International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (1): 697-704
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Knocking Down Meloidogyne Incognita Genes by Plant Delivered siRNA Has Negative Pleiotropic Effects on Nematode Vigor

[1]D Baliyan* and [2]R Kumar
[1]Mewar University, Gangrar, Chittorgarh
[2]Hindu College, University of Delhi, New Delhi

Received: 12 Oct 2018/ Accepted: 14 Nov 2018/ Published online: 01Jan 2019
Corresponding Author Email: deeksha.baliyan@gmail.com

Abstract
RNA interference (RNAi) approaches have provided novel avenues in genomic sequencing of plant parasitic nematodes (PPNs). It provides the opportunity to recognize prospective target genes and subsequently develop new resistant transgenic plants. dsRNA constructs targeting two different *Meloidogyne incognita* genes were evaluated to adjudge their efficiency in reducing infectivity of *M.incognita* in tobacco. Stable homozygous transgenic tobacco lines were engineered, i.e. RNAi-MiTP (expressing dsRNA of MiTP gene) and RNAi-MiRpn-7 (expressing dsRNA of MiRpn-7 gene). The number of knots/plants, number of females/knots, number of egg masses g-1 root wt. and number of eggs g-1 root wt. were reduced by83.6%, 76.9%, 51.6% and 59.5% in RNAi-MiTP lines and by 72.9%, 61.5%, 40.9% and 50.1% RNAi-MiRpn-7 lines respectively in comparison to the empty vector control tobacco lines. The results from this study suggest that silencing of the MiTP and MiRpn-7 genes can significantly decrease the infective potential of this plant parasitic nematode and demonstrates a promising approach for producing broadened resistance in crops against PPNs.

Keywords
Homozygous transgenic lines, *Meloidogyne incognita*, Plant parasitic nematodes (PPNs), resistant transgenic plants, RNA interference (RNAi).

*****

1. INTRODUCTION
Root knot nematodes (RKNs) i.e. *Meloidogyne* spp. are the most damaging of the agricultural pests (Abad et al. 2008; Elling 2013). They are sedentary parasitic nematodes and more than hundred species of this genus spread around the world are infecting almost every plant (Bakhetia et al. 2008; Chitwood 2003). The common *Meloidogyne* species are *M.incognita*, *M.hapla*, *M.arenaria* and *M.javanica (Atamian et al. 2012; Eisenback et al. 1985)*. These nematodes are the most harmful pests of agriculture which produce intensive crop damage and wreckful morphological symptoms (Chen et al. 2012). According to estimates, the world agriculture bears a heavy loss of 12.3% yield and $170 billion annually due to the infestation by the RKNs alone (Banerjee et al., 2017).

In the recent years, many studies have been conducted and some nematode control approaches

