the roots. The plants were replanted in wet sand. Roots were challenged with *M. incognita*; 3000 eggs per plant were pipetted over the plant roots at about one inch deep before covering the roots with the pre-wet sand. The plants were not watered for 48 h to allow the J2 to infect the roots. Before counting the galls, the newly formed non-GFP roots were removed.

2.7. RT-PCR to determine the transcript level in the *M. incognita* inside the plant roots

For each gene RNA was extracted from two different root samples at 100 mg each using the Ultra Clean Plant RNA Isolation Kit (MOBIO, Carlsbad, CA). Roots from three different plants at 14-dai were combined for each sample. Roots having the strongest GFP expression and high root quality were selected. The RNA was treated with DNase I to remove genomic DNA and then used to synthesize cDNA using a SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA) and using an oligo d(T) as a primer according to the manufacturer's directions. Gene specific primers for the four targeted genes were designed using Primer 3 software (Table 2) and were specific to the flanking region of the RNAi to yield PCR-amplified fragments of approximately 100 bp. Constitutively expressed *M. incognita* ribosomal protein S21, histone H1, and β -actin genes were used as positive RT-PCR controls for each experiment as described by Alkharouf et al. (2006), Huang et al. (2004, 2005), Sukno et al. (2007) and Painter

and Lambert (2003). Specific primer sequences were used to yield a 100 bp fragment for the ribosomal protein S21 and histone H1, and a 283 bp fragment for the β -actin gene (Table 2). Other controls for qRT-PCR included reactions containing no template and qRT-PCR reactions containing no reverse transcriptase. These controls resulted in no amplification. qRT-PCR was performed on two biological replicates and each reaction was replicated three times. RT-PCR efficiency of each reaction was 90% or greater. The transcript level of each of the four targeted genes was determined using the Stratagene Mx3000P RT-PCR system as described by the manufacturer (Stratagene, La Jolla, CA). The cDNA concentration used in qRT-PCR was 400 ng per reaction. DNA accumulation was detected by SYBR Green and the C_t value (cycle at which there is the first clearly detectable increases in the fluorescence) was calculated using the software provided by the Stratagene Mx3000P RT-PCR system. The SYBR Green dissociation curve showed amplification for only one product for each primer set. Data analysis was performed according to the sigmoidal method described by Rutledge and Stewart (2008) for absolute quantification of the transcript level. One hundred femtogram of a universal quantitative standard, lambda gDNA, was used in each of four replicates to establish a quantitative scale to conduct an absolute quantification curve by calculating the optical calibration factor "OCF". This functions to obtain the fluorescence intensity units per nanogram of double-stranded DNA in a quantitative manner to normalize quality control component that involved all factors affecting the sensitivity of the assay such as reaction setup. Absolute quantification of the transcript level of the RNAi targeted genes was calculated using specific equations according to Tremblay et al. (2009), which is done in three steps:

- (1) Converting the individual fluorescence into target quantity expressed in fluorescence units

$$F_0 = \frac{F_{\max}}{1 + \left[\frac{F_{\max}}{F_c} - 1 \right] (E_{\max} + 1)^c}$$

where F_0 is the target quantity expressed in fluorescence units; F_{\max} is the maximal reaction fluorescence that defines the end point of the amplification process; F_c is the reaction fluorescence at cycle C and E_{\max} is the maximal amplification efficiency.

- (2) Target mass of the DNA amplicon expressed in nanogram (M_0)

$$M_0 = \frac{F_0}{OCF}$$

where OCF is the optical calibration factor of the Lambda gDNA, the DNA used for standardization

- (2) The number of target molecules (N_0)

$$N_0 = \frac{M_0 \times 9.1 \times 10^{11}}{A_s}$$

9.1×10^{11} is the number of the base pairs per nanogram of dsDNA and the A_s is the amplicon size in base pairs.

Table 2
Primers used in the RT-PCR reaction.

Name	Sequences (5'–3')
MIA (F)	TGACTCTGGAGATGGTGTACG
MIA (R)	GTGATGACTTGACCGTCAGGC
RS-21 (F) (100)	GGATTATTTTCAGCAAAGACCA
RS-21 (R) (100)	GCATAACGTGTCACTTCTCCA
<i>M. Histone 1</i> (F) (100)	GGCATTGGATGAATGAAGG
<i>M. Histone 1</i> (R) (100)	GATCTTTGCCGGGTCTCTC
RT-MSP (F)	GACCGCGATCTGATTAATA
RT-MSP (R)	TCCAACAAGCAAAATTTACG
RT-TP (F)	CACATTGGAGCGAGCATT
RT-TP (R)	CCCAGCTTGTGAAATGGAAT

