Silencing M.incognita Gene through HdRnai In Transgenic Tobacco For Effective Nematode Resistance

[1]Deeksha Baliyan, [2]Ravindra Kumar
[12]Mewar University, Gangrar, Chittorgarh

Abstract: **--**dsRNA construct targeted to Meloidogyne incognita gene Rpn-7 (MiRpn-7) was evaluated to examine its efficiency for reducing M.incognita infection in the host tobacco plants. The MiRpn-7 gene bears great similarity with its homologues in other important nematodes like H. glycines and C. elegans. The transgenic tobacco plants transformed with MiRpn-7 dsRNA construct were challenged with freshly hatched M.incognita juveniles and the feeding assays were performed in ordes to assess the effects on the resistance of the plants and parasitic abilities of the invading nematodes. It was found that the dsRNA construct silenced the MiRpn-7 gene efficiently in the nematodes isolated from the roots of the transgenic tobacco plants expressing requisite dsRNA and it also severely hindered the infectivity of the invading nematodes which showed significant reduction in the number of nematodes and root knots formation. 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 RNAi-MiRpn-7 tobacco lines (carrying M.incognita Rpn-7 gene), in comparison to the empty vector control tobacco lines. The size of the nematode females isolated from the transformed roots was significantly reduced and their shape was also distorted, thus signifying the lack of normal development of nematodes feeding on the transformed tobacco plants. The diameter of M.incognita females isolated from the transformed tobacco roots was reduced by more than 30% compared to the diameter of the females extracted from the roots of the control plants transformed with the empty vector. The results from the present study that host directed RNAi (HD RNAi) mediated gene silencing of the MiRpn-7 gene can significantly reduce the M.incognita infection and it displays an encouraging approach for countering the menace of plant parasitic nematodes. This holds a promising future towards widening the scope of engineering the nematode-resistant crops for sustainable agriculture.

Keywords: **--HD RNAi, Juveniles, Nematode-resistant crops, Plant parasitic nematodes.**

Introduction

Nematodes are minute worms which are amongst the most abundantly present organisms on the planet (Blaxter et al. 1998). While these worms are mostly free living and derive their nourishment from microorganism hosts, few others parasitize plants/animals. PPNs (plant parasitic nematodes) infect almost all the plant species be it grasses, ornamentals or the food-crops and is responsible for extensively damaging these plants every year. Loss to the world agriculture has been predicted to be more than $170 billion each year (Banerjee et al. 2017). The data for the loss estimate is not the same for different crops/varieties and these also vary for different agro-climatic regions. But averaging the data, ~20% of the yearly production losses beared by the crop plants are attributed to the PPNs. The most damaging ones among the PPNs belong to the endoparasites of Tylenchoidea superfamily. Tylenchoidea comprises of the cyst nematodes (Heterodera spp.) and the RKNs/root-knot nematodes (Meloidogyne spp.). The M.incognita is the most harmful RKN species to the agricultural sector globally, chiefly because of its polyphagic nutrition, varied habitat and high reproductivity (Trudgill and Blok 2001). RKNs have obligatory parasitic lifestyle and they have evolved exceptionally sophisticated and exclusive means of infecting the plants. In order to support their sedentary lifestyle, they inoculate a myriad of effectors/proteins into the host feeder cells. The effectors are proteins (cellulases/proteases/glandular proteins) released by the nematodes. These effectors/proteins contribute towards successful puncturing of the host cells by the nematodes and passage into the root cells and perform a crucial function of overcoming the host defence mechanisms, assisting in successful induction and maintaining the feeder sites (Gheysen andMitchum 2011). The infective stage of the RKNs i.e. the J2 juveniles, possess high motility, but they do not feed. The J2s attack the root-tip region and after penetrating the epidermal region, they move via intercellular migration to the stele. After the establishment of the feeder sites through the development of giant cells in the parenchymatic cells of the phloem, they become immotile (Davis et al.2008). These effectors/proteins change the root differentiation that lead to re-differentiation of the root cells, finally resulting in the generation of humongous multi-nucleate cells which possess high metabolic rates. These mammoth cells are known as the giant cells. The multinucleate giant cells/feeder sites are formed after the cell wall goes through massive restructuring and reshaping with dissolution of the cell walls of the adjoin cells. The feeder sites work to collect whole of the nutrients from the host plants and diverts them to the nematode’s feeding tube which provides the nutrition to the invading nematode for driving its metabolism. Simultaneously along with the creation of the feeder sites, the adjoining pericycle/cortex cells undergo enlargement and division leading to the formation of the gall-like knots in the roots which are indicative of RKN infestation.

