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Combined effect of two transgenes performing host delivered RNAi against *Meloidogyne incognita* and its parasitic potential

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Abstract

In present scenario, RKNs are the major threat to the agriculture, causing both direct and indirect damage. Root-knot nematodes (*Meloidogyne incognita*) are exceedingly evolved obligate sedentary plant endoparasites with highly complex parasitism strategy, which makes it hard to manage them. Developing resistance in plants is a promising way to deal with RKNs problem. RNA interference (RNAi) is a novel method through which plants can be modified to develop resistance against specific pest. *Splicing factor* and *integrase* are two housekeeping genes in *Meloidogyne incognita*. Host induced gene silencing (HIGs) of two genes (*splicing factor* and *integrase)* was performed simultaneously in the target organism through crossing the parents which were already transformed to express the dsRNA of each gene individually. The resultant progeny, expressing dsRNA for both of the genes was subjected to bioassay studies. The results showed a significant reduction in pathogenic and reproductive capability of *Meloidogyne incognita*. The number of galls, number of females and number of egg masses were recorded were in the range of 71.81- 74.39%, 76.73- 78.76% and 67.13- 70.27% respectively as compared to the untransformed events. The results here clearly show the enhanced effect of combinatorial gene silencing as compared to the single gene silencing and no gene silencing at all.

Keywords: RNAi, Arabidopsis, RKN, *Meloidogyne incognita*, dsRNA, siRNA, PPNs

Introduction

The Root knot nematodes (*Meloidogyne* species) are the most devastating group of nematodes affecting the yield and quality of agricultural and horticultural produce. Their cosmopolitan nature and complex strategy of parasitism makes them distinguished parasites. Total damage caused by plant parasitic nematodes in monetary terms is US$173 billion [1].

RKNs are believed to be the major contributor to this loss. About 5% of the total world crop yield is destroyed due to RKNs[2][3][4].Use of management practices *viz*. crop rotation unfortunately is not efficient for RKNs because many vegetables and weeds comes under the host range of this parasite, fallowing is not affordable for many farmers, biological control is limited in the sense that it is inconsistent, slow and less effective as compared to other methods and the consequences of indiscriminate use of pesticides in last few decades have been realized and use of pesticides in current agriculture practices is not desirable. This scenario creates a window for developing the resistant plant cultivars for crop production and RNAi is one of the promising way to develop resistant plant cultivars.

RNA interference (RNAi) is a conserved phenomenon of post transcriptional gene silencing through which explicit genes can be silenced in eukaryotes which may govern the vital activities in target organism. RNAi is being used as an effective method for gene function analysis and engineering of resistance in different plant against various pathogens [5]. In the process of RNAi, an exogenous dsRNA is introduced into the eukaryotic organism, the RNAi pathway is started by the RNAse III enzyme which is also called dicer enzyme, Dicer is joined with RNA-binding proteins, the TAR-RNA-binding protein (TRBP), PACT, and Ago2 [6] which disintegrate the dsRNA in 21-25 small nucleotides sequence called small interfering RNA (siRNA)[7].The sienna incorporate into the protein complex (RISC: RNA induced silencing complex), afterwards the RISC is guided to the targeted mRNA and the siRNA complementary to the mRNA starts degrading it. The target recognition and the cleavage is achieved with the help of the argonaute protein in the RISC [8].

Host delivered RNAi has been an effective way for targeted gene silencing in nematodes. The host can be modified genetically to produce the explicit dsRNA which upon ingestion by the nematode will initiate the targeted silencing of a particular gene. Plant resistance as a result of silencing *splicing factor* and *integrase* genes in host delivered RNAi in tobacco was also gained[9].The genes mentioned above are housekeeping genes. Housekeeping genes are typically constitutive genes that are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and physio pathological conditions [10]. Although some housekeeping genes are expressed at relatively constant rates in most non-pathological situations, the expression of other housekeeping genes may vary depending on experimental conditions. Results of host delivered RNAi of *splicing factor* and *integrase* gene in Arabidopsis was significant with number of galls, females and egg masses reduced by 59.5, 66.8 and 63.4%, in lines silenced for *splicing factor* while in the lines silenced for

integrase gene, the number of galls, females and egg masses was reduced up to 59.5, 66.8 and 63.4%, respectively [11]. This current study was undertaken to observe effect of silencing both these genes on RKN parasitism in Arabidopsis.