3. Results

3.1. Gene silencing effect on *M. incognita* development inside the roots

To examine the effect of silencing of the four *M. incognita* genes, we measured the size and monitored the development of the nematode inside the roots and their ability to form galls on the

roots. At 28 dai, 20 different plants were selected out of 50 according to the healthiest roots and strongest GFP expression. The number of galls was counted on the RNAi-expressing roots and control roots. Under blue light, *M. incognita* galls were easily identified as solid, thick green regions on the transformed roots (Fig. 2). At this time, nematodes in control roots reached the final stage in their life cycle. The decreases in the number of galls formed on the roots transformed with ATP, LDH, MSP and TP were 2.7-, 2.3-, 12.4- and 19-fold compared to the control, respectively (Table 3; Fig. 4). RNAi targeted to the mitochondrial stress protein and the tyrosine phosphatase genes showed the highest effect on nematode development inside the roots. The number of galls formed on these transformed roots average 2.3 per plant for the mitochondrial stress protein experiment and 1.5 per plant for the tyrosine phosphatase roots, while the number of galls formed on the pRAP17 control was 28.5 per plant. Although the number of galls formed on the RNAi transformed roots that targeted LDH and ATP (12.2 and 10.35, respectively) was higher than those on roots transformed to silence MSP and TP, they were less than 50% of the number of galls formed on the pRAP17 control.

The development of *M. incognita* inside roots was monitored by measuring the diameter of 20 nematodes in the RNAi-expressing roots and comparing it with the diameter of nematodes in the pRAP17 control roots at 28 dai (Table 3; Fig. 3). The decrease in the diameter of *M. incognita* inside the roots transformed with RNAi directed against MSP, TP, LDH, and ATP was 6.5, 5.4, 4.3, and 2.6 times less than the diameter of *M. incognita* in the empty vector control roots, respectively.

Many of the nematodes reached the adult stage in roots transformed with the empty pRAP17 control vector and were able to form galls and produce egg masses. In contrast, in RNAi-expressing roots the nematodes were still thin and did not develop to the adult stage (Fig. 4). These results indicated that the RNAi fragment interfered with the life cycle of the nematode and interrupted

Table 3

Number of galls formed on the controls and the RNAi-expressing roots and nematode diameter inside the roots after 28 dai.

Experiments	No. of galls/plant root (simple mean)	Nematode diameter inside the roots \pm SD (μ m)
pRAP17	28.5	85.6 \pm 24.02
LDH	12.2	20.05 \pm 14.46
MSP	2.3	13.2 \pm 4.18
ATP	10.35	32.39 \pm 21.15
TP	1.5	15.75 \pm 8.48

nematode development. Furthermore, silencing the tyrosine phosphatase and mitochondrial stress protein genes strongly interrupted the development of the nematode. Silencing the other two genes, lactate dehydrogenase and ATP synthase, did not have the same strong effect on the nematode development. However, 50% fewer galls formed on the roots expressing lactate dehydrogenase and ATP synthase RNAi and the nematodes were at least 2.6 times less in diameter than those of the pRAP17 control.

3.2. Messenger-RNA transcript level in the RNAi-expressing roots

The mRNA transcript levels of the genes that were targeted with the RNAi in roots at 14 dai were determined by Quantitative Real Time-PCR using gene specific primers (Table 2). The absolute quantification of the transcripts (number of target molecules) was calculated using the sigmoidal method described by Rutledge and Stewart (2008) and Tremblay et al. (2009). The levels of the transcripts in the RNAi-expressing roots were noticeably down-regulated compared to the control lines. Transcripts of TP in the nematode that fed on roots transformed with the construct designed to silence TP were not detectable, but was 175.5 molecules in the nematode that fed on pRAP17 empty vector control roots. In