have been used such as agricultural practices, nematicides and biological agents (Chandel et al., 2010). Although the different control approaches were found to be effective at different level for nematode control, still each strategy has its own limitations. This created a need for a more holistic and sustainable strategy to be devised against the nematode infection. One such promising new strategy is RNA interference (RNAi) based nematode control, which has been extensively studied in free living nematode *C.elegans* (Kamath et al. 2003). RNAi is a post transcriptional gene silencing mechanism in which the siRNA (small interfering RNA) are generated from dsRNA (double stranded RNA) corresponding to the target gene through processing by Dicer (Fire et al. 1998). Following this, the siRNAs thus generated trigger the process of gene silencing by activating the RISC (RNA induced silencing complex) which identify and degenerate the target mRNA hence preventing the mRNA from translating into protein (Rosso et al. 2009). The RNAi approach could be effectively used to combat the nematode infection in agricultural crops by adopting a strategy known as HD RNAi (host delivered RNAi), in which the dsRNAs that corresponds to the target nematode gene/genes are expressed in the transgenic host plant (Tamilarasan et al. 2013). The process of RNA interference is initiated based upon the siRNA-mRNA (target) homology when the nematodes feed upon the transgenic host plant. The selection of the target genes is generally based on the physiological role they play in fundamental metabolic processes including reproduction, nutrition, locomotion, development, neurological process, recognition etc. The choice of the target gene/genes is the most crucial step in the process of HD RNAi, because it is important to avoid off target silencing (Fairbairn et al. 2007). The main paradigms for selecting an efficient target for HD RNAi are: (i) the sequence should be conserved so that silencing is nematode-specific and (ii) the sequence should be homologically discreet enough so that they are present in different nematode species but should not be present in non-target organisms (Danchin et al. 2013). A plethora of studies have been conducted successfully utilizing RNA interference for nematode control (Kumar et al. 2017; Dong et al. 2016; Niu et al. 2016; Dutta et al. 2015, Lourenco-Tessutti et al. 2015; Dinh et al. 2014a, b; Jaouannet et al. 2013; Papolu et al. 2013; Xue et al. 2013). Variable results with different intensities of resistance against the RKNs have been established through various research groups, showing promising outcomes in further studies. A number gene targets have been exploited for RNAi in numerous plant systems like soybean, potato, tobacco, wheat, tomato and demonstrated with nematode genes of various metabolic processes like development, movement, parasitism etc. Kumar et al. (2017) successfully demonstrated the potential of HD RNAi for resistance against *M.incognita* in transgenic Arabidopsis plants which expressed the dsRNAs for the *M.incognita* splicing factor and integrase. These two genes were shown to be involved in the nematode development and severely affected the parasitic potential of the nematode. The engineered *Arabidopsis* plants showed significant reduction in the number of galls and females.

In the present study, two genes of *M.incognita* viz. MiTP (Tyrosine phosphate) and MiRpn-7 have been evaluated in transgenic tobacco for assessing their resistance potential against *M.incognita* infection. Ibrahim et al. (2011) engineered transgenic soybean lines expressing dsRNA of tyrosine phosphate of *M.incognita* which exhibited significant resistance to the nematode. Similarly, Niu et al., (2012) displayed RNAi silencing of Rpn-7 gene in soybean displayed reduced parasitic infection. Still, these two genes have not yet been examined in tobacco for evaluating resistance against nematode infection using the HD RNAi method. The commercial significance and wide use of tobacco as a model plant in a number of studies encouraged us towards evaluating these genes in tobacco, which have so far been shown to effectively induce nematode resistance in soybean roots. Hence, this study was conducted to engineer and examine the stable transgenic tobacco lines RNAi-MiTP and RNAi-MiRpn-7, expressing dsRNA of MiTP and MiRpn-7 genes respectively, for their potential resistance against *M.incognita* infection.

2. MATERIALS AND METHODS

2.1 Nematode culture

A pure culture of RKN *M.incognita* was maintained on tomato plants under green house conditions. *M. incognita*s were sequestered from the tomato plants growing in the laboratory field of Department of Molecular Biology and Genetic Engineering, College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut. The singular egg masses were handpicked and were let to hatch separately. The female nematodes were collected and after washing them their perineum were intersected to correctly identify the species (Ebhad et al. 2013, Eisenback 2010). PCR assays using

sequence characterized amplified region (SCAR) primers were also used to correctly identify the nematode species (Adam et al. 2007). Once the correct species was identified and hatching of the corresponding egg masses, the stage 2 juveniles (J2) were maintained on tomato plants (Solanum lycopersicum Cv.Pusa Hybrid 4) in greenhouse. The tomato seeds were sown in vermicutile: sand mixture (ratio 1:1) and after 15 days these were inoculated with J2's of the nematodes. For the purpose of the present study, egg masses were handpicked 6 weeks post infection (wpi), washed and thereafter left to hatch on wire-mesh laid on a petri plate having 15 ml of ddH_2O at 27-30^{0}C.

2.2 Scheming the RNAi vector

For expressing dsRNA cassettes of the *M.incognita* genes in tobacco, a 400 bp fragment of the MiTP gene and a 408 bp fragment of the MiRpn-7 gene were amplified in both sense and antisense orientations using gene specific primers and were cloned in pRB7 vector (the destination vector) driven by tobacco root specific promoter TobRb7 for driving its expression in plants. The promoter was positioned upstream of the intron (from *Arabidopsis thaliana*) flanked by 2 MCSs (multiple cloning sites).