The prominent symptoms of nematode infection in the crops are stunted growth, wilted stems and increased susceptibility to other pests/diseases. Some crop varieties of tomato/potato/soybean contain natural nematode-resistance genes/loci which bear inherent resistance to some nematode species, but the expanse of such resistance is very narrow, effective merely against some particular pathotypes. Besides this, such resistance genes/loci are not present for many plants like sugar/pineapple. Other approaches for controlling the nematode infection like crop rotation fall short due to the immense potential of the RKNs to infest thousands of crops. Subsequently, attempts for eliminating or minimizing the production loss incurred due to nematode infection diverted towards the utilization chemicals known as nematicides. Despite the fact that these chemical agents including carbon disulfide, organophosphorus compounds, acetoprole, carbamates, oxime-carbamates and fumigants is quite efficient in curtailing the PPN infection; their high cost and toxic effects on environmental and human health render them unsafe and unsubstantial as a nematode controlling strategy. Infact most of the chemical agents used for nematode control have been prohibited by legal bans in many countries because of their toxic effects on the human health and the environment including depletion of ozone layer. The limitations of the current control strategies have created a huge and urgent need for developing new and unconventional measures for nematode control.

The knowledge of the fact that feeding dsRNA/siRNA/miRNA to C.elegansdownregulates the nematode's genes and discovery of the RNAi (RNA interference) mechanism (Fire et al. 1998) paved way for a plethora of such studies in other nematodes also to scrutinize the role that different genes play. Thereby RNAi has been recognized as a potent means for analyzing and controlling the gene functions. Whereas, the experiments on RNAi induction in C.elegans by feeding dsRNA/siRNA have been extremely successful, such intake is not adequately efficient for the PPNs. Such an obstinate behaviour shown by the PPNs might be attributed to their obligatory parasitic nature. Hence these nematodes are unable to feed outside the host, inhibiting the dsRNA/siRNA intake (Rosso et al. 2009). First suchsuccessful attempt accomplished in the cyst nematode H.glycines and G.pallida utilized a neuro-transmitter octopamine for stimulating dsRNA uptake (Urwin et al. 2002). A number of manuscripts emerged on the investigations conducted on the RKN M.incognitadownregulating different genes successfully by employing the same procedure (Dubreuil et al. 2011, Shingles et al. 2007, Huang et al. 2006, Rosso et al. 2005). In addition several reports are available in literature based on the host directed RNAi (HD RNAi) i.e. expressing nematode dsRNA/siRNA in the host plants for charactering the functions of various genes (Hewezi et al. 2008 and Patel et al. 2010) and for identifying the potent target genes for control nematode in crops (Youssef et al. 2013, Charlton et al. 2010, Li et al. 2010, Klink et al. 2009, Sindhu et al. 2009). The present study was undertaken to examine the credibility of silencing the MiRpn-7 gene of M.incognita by expressing dsRNA construct of the gene in the host tobacco plant through HD RNAi (host directed RNAi) mediated gene silencing in nematodes. To evaluate the effects of the treatment on nematode infection we examined the morphological characteristics of the transformed tobacco plants and evaluate these lines with regards to resistance against the root knot nematode M.incognita.

Nematode Culture

A pure culture of RKN M.incognita was maintained on tomato plants under green house conditions. M. incognita was sequestered from the tomato plants growing in the laboratory field of Department of Molecular Biology and Genetic Engineering, College of Biotechnology, SardarVallabhbhai 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 (SolanumlycopersicumCv.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 ddH2O at 27-30 0C.

Scheming TheRnai Vector

For expressing dsRNA of the MiRpn-7 gene in tobacco, a 400 bp fragment of the gene was 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).

Transferring the Construct from E.ColiToA.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).

Generating Transgenic Tobacco Expressing Mirpn-7 Gene

Tobacco plants were transformed with 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 0C 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.

Nematode 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 5 weeks in the green house. 5wpi 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 were 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).

CDNASynthesis 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-cDNATM Kit (Applied Biosystems Cat #4387406). The target sequence was amplified from cDNA by RT 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).