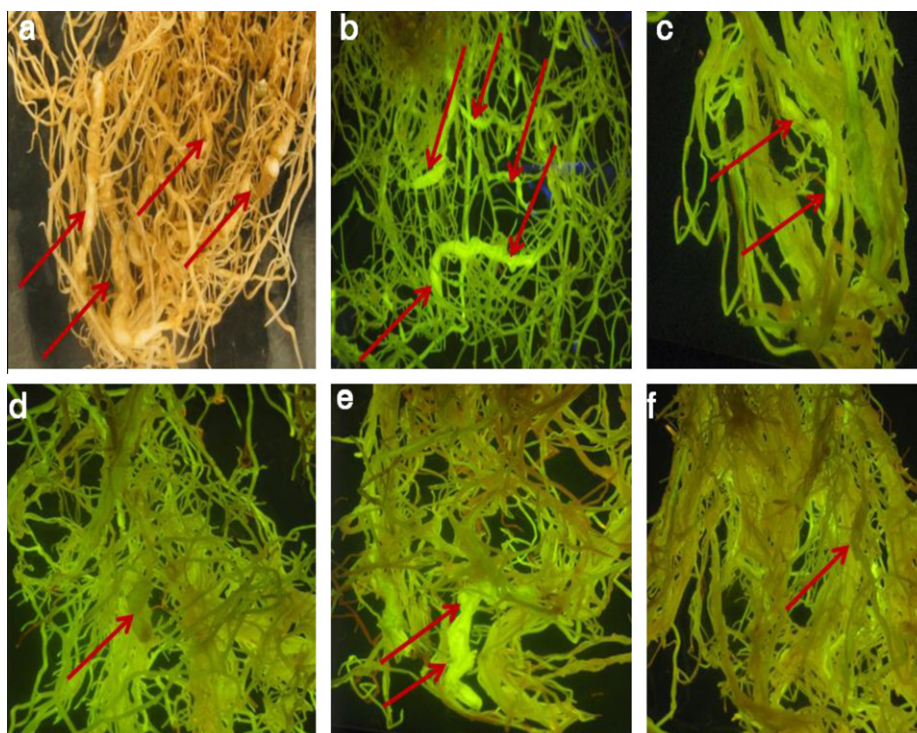


Fig. 2. Micrograph showing the numerous galls formed by *M. incognita* on the soybean control roots and the fewer galls on the RNAi transformed roots 28 dai. The arrows point to galls. (a) Non-transgenic control roots under normal light; (b) pRAP17 control; (c) LDH-RNAi root; (d) MSP-RNAi root; (e) ATP-RNAi root and (f) TP-RNAi root. (b–f) Under blue light.

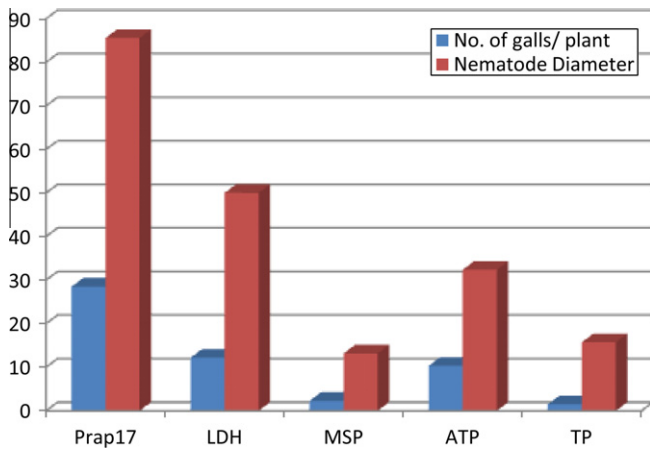


Fig. 3. The bar graph shows the number of galls formed and nematode diameter in the control roots and in the roots of different RNAi transformed lines.

the nematode the fed on roots transformed to silence the nematode MSP, the MSP transcript level was 4.3, while the level of the same transcripts in the nematode the fed on pRAP17 empty vector control roots was 169.89 (Fig. 5). We used three different house-keeping genes as endogenous standard genes for qRT-PCR, ribosomal protein S21, histone H1, and β -actin. The transcript levels of the three control genes were very low in the nematodes that fed on roots expressing RNAi targeted MSP and TP expression, 35 molecules and zero molecules for the ribosomal Protein S21, 0.7,

and 0.1 molecules for the histone H1, and 4.7 molecules and 0.3 for β -actin, respectively (Fig. 6). The expression level of those genes in the nematodes that fed on empty vector control roots were very high, 543.7, 42.9, and 194.4, respectively. We could not detect LDH and ATP transcripts using qRT-PCR; apparently their transcript levels were too low.

4. Discussion

The application of RNA interference to genomic studies of *C. elegans* has been very successful in helping to determine the function of genes and to identify genes that play an important role in the life cycle of *C. elegans* (Fire et al., 1998; Kamath et al., 2003). In our studies, we focused on transforming the soybean roots with DNA constructs encoding gene fragments of approximately 400 bp (132 kDa) RNAi fragments and on challenging the transformed roots with *M. incognita*. The high efficiency of *M. incognita* to take up a molecule of GFP (28 kDa) from transformed *Arabidopsis* plants has been reported by Urwin et al. (1997). In addition, Shingles et al. (2007) silenced the *M. incognita* gene encoding cathepsin L-cysteine, mi-cpl-1, and reduced the number of females that were able to produce eggs by 60% using an 800 bp fragment of this gene by soaking the nematode in octopamine solution at 21 dai. Thus, it is reasonable that gene fragments that we used would also be taken up by the nematode.