2.3 Transferring the construct from *E.coli* to *A.tumefaciens*

Up to this point the entire recounted work was accomplished in DH5-α strain of E.coli; the entire construct having both sense and antisense orientations of the GOI was maintained in the said bacterial strain adhering to the standard cloning protocols. However for transferring the expression cassette from bacterial cells to tobacco, the cassette was first transformed to *A.tumefaciens* and then from *A.tumefaciens* the cassette was finally transferred to the tobacco plants. The dsRNA cassette transfer was accomplished through "Triparental Mating" transformation (Adapted from Shaw, 1995).

2.4Generation of transgenic tobacco expressing MiTP and MiRpn-7 gene

Tobacco plants were transformed with MiTP and MiRpn-7 RNAi constructs (expressed in *A.tumefaciens*) using the Agrobacterium mediated gene transformation. T1 seeds were screened on antibiotic plate with MS medium supplemented with kanamycin (50µg/ml). Kanamycin-resistant tobacco plants were transferred to sterile vermicutile: sand mixture (ratio 1:1) and grown in green house conditions: a temperature of 27-30 ^{0}C temperature, a photoperiod of 16hour L/8hour D (L/D = Light period/Dark period) and a relative humidity of about 75-80 %. The seeds from T2 generation were utilized for raising homozygous tobacco transgenic tobacco lines of T3 generation. The morphological characters of the transgenic tobacco lines viz. root, shoot and leaf morphologies were compared with those of the negative control tobacco lines assess any deviation/variation in the phenotypes which may accidentally alter/reduce PPN infection.

2.5Nematode feeding assay

For evaluating the transformed tobacco plants (RNAi lines), at least 4 weeks old PCR-confirmed plants were used for infecting with *M.incognita*. The plants were expelled from agar plates and agar was entirely removed by washing with ddH2O and thereafter they were transferred to sand: vermiculite (1:1) mixture contained in pots for 3 days. After this, the soil beside the roots was taken out and the roots and the roots were inoculated with ~2000 freshly hatched J2 nematodes. The plants were nurtured by requisite watering for 6 weeks in the green house. 6wpi the plant roots were removed from soil and were washed with running water in order to analyze them further for estimating the response of transgenic as well wild type tobacco lines. The roots were dissected and the number of knots per plant, females per knot, egg masses per plant, eggs hatched per egg mass was carefully counted as a measure of nematode infection and the mean values were calculated from at least 30 tobacco plants per transgenic line. All the photographs were taken with a Nikon D300 digital camera attached to a stereomicroscope (SMZ-1000).

2.6cDNA synthesis from RNAi female nematodes and expression analysis

For finding out the efficiency and effectiveness of dsRNA expression (and resulting RNA interference) on *M.incognita* infection, females were separated out from transgenic as well as control plants by dissecting the roots. Considering that plants from RNAi plant lines have very less number of knots, all the knots were cut for picking out as much number of females as possibly feasible for isolating nematode RNA. The females were also collected from the roots of control plants in a similar manner. Total RNA was isolated from the isolated females using the RN Easy kit (Qiagen Cat. #74104) as per the prescribed protocol. cDNA synthesis was carried out High-Capacity RNA-to-cDNA^{TM} Kit (Applied Biosystems Cat #4387406). The target sequence was amplified from cDNA by qRT PCR. Several cycles of amplification (~50) were carried out in PCR. The *M.incognita* actin gene (which is a constitutive gene) was used as a control for normalizing the transgene expression data. The PCR products were separated by agarose

gel electrophoresis and visualized using the Gel Doc UV- Transilluminator (Dnr Bio-Imaging Systems).