Materials and Method Culture of Transgenic Arabidopsis parents

The seeds were first surface sterilized inside laminar air flow. A wash of double distilled water followed by mercuric chloride (0.1%) + SDS followed by final wash in 70% ethanol was performed. The process was repeated 5 times. The seeds were then added with 1% agar and was kept at 4°C for 48 hours. The seeds after 48 hours were carefully placed on the agar gel with the help of autoclaved microtips. The Petri plates were then sealed with parafilm and the plates were kept in the culture room by maintaining a temperature of 22°C and 16 hours of continue light and 8 hours of darkness. (Fig1A). Half MS media was used for culturing the plants in petri plates.

Transfer of young plants from agar medium to small pot

The 7 days old plants were transferred to the 4 inches' pot containing soilrite. The pot were kept in a plate. After the transfer in pot, the plates were covered with polyfilm. The pot trays were then transferred to the National phytotron facility for better flowering and seed setting. The nutritional requirements of plants meet through Hoagland's solution. (Fig1B)

Crossing of two different parental line

The Arabidopsis plants were allowed to flower (Fig. 1C). Mature siliques and open flowers were removed from the mother plant. The unopened inflorescence was gently fixed under a binocular microscope with 10x magnification. Flower bud was opened by inserting the tip of forceps between petals and sepals and all immature anthers were removed. This procedure was repeated for all remaining buds of the inflorescence. The emasculated inflorescences were marked (Fig. 1D). Plant were let to grow for 2-3 days. The emasculated inflorescence was fixed again under the binocular. The stigmata had developed a rough, sticky surface and was ready to be pollinated. An open, mature flower was taken from the father plant with one pair of forceps and brought under the binocular. With the other forceps, the filament of an anther with visible pollen shedding was hold. Anther was tapped on the stigma to cover it with pollen as much as possible. Pollinated flowers were marked. Mature siliques were harvested after 25 days. (Fig. 1E)

Culture of T1 seeds

The harvested seeds were taken and kept in 4°C for storage, the seeds were subjected to surface sterilization in the laminar air flow. The seeds were then added with 1% agar and was kept at 4°C for 48 hours. The media for Arabidopsis culture was prepared by using MS salt, sucrose, clerigel and kanamycin (Fig. 1F).

Molecular confirmation for required transformants with both Genes

The primers were designed using primer designing tool in NCBI. The primers were diluted in 1x TE buffer and vortexed. Primers were incubated for 1 hour in 37°C. Stock solution was made by taking 10µl of diluted primers and 90µl of DDW water. CTAB (Cetyl trimethyl ammonium bromide) method was used for total DNA was isolation from transgenic and control Arabidopsis plants with minor modification and used for polymerase chain reaction [12]. To check purity, the sample DNA was run on an agarose gel (0.8%) and quantification was done using NanoDrop (ND-1000 spectrophotometer). PCR reactions were done using BioRad MyCycler machine. Amplified PCR products were analyzed by electrophoresis on 1% agarose gel, and photographed with gel documentation system.

Nematode Culture

The root-knot nematode, *M. incognita*, population was taken from the culture plants maintained at the Division of Nematology, IARI, New Delhi. The tomato plants were uprooted, roots washed with double distilled water until absolutely clean and egg masses handpicked in a cavity block. Egg masses were surface sterilized with 0.1% $HgCl_2$ for one min. and then rinsed with sterile distilled water three times so as to wash off the surface sterilizing agent. The egg masses were placed in the BOD for hatching at 26-28°C. The freshly hatched J2s were collected in a beaker in suspension and were counted and calibrated per ml. An average of three aliquots was taken and used further inoculums.