Here, we seek to broaden resistance of soybean against RKN, *M. incognita*, using RNAi gene silencing to interrupt four different *M. incognita* genes. Four *M. incognita* genes were chosen based on their high DNA sequence similarity with *C. elegans* genes that are

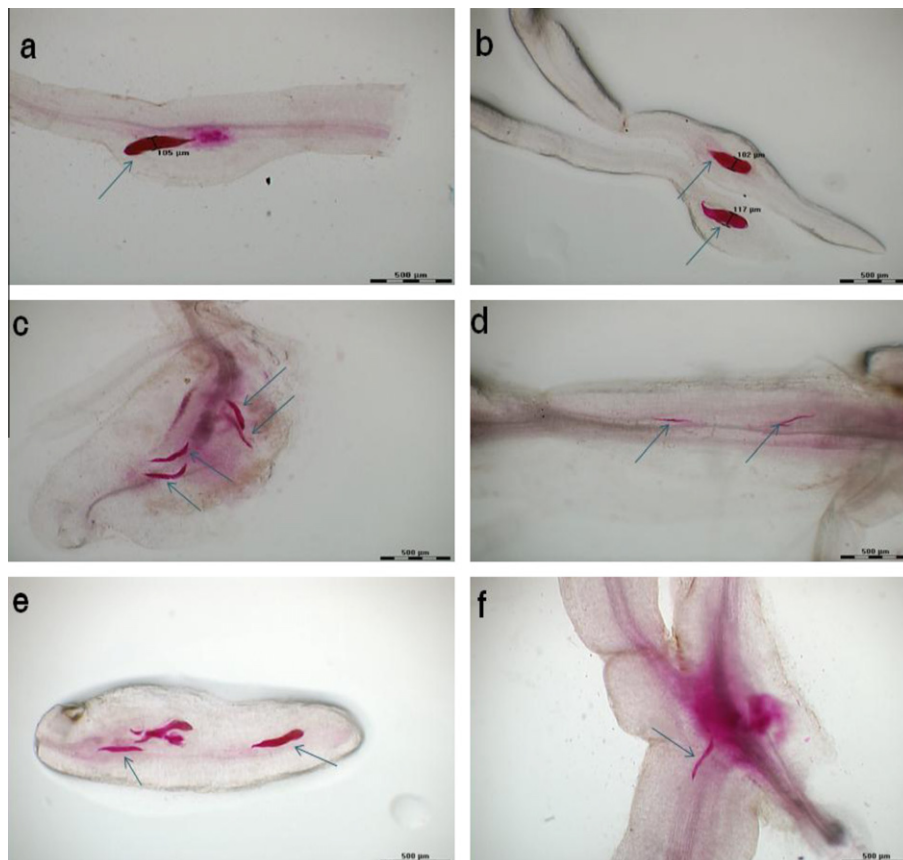


Fig. 4. Micrograph showing *M. incognita* inside soybean transgenic and control roots. The diameter of *M. incognita* is larger and the nematode is more mature in control roots than in the RNAi transformed roots after 28 dai. The arrows point to the nematode. (a) *M. incognita* in the non-transgenic control; (b) pRAP17 control; (c) LDH-RNAi root; (d) MSP-RNAi root; (e) ATP-RNAi root and (f) TP-RNAi root. Bar = 500 μ m.