3. RESULTS

3.1 *M.incognita* feeding assay: Effects on resistance of host plants against nematode infection

Roots in control tobacco plants show a considerably large number of knots per plant; on an average 140 (±4) knots were present per control plant. The number of knots/plant were substantially reduced in the case of transgenic plants carrying dsRNA cassettes in contrast to the empty vector carrying control tobacco lines (as depicted in **Figure 1**). Only few (3 out of 30) RNAi-MiTP tobacco lines which were carrying dsRNA of Tyrosine phosphate gene of *Meloidogyne incognita*, had knots in their roots, but these knots were highly reduced in their number (~23 knots/plant) as compared to the control. It is also noteworthy that these knots were comparatively much smaller in size and quantity in comparison to the root-knots in control tobacco plants. Most of the RNAi-MiTP tobacco lines (i.e. the remaining 27 lines) did not develop any observable root-knots. Likewise, the RNAi-MiRpn-7 tobacco lines bearing dsRNA of Rpn-7 gene of *Meloidogyne incognita* upon examination showed root-knots present in just 9 lines out of total 30, while the remaining 21 lines did not develop any root-knot. Also the knot size was significantly smaller in comparison to those present on the control tobacco lines.

While the number of females/knot in control plants was 13 (±4), the number was reduced to 3 (±1.3) in case of RNAi-MiTP lines and it was 5 (±2.4) females/knot in case of RNAi-MiRpn-7 tobacco lines. A similar significant reduction was observed in the number of egg masses per gram root weight and number of eggs per gram root weight between the control and RNAi tobacco lines carrying dsRNA cassettes. In conclusion, it was observed that in RNAi-MiTP tobacco lines (carrying *M.incognita* Tyrosine phosphate gene), the number of knots/plant, number of females/knot, number of egg masses g-1 root wt. and number of eggs g-1 root wt. were reduced by 83.6%, 76.9%, 51.6% and 59.5% respectively in comparison to the empty vector control tobacco lines. Similarly, in RNAi-MiRpn-7 tobacco lines (carrying *M.incognita* Rpn-7 gene), the number of knots/plant, number of females/knot, number of egg masses g-1 root wt. and number of eggs g-1 root wt. were reduced by 72.9%, 61.5%, 40.9% and 50.1% respectively in comparison to the empty vector control tobacco lines (**Table 1**).

3.2 *M.incognita* feeding assay: Effects on nematode development

With the intention to detect the effect of feeding of transgenic plants on the development of nematodes, a few knots from transgenic control as well as RNAi tobacco lines carrying either of the dsRNA cassettes were dissected for taking out *M.incognita* females. The shapes and sizes of 6wpi females were compared. It was found that the control tobacco lines bear *M.incognita* females displaying the peculiar saccate/tear-drop shape and dark color. While the shape of females isolated from RNAi tobacco lines (carrying dsRNA of either of the *M.incognita* genes) were disformed; the size of these females was small in comparison to those of control and were fusiform in shape. Also the gut granules (dark stained females) which are indicative of actively feeding RKN females were found to be present in females isolated from control plants. While the females isolated from the RNAi tobacco lines carrying dsRNA cassette of either of the GOI were transparent in appearance, signifying the absence of gut granules and active feeding in such females (**Figure 2**). The females isolated from the roots of RNAi-MiTP lines showed a 31.8% reduction, while those from RNAi-MiRpn-7 tobacco lines showed 62.6% reduction in size in comparison to the control (**Table 2**). Thus it can be concluded from the above observations that the *M.incognita* that fed on the roots of RNAi tobacco plants bearing dsRNA cassettes did not develop normally and faced difficulty in their proper development. Hence we can conclude with conviction that *M.incognita* development is severely impaired by the dsRNA of Tyrosine phosphate and/or Rpn-7 gene from *M.incognita*.

3.3 Evaluating transgene expression in transgenic tobacco lines by RT-PCR

The nematodes were picked from the roots of RNAi tobacco plants bearing dsRNA cassettes of Tyrosine phosphate/Rpn-7 genes of *M.incognita* and total RNA was extracted and then cDNA was synthesized from RNA. The synthesized *M.incognita* cDNA was then subjected to qRT-PCR. Even after several (~50) cycles of PCR amplifications, we could not amplify the Tyrosine phosphate transcript from the cDNA from the cDNA of nematodes isolated from RNAi-MiTP lines, i.e. the RNAi tobacco plants bearing dsRNA of Tyrosine phosphate gene from *M.incognita* (**Figure 3**). Similarly, Rpn-7 transcript could not be amplified from cDNA of the nematodes isolated from RNAi-MiRpn-7 lines, i.e. the RNAi tobacco plants bearing dsRNA of Rpn-7 gene from *M.incognita*.