Results

(a) Effects on Resistance of Host Plants Against Nematode Infection

For the feeding assay, the tobacco lines (negative control lines and RNAi-MiRpn-7 lines) were inoculated with 2000 freshly hatched J2 juveniles of M.incognita per plant. Each data represents 30 independent line with 10 replications for each transgenic event and 3 repetition of each of the experiments. We opted for a specified period of 6 weeks post infection (wpi) for evaluating the effect of host-delivered dsRNA cassette on M.inc-ognita infection by evaluating the numbers of knots/plant, number of females/knot, number of egg masses g-1 root wt. and number of eggs g-1 root wt. in comparison to empty vector control tobacco lines (Table 1). The transgenic tobacco lines expressing the Rpn-7 gene from M.incognita (RNAi-MiRpn-7 lines) displayed a substantial reduction in the numbers of knots/plant (72.9%), number of females/knot (61.5%), number of egg masses g-1 root wt. (40.9%) and number of eggs g-1 root wt. (50.1%) in comparison to empty vector control tobacco lines. The results clearly indicate the potent effect of silencing the MiRpn-7 gene on the overall nematode infection in terms of the growth and developmental characteristics of these worms.

Table 1: Comparison of transgenic tobacco plants with negative control after nematode feeding

RNAiLine	Number of			
	Knots/ plant	Female/ knot	Egg masses g1- root wt.	Eggs g1- root wt.
Control	140 ± 4	13 ± 4	38.4 ± 17.2	7240 ± 1256
RNAi-MiRpn-7	38 ± 5.4	5 ± 2.4	22.7 ± 15.3	3610 ± 879
% Change	-72.86%	-61.54%	-40.88%	-50.14%

The means, standard error of mean and statistically significant values were determined by 1way-ANOVA and Tukey HSD test at 5% level of significance.

(b) Effects on Nematode Development

For evaluating the effect of host delivered RNAi (HD RNAi) on the root knot nematodes, the female M.incognita were isolated from transgenic tobacco lines expressing dsRNA of MiRpn-7 gene (RNAi-MiRpn-7) and stained with acid fuchsin. It was observed that the female nematodes isolated from the roots of RNAi-MiRpn-7 tobacco lines were of very small size and showed a 31.8% reduction in diameter in comparison to the control (Table 2). These females also displayed a distorted or fusiform shape in contrast with the peculiar tear-drop shape of the females isolated from the control plants.

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

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

The mean and statistically significant values were determined by 1way-ANOVA and Tukey HSD test at 5% level of significance.

Transcript DownregulationInM.Incognita Feeding Transgenic Tobacco

At 6 wpi, female M.incognitawere isolated from the roots of the plants from MiRpn-7 dsRNA expressing transgenic lines (RNAi-MiRpn-7). Females were taken out in 3 batches each containing 15 individuals for each transgenic line along with the females from the roots of the control tobacco lines. The females thus isolated were stored in liquid nitrogen to be used later for isolating total RNA from them. qRT PCR was employed for quantification of the transcript levels in the females isolated from the roots of the plants belonging to RNAi and control lines. The M.incognita actin gene (which is a constitutive gene) was used as a control for normalizing the transgene expression data. For each of the RNAi-MiRpn-7 lines, the expression of the Rpn-7 (MiRpn-7) gene was found to be substantially reduced for the nematodes isolated from RNAi plants compared with the control.

Conclusion

Our transgenic tobacco (RNAi-MiRpn-7) lines showed potent reduction in the number of knots/plant (83.6%) and number of females/knot (76.9%) in comparison to the empty vector control tobacco lines. Earlier researchers have shown up to 90% reduction in the number of nematodes on silencing other genes in different 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.

References

1) Banerjee, S., Banerjee, A., Gill, S.S., Gupta, O.P., Dahuja, A., Jain, P.K. and Sirohi, A. (2017). "RNA Interference: A Novel Source of Resistance to Combat Plant Parasitic Nematodes". Frontiers in Plant Science 8: 834. doi: 10.3389/fpls.2017.00834

2) Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM et al.: A molecular evolutionary framework for the phylum Nematoda. Nature 1998, 392:71-75.