Study of root-knot nematode infection analysis

The infective juvenile of *Meloidogyne incognita* were inoculated at the rate of 500IJs per plant (Fig. 1G). Nematode penetration and development studies were carried after staining using Acid-Fuchsin[13]. Observations were recorded after 30 days of inoculation. For different treatments factor like Number of root knots, Number of females and Number of egg masses were recorded.

For the study 7 treatment were taken. Four of them were Arabidopsis plants expressing dsRNA for both integrase and splicing factor gene (SFIT1, SFIT2, SFIT3 and SFIT4). One untransformed plant was taken as control. For a comparative study, two other treatments SPL and INT were taken which singly silence splicing factor gene and integrase gene respectively. Five replications were taken for each treatment. The data taken from every replicate of each treatment were analyzed for variance (ANOVA) and CRD test was done to verify the data as significant or non-significant. The means were calculated by using Tukey's test at the $P < 0.01$ significance level using software, SAS for Windows (V 9.3 Chicago, USA)

Results and discussion

Confirmation of T1 progenies to have genes for dsRNA expression for both *integrase* and *splicing factor* through PCR.

The first generation crossed plants were grown and the 4 events were generated (SFIT1, SFIT2, SFIT3 and SFIT4) and tested for the presence of both the genes *viz splicing factor* and *integrase*. The 4 events were grown in Petri plate with agar media. After 10 days of growth leaf samples were taken from each event and the plant genomic DNA was isolated using the CTAB method. The primers were designed specifically to the target genes. The PCR was carried out followed by the gel electrophoresis. Presence of amplicons with the expected band size confirmed the presence of both the genes in the crossed lines. (Fig. 2)

Effect of combinatorial gene silencing on the parasitic ability of *Meloidogyne incognita*

10 days old plants were inoculated with 500 IJs per plant (Fig. 1F). 30 days post inoculation the readings were taken for the number of galls. Compared to the untransformed Arabidopsis plants the individual plants expressing dsRNA of and *splicing factor integrase* gene had significantly less number of galls. Reduction in the number of galls for RNAi silenced lines of Arabidopsis for *splicing factor* gene was recorded to be 69.24 % and plant silenced for *integrase* gene showed a reduction by 58.13% as compared to the untransformed line (Table 1) (Fig 4). The combinatorial effect of silencing of both the genes was observed in the crossed lines and the plants expressing dsRNA of both the genes came up with less number of galls as compared to the individual plants expressing dsRNA of splicing factor and integrase and untransformed as well. The crossed line SFIT 2 showed the most promising result with the reduction of the number of galls by 74.39% as compared to the untransformed lines (Table 1) (Fig4**).** The acid-fuchsin stained roots were examined for spotting the number of

females per gall. The effect of combinatorial host delivered silencing of nematode genes on the reproductive potential of *Meloidogyne incognita* can be judged by the number of egg masses produced by the female root knot nematode. The egg masses were counted and the Arabidopsis plants modified for individual gene HIGs were noted to bear significantly less egg mass as compared to the untransformed ones. The crossed Arabidopsis plants resulting in HIGs of both the genes showed a significant reduction in the number of egg mass ranging with a decrement of egg mass from 80.27% to 82.18% as compared to the untransformed plants (Table 1) (Fig. 4).

This research has targeted the study of combined behaviour of silencing two genes together which earlier have given promising results on individual silencing in *Meloidogyne incognita* through HIGs. The individually transformed plants of Arabidopsis, expressing the dsRNA of *splicing factor* and *integrase* gene were taken as parent. In order to bring both of the constructs together which expresses ds RNA together those two parental lines were crossed. The obtained progenies were confirmed for having construct for both silencing for the gene were done by PCR and gel electrophoresis. Four different events were subjected to bioassay studies.