These findings clearly indicate that the dsRNA generated in the transgenic tobacco plants trigger RNAi mediated degradation of target mRNA in *M.incognita*; and as a consequence of the target transcript degradation, the *M.incognita* worms feeding on these transgenic plants bearing the dsRNA of the above genes did not show normal development. The resistance thus perceived in the transgenic tobacco plants was actually a consequence of RNAi mediated silencing of critical housekeeping genes of the nematode; they failed to develop normally on the roots of transgenic plants bearing the dsRNA cassettes.

Table 1: Comparison of transgenic tobacco plants with negative control

Tobacco Line	No. of Replicates	Number of			
		Knots/plant	Females/knot	Egg masses g1- root wt.	Eggs g1- root wt.
Control[a]	30	140 ± 4 (30)	13 ± 4	38.4 ± 17.2	7240 ± 1256
RNAi-MiTP[b]	30	23 ± 3.2 (3) [-83.57%]	3 ± 1.3 [76.92%]	18.6 ± 13.4 [51.56%]	2930 ± 657 [59.53%]
RNAi-MiRpn-7[c]	30	38 ± 5.4 (9) [-72.86%]	5 ± 2.4 [61.54%]	22.7 ± 15.3 [40.88%]	3610 ± 879 [50.14%]

The alphabets a,b,c marked as superscripts indicate the dsRNA treatment given to different transgenic tobacco lines. a = Root sinoculated with empty vector pRB7 as negative control, b = Roots inoculated with pRB7-MiTP dsRNA onstruct, c = Roots inoculated with pRB7-MiRpn-7 dsRNA construct. ~2000 freshly hatched J2 nematodes were used for infecting the transgenic tobacco lines (RNAi-MiTP and RNAi-Rpn-7 lines) as well as empty vector control plants. The roots were observed under a stage microscope 6wpi for counting the number of knots. 30 replications per transgenic tobacco line were used for the analysis and data here show the mean ± SE values. The means, standard error of mean and statistically significant values were determined by 1way-ANOVA and Tukey HSD test at 5% level of significance. The figures in round brackets () represent the number of plants. The figures in square brackets [] represent the percentage change in comparison to control.

Table 2: Comparison of sizes *M,incognita* females isolated from transgenic tobacco lines and control 6wpi.

Tobacco Line	Diameter±SE (µm)	Percentage change
Control	289 ± 21.04	
RNAi-MiTP	197 ± 16.36	-31.8%
RNAi-MiRpn-7	108 ±24.02	-62.6%

Ten tobacco plants per line were used for analysis. The mean and statistically significant values were determined by 1way-ANOVA and Tukey HSD test at 5% level of significance.

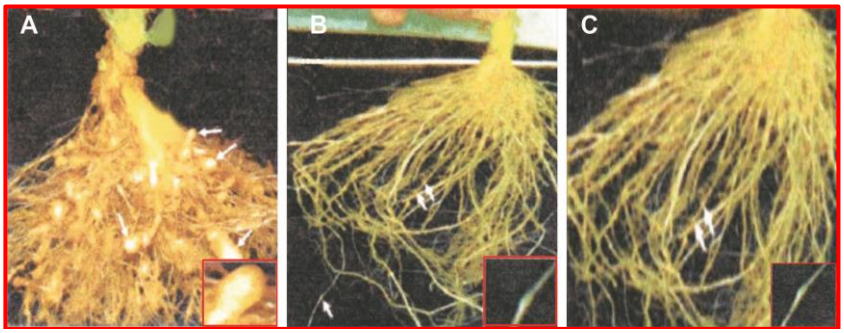

Figure 1: Comparison of roots of transgenic tobacco plants with that of negative control (6wpi).