3) Charlton WL, Harel HY, Bakhetia M, Hibbard JK, Atkinson HJ et al. (2010) Additive effects of plant expressed double-stranded RNAs on root-knot nematode development. Int J Parasitol 40: 855-864. doi: 10.1016/j.ijpara.2010.01.003. PubMed: 20100489.

4) Davis EL, Hussey RS, Mitchum MG, Baum TJ (2008) Parasitism proteins in nematode-plant interactions. CurrOpin Plant Biol 11:360-366. doi:10.1016/j.pbi.2008.04.003. PubMed: 18499507.

5) Dubreuil G, Deleury E, Magliano M, Jaouannet M, Abad P et al. (2011) Peroxiredoxins from the plant parasitic root-knot nematode, Meloidogyne incognita, are required for successful development within the host. Int J Parasitol 41: 385-396. doi:10.1016/j.ijpara.2010.10.008. PubMed: 21145323.

6) Gheysen G, Mitchum MG (2011) How nematodes manipulate plant development pathways for infection. CurrOpin Plant Biol, 14: 415–21. PubMed: 21458361.

7) Hewezi T, Howe P, Maier TR, Hussey RS, Mitchum MG et al. (2008) Cellulose binding protein from the parasitic nematode Heteroderaschachtii interacts with Arabidopsis pectin methylesterase: cooperative cell wall modification during parasitism. Plant Cell 20: 3080-3093. doi: 10.1105/tpc.108.063065. PubMed: 19001564.

8) Huang G, Allen R, Davis EL, Baum TJ, Hussey RS (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. ProcNatlAcadSci U S A 103: 14302-14306. doi:10.1073/pnas.0604698103. PubMed: 16985000.

9) Huang G, Gao B, Maier T, Allen R, Davis EL et al. (2003) A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode Meloidogyne incognita. Mol Plant Microbe Interact 16: 376-381. doi:10.1094/MPMI.2003.16.5.376. PubMed: 12744507.

10) Klink VP, Kim KH, Martins V, Macdonald MH, Beard HS et al. (2009) A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of development of female Heteroderaglycines cyst formation during infection of Glycine max. Planta 230: 53-71. doi:10.1007/s00425-009-0926-2. PubMed: 19347355.

11) Li J, Todd TC, Trick HN (2010) Rapid in planta evaluation of root expressed transgenes in chimeric soybean plants. Plant Cell Rep 29: 113-123. doi:10.1007/s00299-009-0803-2. PubMed: 20012965.

12) Patel N, Hamamouch N, Li C, Hewezi T, Hussey RS et al. (2010) A nematode effector protein similar to annexins in host plants. J Exp Bot 61: 235-248. doi:10.1093/jxb/erp293. PubMed: 19887499.

13) Rosso MN, Jones JT, Abad P (2009) RNAi and functional genomics in plant parasitic nematodes. Annu Rev Phytopathol 47: 207-232. doi: 10.1146/annurev.phyto.112408.132605. PubMed: 19400649.

14) Rosso MN, Dubrana MP, Cimbolini N, Jaubert S, Abad P (2005) Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. Mol Plant Microbe Interact 18: 615-620. doi: 10.1094/MPMI-18-0615. PubMed: 16042006.

15) Shingles J, Lilley CJ, Atkinson HJ, Urwin PE (2007) Meloidogyne incognita: molecular and biochemical characterisation of a cathepsin L cysteine proteinase and the effect on parasitism following RNAi. ExpParasitol 115: 114-120. doi:10.1016/j.exppara.2006.07.008. PubMed: 16996059.

16) Sindhu AS, Maier TR, Mitchum MG, Hussey RS, Davis EL et al. (2009) Effective and specific in plantaRNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success. J Exp Bot 60: 315-324. PubMed: 19015219.

17) Trudgill DL, Blok VC (2001) Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. Annu Rev Phytopathol 39: 53-77. doi:10.1146/annurev.phyto.39.1.53. PubMed: 11701859.

18) Urwin PE, Lilley CJ, Atkinson HJ (2002) Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. Mol Plant Microbe Interact 15: 747-752. doi:10.1094/MPMI.2002.15.8.747. PubMed: 12182331.

19) Youssef RM, Kim KH, Haroon SA, Matthews BF (2013) Posttranscriptional gene silencing of the gene encoding aldolase from soybean cyst nematode by transformed soybean roots. ExpParasitol 134: 266-274. doi:10.1016/j.exppara.2013.03.009. PubMed: 23541467.