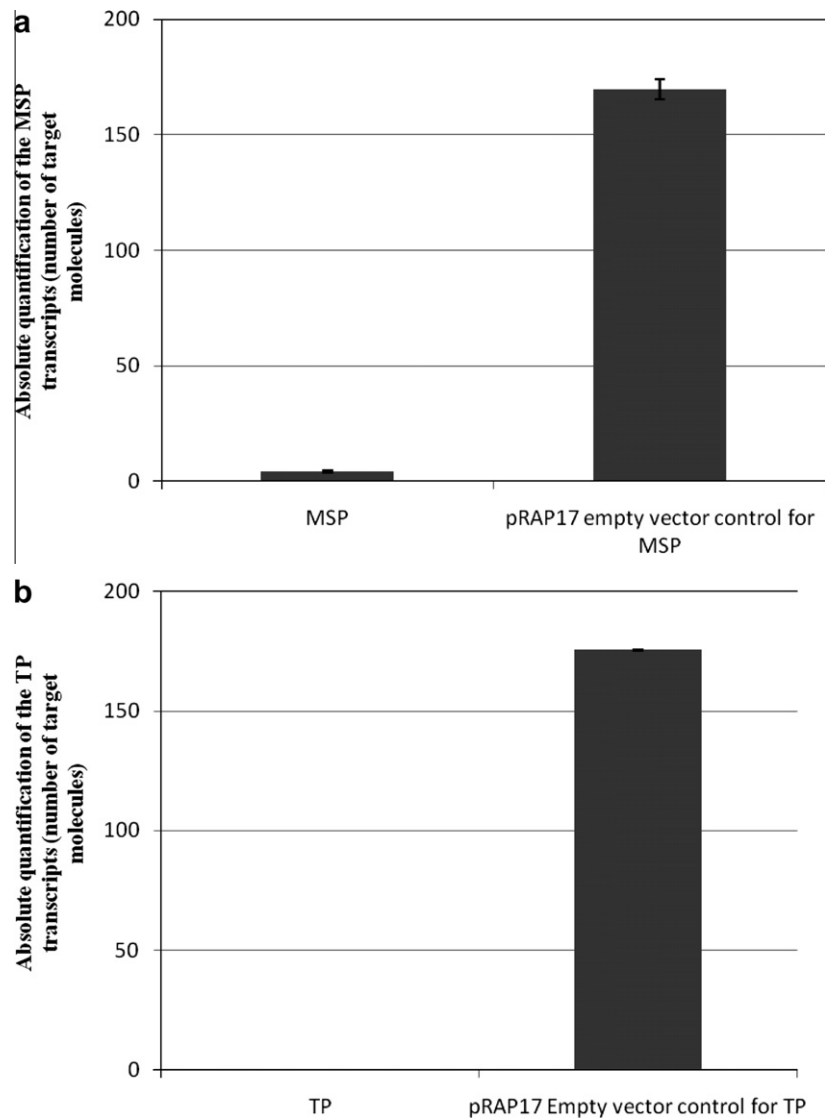


Fig. 5. Quantitative Real Time-PCR results showing the mRNA transcript level of the (a) MSP and (b) TP genes in the RNAi-expressing roots and empty vector (control). The x-axis represents the experiment type. The y-axis represents the absolute quantification of the mRNA transcript of different genes (number of target molecules) \pm standard deviation of the simple mean based on duplicate qRT-PCR analysis of three independent replicas each.

predicted to have RNAi lethal effect. Two of these genes are increased in expression in *H. glycines*, when it is sedentary and feeding as compared to the motile J2 stage (Alkharouf et al., 2007; Klink et al., 2009b). Several laboratories have reported the effects of RNAi on nematode development. Nematodes soaked in dsRNAi solution with resorcinol showed a phenotypic response, which was time-limited. The effect decreased gradually after moving the nematode out of the RNAi solution (Rosso et al., 2005; Bakhietia et al., 2007; Lilley et al., 2007). Klink et al. (2009a,b) used four different RNAi gene silencing constructs to transform soybean roots. All four constructs decreased the number of soybean cyst nematodes reaching maturity 30 dai by over 75%.

Lilley et al. (2007) reported that *in planta* delivery of the RNAi fragment to the nematode provides continued exposure of the nematode to the RNAi fragments as the nematode feeds. This may provide an effective way to use RNAi gene silencing technology to broaden resistance of plants against plant-parasitic nematodes. In addition, Yadav et al. (2006) were successful in silencing two *M. incognita* genes (splicing factor and integrase) by having the nematode feed on RNAi transformed tobacco roots. The galls formed on the RNAi transformed roots of those constructs

were significantly smaller in size and number compared to control plants.

Two of our constructs were targeted against tyrosine phosphatase and mitochondrial stress-70 protein precursor, respectively. Production of the RNAi to TP and MSP in the root resulted in a considerable reduction in gall formation, 94.7% and 91.9%, respectively. Furthermore, nematode development was suppressed by 81.6% and 84.6%, respectively, as indicated by measurements of the diameter of the nematode. Similarly, Yadav et al. (2006) examined the development of the nematode, *M. incognita*, after they fed on roots expressing RNAi targeted to a gene encoding a splicing factor and a gene encoding integrase by counting the number of mature females in the galls. They found many mature female nematodes in the control plant, while there were very few healthy females in the transformed roots.

Our qRT-PCR results indicate that the RNAi constructs strongly affect the expression level of targeted genes in RKN that fed on RNAi-expressing roots, while the level of the transcripts in the nematodes that fed on empty vector control roots is very high. The transcript level of the TP gene was undetectable in the nematode that fed on RNAi transformed roots targeted TP, while the level of the