The host delivered dsRNAs initiate RNA interference mediated gene silencing in *Meloidogyneincognita*. The figure (Scale bar: 1cm) shows 6 wpi roots of: A. Negative control (empty vector) tobacco plant, B. RNAi-MiTP (Tyrosine phosphate) tobacco plant, C. RNAi-MiRpn-7 tobacco plant. The arrows point

towards the knots acquired because of *M.incognita* infection. A single root-knot has been magnified for a clearer view shown in the inset (Scale bar: 2mm).

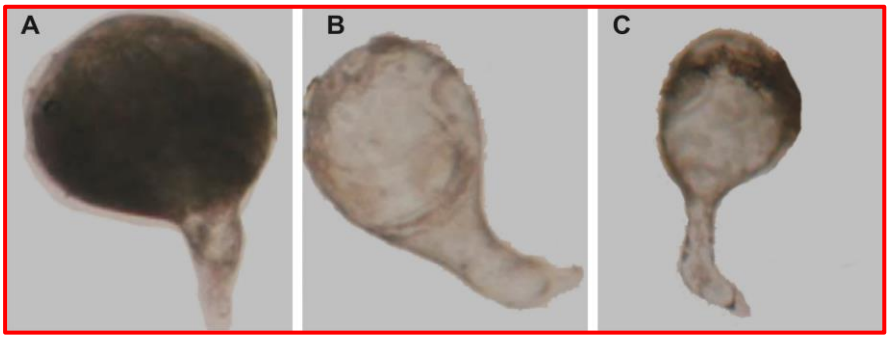

Figure 2: *M.incognita*females isolated from root-knots of.

A. Negative control (empty vector) tobacco plant, B. RNAi-MiTP (Tyrosine phosphate) tobacco plant, C. RNAi-MiRpn-7 tobacco plant. Scale bar: 300µm.

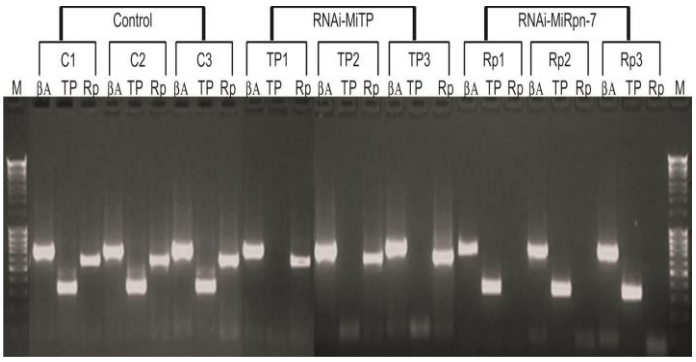

Figure 3: RT-PCR amplification of target transcripts from cDNA of transgenic tobacco plants.

Shown above the gel photograph: M = Molecular ladder, C = Negative control (empty vector), RNAi-MiTP = Trangenic tobacco expressing Tyrosine phosphate gene from *M.incognita,* RNAi-MiRpn-7 = Trangenic tobacco expressing Rpn-7 gene from *M.incognita.* Shown on the gel: βA = β-Actin gene from *M.incognita,* TP = Tyrosine phosphate gene from *M.incognita,* Rpn-7 = Rpn-7 gene from *M.incognita.*

4. DISCUSSION

The RKNs are the most damaging of the crop pests and infest almost all vascular crops worldwide. Since the starting of the research on creating nematode resistant plants using RNAi began almost a decade ago, this technique has garnered quite a success to become a promising tool against the nematode menace. Luckily there have been a few studies in different plant systems which reported even upto 90% reduction in nematode infection (Yadav et al. 2006). The earlier studies targeted various genes such as kinase, transcription factors, secretory enzymes and metabolic genes. For the present study, two M.incognita genes (MiTP and MiRpn-7) were targeted through HD RNAi approach. Our transgenic tobacco lines (RNAi-MiTP and RNAi-MiRpn-7) showed significant reduction (43-84%) in the number of knots/plants, number of females/knot, number of egg masses g-1 root wt. and number of eggs g-1 root wt as compared to the control tobacco plants. Obviously, every plant system is unique in itself and show variable resistance to the pests and various other responses and variable interactions. Nevertheless, this study and other such studies contribute towards generating good target genes for HD RNAi. Such extensive knowledge on valuable candidate genes when brought forth through such studies would create a resourceful library of sorts and the cautious use of such candidates alone/combined holds promising results towards generating nematode resistant crop plants. Gene pyramiding utilizing MiTP and MiRpn-7 genes along with some other reported useful gene/s might

improve the resistance of crops to the nematodes in the field.

REFERENCES

1) Abad P, Gouzy J, Aury JM, Castagnone-Sereno P, Danchin EG, Deleury E, Perfus-Barbeoch L, Anthouard V, Artiguenave F, Blok VC, Caillaud MC, Coutinho PM, Dasilva C, De Luca F, Deau F, Esquibet M, Flutre T, Goldstone JV, Hamamouch N, Hewezi T, Jaillon O, Jubin C, Leonetti P, Magliano M, Maier TR, Markov GV, McVeigh P, Pesole G, Poulain J, Robinson-Rechavi M, Sallet E, Ségurens B, Steinbach D, Tytgat T, Ugarte E, van Ghelder C, Veronico P, Baum TJ, Blaxter M, Bleve-Zacheo T, Davis EL, Ewbank JJ, Favery B, Grenier E, Henrissat B, Jones JT, Laudet V, Maule AG, Quesneville H, Rosso MN, Schiex T, Smant G, Weissenbach J, Wincker P. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nature Biotechnology 26:909–915, (2008)
2) Atamian HS, Roberts PA, Kaloshian I. High and low throughput screens with root-knot nematodes Meloidogyne spp. J Vis Exp 61:e39, (2012)
3) Bakhetia M, Urwin PE, Atkinson HJ. Characterisation by RNAi of pioneer genes expressed in the dorsal pharyngeal gland cell of *Heteroderaglycines*and the effects of combinatorial RNAi. Int J Parasitol 38:1589–1597, (2008)
4) Banerjee S, Banerjee A, Gill SS, Gupta OP, Dahuja A, Jain PK, and Sirohi A. RNA Interference: A Novel Source of Resistance to Combat Plant Parasitic Nematodes. Frontiers in Plant Science. 8: 834, (2017)
5) Chandel YS, Kumar S, Jain RK, and Vashisth S. An analysis of nematode problem in greenhouse cultivation in Himachal Pradesh and avoidable losses due to Meloidogyne incognita in tomato. Indian Journal of Nematology. 40: 98-203,(2010)
6) Chen SY, Sheaffer CC, Wyse DL, Nickel P and Kandel H. Plant-parasitic Nematode Communities and Their Associations with Soil Factors in Organically Farmed Fields in Minnesota. Journal of Nematology, 44(4): 361-369, (2012)
7) Chitwood DJ. Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture-Agricultural Research Service. Pest ManagSci 59:748–753, (2003)
8) Danchin EG, Arguel MJ, Campan-Fournier A, Perfus-Barbeoch L, Magliano M, Rosso MN, Da Rocha M, Da Silva C, Nottet N, Labadie K, Guy J, Artiguenave F, Abad P. Identification of novel target genes for safer and more specific control of root-knot nematodes from a pan-genome mining. PLoSPathog 9:e1003745, (2013)
9) Dinh PTY, Brown CR, Elling AA. RNA interference of effector gene *Mc16D10L* confers resistance against *Meloidogynechitwoodi*in *Arabidopsis* and potato. Phytopathology 104:1098–1106, (2014a)
10) Dinh PTY, Zhang L, Brown CR, Elling AA. Plant-mediated RNA interference of effector gene *Mc16D10L* confers resistance against *Meloidogynechitwoodi*in diverse genetic backgrounds of potato and reduces pathogenicity of nematode offspring. Nematology 16:669–682, (2014b)
11) Dong L, Xu J, Chen S, Li X, Zuo Y. *Mi-flp-18* and *Mi-mpk-1* genes are potential targets for *Meloidogyne incognita* control. J Parasitol 102:208–213, (2016)
12) Dutta TK, Papolu PK, Banakar P, Chaudhary D, Sirohi A, Rao U. Tomato transgenic plants expressing hairpin construct of a nematode protease gene conferred enhanced resistance to root-knot nematodes. Front Microbiol 6:260, (2015)
13) Elling AA. Major emerging problems with minor *Meloidogyne*species. Phytopathology 103:1092–1102, (2013)
14) Eisenback JD, Sasser JN, and CC. Diagnostic characters useful in the identification of the four most common species of root-knot nematodes (*Meloidogyne*spp.) In: Sasser JN, Carter CC (eds.) An advanced treatise on *Meloidogyne*1: Biology and control, North Carolina State University Graphics, Raleigh, 95–112, (1985)
15) Fairbairn DJ, Cavallaro AS, Bernard M, Mahalinga-Iyer J, Graham MW, Botella JR. Host-delivered RNAi: an effective strategy to silence genes in plant parasitic nematodes. Planta 226:1525–1533, (2007)
16) Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811, (1998)
17) Ibrahim HMM, Alkharouf NW, Meyer SLF, Aly MA, Gamal El-Din Ael K, Hussein EH, Matthews BF. Post-transcriptional gene silencing of root-knot nematode in transformed soybean roots. ExpParasitol 127:90–99, (2011)
18) Jaouannet M, Magliano M, Arguel MJ, Gourgues M, Evangelisti E, Abad P, Rosso MN. The root-knot nematode calreticulin *Mi-CRT* is a key effector in plant defense suppression. Mol Plant Microbe Interact 26:97–105, (2013)
19) Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J. Systematic functional analysis of the *Caenorhabditis elegans*genome using RNAi. Nature 421:231–237, (2003)
20) Kumar A, Kakrana A, Sirohi A, Subramaniam K, Srinivasan R, Abdin MZ, Jain PK. Host-delivered RNAi-mediated root-knot nematode resistance in Arabidopsis by targeting splicing factor and integrase genes. Journal of General Plant Pathology 83(2): 91-97, (2017)
21) Lourenço-Tessutti IT, Souza Junior JDA, Martins-de-Sa D, Viana AA, Carneiro RM, Togawa RC, de Almeida-Engler J, Batista JA, Silva MC, Fragoso RR, Grossi-de-Sa MF. Knockdown of heat-shock protein 90 and isocitratelyase gene expression reduced root-knot nematode reproduction. Phytopathology 105:628–637, (2015)
22) Niu J, Jian H, Xu J, Chen C, Guo Q, Liu Q, Guo Y. RNAi silencing of the *Meloidogyne incognita Rpn7* gene