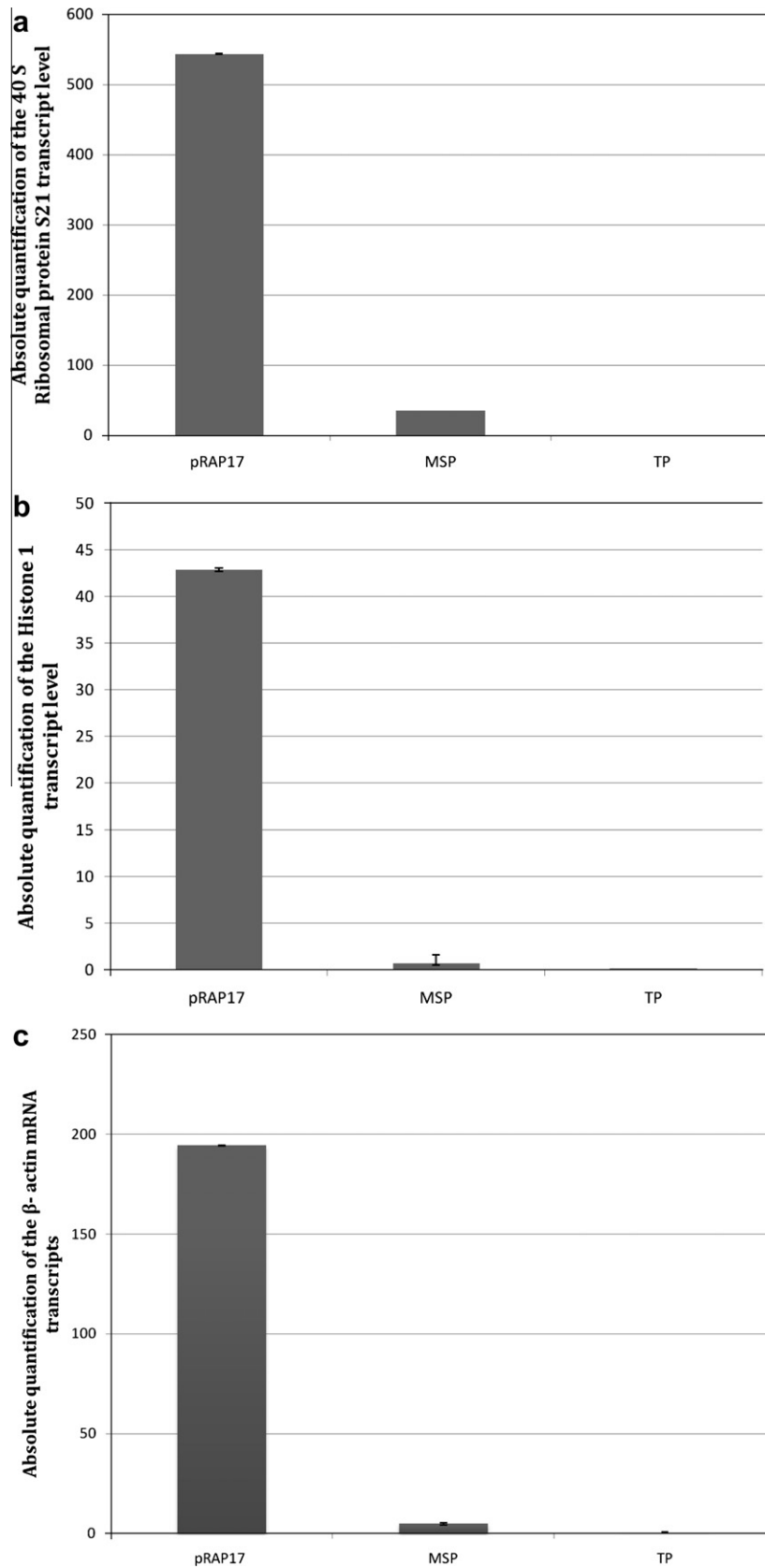


Fig. 6. qRT-PCR results showing the mRNA transcript level of the ribosomal protein S21 and histone H1 and β -actin in the nematode that fed on RNAi-expressing roots and empty vector (control). The x-axis represents the treatments. The y-axis represents the absolute quantification of the mRNA transcript of ribosomal protein S21, histone H1, and β -actin genes in TP- and MSP-RNAi-expressing roots and the empty vector control (number of target molecules \pm standard deviation of the simple mean based on qRT-PCR analysis of three independent replicas each).

TP transcripts in the nematode that fed on empty vector control roots was very high. The level of the MSP transcript was greatly reduced to 4.3 molecules in the MSP-targeted nematode, while in the nematode that fed on the empty vector control roots was very high, 169.9 molecules. Transcript levels of the genes encoding ribosomal protein S21, histone H1, and β -actin were measured and used as a control reference for constitutively expressed house-keeping genes for normalization. Interestingly, the levels of the three different transcripts were very low and similar to the levels of transcript of the MSP and TP genes in the MSP and TP-RNAi targeted nematode, 35 and zero for the ribosomal protein S21, 0.7, and 0.11 for the histone H1, and 4.7 and 0.3 for the β -actin, respectively, while the transcript level of the ribosomal protein S21, histone H1, and β -actin genes in the nematode that fed on the control roots was very high, 543.7, 42.9, and 194.4, respectively. In contrast, Yadav et al. (2006) were able to amplify a control transcript for their nematodes that had been fed on plants transformed to produce RNAi targeting *M. incognita* splicing factor and integrase genes. In our experiments, the transcript levels of the three control genes in nematodes that fed on the roots transformed with RNAi gene silencing constructs may be reduced because the *M. incognita* were dying. These results indicate that the constructs decreased the number of transcripts of the targeted gene and greatly interfered with nematode development.