reduces nematode parasitic success. Eur J Plant Pathol 134:131–144, (2012)

23) Niu J, Liu P, Liu Q, Chen C, Guo Q, Yin J, Yang G, Jian H.*Msp40* effector of root-knot nematode manipulates plant immunity to facilitate parasitism. Sci Rep 6:19443, (2016)
24) Papolu PK, Gantasala NP, Kamaraju D, Banakar P, Sreevathsa R, Rao U. Utility of host delivered RNAi of two FMRF amide like peptides, *flp-14* and *flp-18*, for the management of root knot nematode, *Meloidogyne incognita*. PLoS One 8:e80603, (2013)
25) Rosso MN, Jones JT, Abad P. RNAi and functional genomics in plant parasitic nematodes. Annu Rev Phytopathol 47:207–232, (2009)
26) Shaw, C.H. Introduction of Cloning Plasmids into Agrobacterium tumefaciens; Agrobacterium tumefaciens Chemotaxis Protocols. Methods in Molecular Biology; 44: 29-36, (1995)
27) Tamilarasan S, Rajam MV. Engineering crop plants for nematode resistance through host-herived RNA interference. Cell Dev Biol 2:114, (2013)
28) Xue B, Hamamouch N, Li C, Huang G, Hussey RS, Baum TJ, Davis EL. The *8D05* parasitism gene of *Meloidogyne incognita* is required for successful infection of host roots. Phytopathology 103:175–181, (2013)
29) Yadav BC, Veluthambi K, Subramaniam K. Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. MolBiochemParasitol 148:219–222, (2006)