Our results demonstrate that silencing of the four *M. incognita* genes had a substantial detrimental effect on *M. incognita* development, which prevented root damage through suppression of gall formation. The best results were achieved by silencing TP and MSP genes. Although the transformed roots on each plant were not necessarily formed from one transformation event and it is likely that the levels of RNAi produced in each transformation event due to position effects, this approach provides a rapid method for screening a number of genes quickly and gives an indication about the importance of the gene in the nematode life cycle. Furthermore, these results indicate that resistance of soybean plants to RKN can be increased by silencing the TP and MSP genes in the nematode.

Acknowledgments

The authors thank Peggy MacDonald for her excellent technical assistance and gratefully acknowledge support from United Soybean Board Project No. Y9254 and US-Egypt joint Project No. BIO8-001-002.

References

- Alkharouf, N.W., Klink, V.P., Chouikha, I.B., Beard, H.S., MacDonald, M.H., Meyer, S., Knap, H.T., Khan, R., Matthews, B.F., 2006. Time course microarray analyses reveal global changes in gene expression of susceptible *Glycine max* (soybean) roots during infection by *Heterodera glycines* (soybean cyst nematode). *Planta* 224, 838–852.
- 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.
- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.
- Bakhtia, M., Charlton, W.L., Urwin, P.E., McPherson, M.J., Atkinson, H.J., 2005. RNA interference and plant parasitic nematodes. *Trends in Plant Science* 10 (8), 362–367.
- Bakhtia, 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 (3), 306–312.
- Bernard, P., Couturier, M., 1991. The 41 carboxy-terminal residues of the Mini-F plasmid ccdA protein are sufficient to antagonize the killer activity of the CcdB protein. *Molecular and General Genetics* 226, 297–304.
- Byrd, D.W., Kirkpatrick Jr., T., Barker, K.R., 1983. An improved technique for clearing and staining plant tissues for detection of nematodes. *Journal of Nematology* 15 (1), 142–143.
- Caillaud, M.C., Abad, P., Favery, B., 2008. Cytoskeleton reorganization, a key process in root-knot nematode induced giant cell ontogenesis. *Plant Signaling and Behavior* 3 (10), 816–818.
- Charlton, W.L., Harel, H.Y.M., Bakhtia, M., Hibbard, J.K., Atkinson, H.J., McPherson, M.J., 2010. Additive effects of plant expressed double-stranded RNAs on root-knot nematode development. *International Journal for Parasitology* 40 (7), 855–864.
- Collier, R.k., Fuchs, B., Walter, N., Lutke, W.K., Taylor, C.G., 2005. *Ex vitro* composite plants: an inexpensive, rapid method for root biology. *The Plant Journal* 43, 449–457.
- Dalzell, J.J., McMaster, S., Fleming, C.C., Maule, A.G., 2010. Short interfering RNA-mediated gene silencing in *Globodera pallid* and *Meloidogyne incognita* infective stage juveniles. *International Journal for Parasitology* 40, 91–100.
- Davis, E.L., Mitchum, M.G., 2005. Nematodes sophisticated parasites of legumes. *Plant Physiology* 137, 1182–1188.
- Davis, E.L., Hussey, R.S., Baum, T.J., 2004. Getting to the roots of parasitism by nematodes. *Trends in Parasitology* 20 (3), 134–141.
- Faneli, E., Vito, M.D., Jones, J.T., Giorgi, C.D., 2005. Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia*, using RNAi. *Gene* 349, 87–95.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391 (19), 806–811.
- Huang, X., Madan, A., 1999. CAP3: a DNA sequence assembly program. *Genome Research* 9, 868–877.
- Huang, G., Dong, R., Maier, T., Allen, R., Davis, E.L., Baum, T.J., Hussey, R.S., 2004. Use of solid-phase subtractive hybridization for the identification of parasitism gene candidates from the root-knot nematode *Meloidogyne incognita*. *Molecular Plant Pathology* 5 (3), 217–222.
- Huang, G., Dong, R., Allen, R., Davis, E.L., Baum, T.J., Hussey, R.S., 2005. Developmental expression and molecular analysis of two *Meloidogyne incognita* pectate lyase genes. *International Journal for Parasitology* 35, 685–692.
- Huang, G., Allen, R., Davis, E.L., Baum, T.J., Hussey, R.S., 2006a. Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *PNAS* 103 (39), 14302–14306.
- Huang, G., Dong, R., Allen, R., Davis, E.L., Baum, T.J., Hussey, R.S., 2006b. A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. *Molecular Plant–Microbe Interactions* 19 (5), 463–470.
- Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapik, A., Bot, N.L., Moreno, S., Sohrmann, M., Welchman, D.P., Zipperlen, P., Ahringer, J., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421 (16), 231–237.
- Klink, V.P., Hosseini, P., MacDonald, M.H., Alkharouf, N.W., Matthews, B.F., 2009a. Population-specific gene expression in the plant pathogenic nematode *Heterodera glycines* exists prior to infection and during the onset of a resistant or susceptible reaction in the roots of the *Glycine max* genotype peking. *BMC Genomics* 10.
- Klink, V.P., Kim, K.H., Martins, V., MacDonald, M.H., Beard, H.S., Alkharouf, N.W., Lee, S.K., Park, S.C., Matthews, B.F., 2009b. 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.
- Koenning, S.R., Overstreet, C., Noling, J.W., Donald, P.A., Becker, J.O., Fortnum, B.A., 1999. Survey of Crop losses in response to phytoparasitic nematodes in the United States for 1994. *Journal of Nematology* 31 (4S), 587–618.
- Lilley, C.J., Bakhtia, M., Charlton, W.L., Urwin, P.E., 2007. Recent progress in the development of RNA interference for plant parasitic nematodes. *Molecular Plant Pathology* 8 (5), 701–711.
- Lilley, C.J., Goodchild, S.A., Atkinson, H.J., Urwin, P.E., 2005. Cloning and characterisation of a *Heterodera glycines* aminopeptidase cDNA. *International Journal for Parasitology* 35, 1577–1585.
- Meyer, S.L.F., Massoud, S.I., Chitwood, D.J., Roberts, D.P., 2000. Evaluation of *Trichoderma virens* and *Burkholderia cepacia* for antagonistic activity against root-knot nematode, *Meloidogyne incognita*. *Nematology* 2, 871–879.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15, 473–497.
- Nitao, J.K., Meyer, S.L.F., Oliver, J.E., Schmidt, W.F., Chitwood, D.J., 2002. Isolation of flavipin, a fungus compound antagonistic to plant-parasitic nematodes. *Nematology* 4, 55–63.
- Painter, J.E., Lambert, K.N., 2003. *Meloidogyne javanica* chorismate mutase transcript expression profile using real-time quantitative RT-PCR. *Journal of Nematology* 35 (1), 82–87.
- Rosso, M.N., Dubrana, M.P., Cimbolini, N., Jaubert, S., Abad, P., 2005. Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. *Molecular Plant–Microbe Interactions* 18 (7), 615–620.
- Rosso, M.N., Jones, J.T., Abad, P., 2009. RNAi and functional genomics in plant parasitic nematodes. *The Annual Review of Phytopathology* 47, 207–232.
- Rutledge, R.G., Stewart, D., 2008. A kinetic-based sigmoidal model for the polymerase chain reaction and its application to high-capacity absolute quantitative real-time PCR. *BMC Biotechnology* 8 (47).
- Salmon, M.A., Van Melderen, L., Bernard, P., Couturier, M., 1994. The antidote and autoregulatory functions of the F plasmid ccdA protein: a genetic and biochemical survey. *Molecular and General Genetics* 244, 530–538.
- Sasser, J.N., Freckman, D.W., 1987. A world perspective on nematology: the role of the society. In: Veek, J.S., Dickson, D.W. (Eds.), *Vistas on Nematology*. Society of Nematologists, Hyattsville, MD, pp. 7–14.

- Shingles, J., Lilley, C.J., Atkinson, H.J., Urwin, P.E., 2007. *Meloidogyne incognita*: molecular and biochemical characterisation of a cathepsin L-cysteine proteinase and the effect on parasitism following RNAi. *Experimental Parasitology* 115, 114–120.
- Sindhu, A.S., Maier, T.R., Mitchum, M.G., Hussey, R.S., Davis, E.L., Baum, T.J., 2009. Effective and specific *in planta* RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success. *Journal of Experimental Botany* 60 (1), 315–324.
- Steeves, R.M., Todd, T.C., Essig, J.S., Trick, H.N., 2006. Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. *Functional Plant Biology* 33, 991–999.
- Sukno, S.A., McCuiston, J., Wong, M.Y., Wang, X., Thon, M.R., Hussey, R., Baum, T., Davis, E., 2007. Quantitative detection of double-stranded RNA-mediated gene silencing of parasitism genes in *Heterodera glycines*. *Journal of Nematology* 39 (2), 145–152.
- Tremblay, A., Li, S., Scheffler, B.E., Matthews, B.F., 2009. Laser capture microdissection and expressed sequence tag analysis of uredinia formed by *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust. *Physiological and Molecular Plant Pathology* 73, 163–174.
- 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 (8), 747–752.
- Urwin, P.E., Moller, S.G., Lilley, C.J., McPherson, M.J., Atkinson, H.J., 1997. Continual green-fluorescent protein monitoring of cauliflower mosaic virus 35S promoter activity in nematode-induced feeding cells in *Arabidopsis thaliana*. *Molecular Plant–Microbe Interactions* 10, 394–400.
- Voinnet, O., 2001. RNA silencing as a plant immune system against viruses. *Trends in Genetics* 17 (8), 449–459.
- Williamson, V.M., Gleason, C.A., 2003. Plant–nematode interactions. *Current Opinion in Plant Biology* 6, 327–333.
- 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.