The bioassay studies revealed that the combinatorial silencing of the two genes have provided enhanced results as compared to the events which were individually silenced. The events expressing dsRNA for both of the genes resulted in reduced number of number of galls, number of females, number of egg masses and number of eggs per egg mass. The event where only *splicing factor* gene was silenced through HIGs showed reduction in number of galls, number of females, number of egg masses, number of eggs per egg mass by 69.24%, 71.84%, 74.30% and 70.27% respectively as compared to the untransformed Arabidopsis. The events singly silencing the integrase gene through HIGs showed a reduction in number of galls, number of females, number of egg masses, no of eggs per egg masses by 58.13%, 64.43%, 58.6% and 45.38% respectively as compared to the untransformed Arabidopsis plant. Whereas, the events expressing the dsRNA for both of the genes showed number of galls, number of females, number of egg masses, number of eggs per egg mass in the range of 71.81- 74.39%, 76.73- 78.76% and 67.13- 70.27% respectively

Conclusions

The combinatorial host delivered silencing of the genes *splicing factor* and *integrase* in *Meloidogyne incognita* has performed well than the individual silencing of both the genes. The reduction in the number of galls , females, eggmasses observed was significantly greater than that of events individually silenced for the genes *splicing factor* and *integrase* as well as untransformed events .The silencing of the gene *integrase* has been effective in the company of silenced *splicing factor* and hence giving better results. Combinatorial silencing of taken genes has shown a positive interaction which can be exploited in agriculturally important crops.

Table 1. Efficacy of Host induced gene silencing on parasitic capability of *Meloidogyne*

Treatments	Number of galls	% Decrease	Number of females	% Decrease	Number of egg masses	% Decrease
SFIT 1	23.28	73.71	27.85	76.73	5.85	80.76
SFIT 2	22.71	74.39	25.42	78.76	5.42	82.18
SFIT 3	22.85	74.24	26.42	77.92	6.00	80.27
SFIT 4	25	71.81	27.57	76.96	5.57	81.68
SPL	27.28	69.24	33.71	71.84	7.71	74.3
INT	37.14	58.13	42.57	64.43	12.57	58.6
Untransformed	88.71	0	119.71	0	30.42	0
Cd (p=0.05)	2.166		3.169		1.258	
F (cal)	1007.7		948.92		429.62	
F (table) at 1%	3.26		3.2657		3.26	

***incognita* in different treatments**

***Significant Values at P= 0.01**

List of Figures

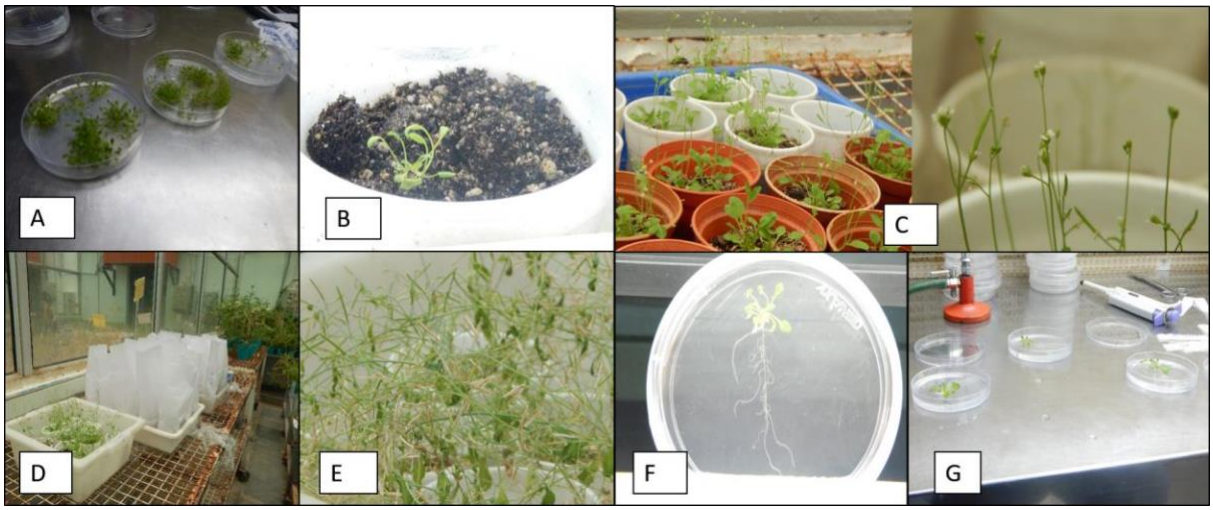

Figure 1. Different stages of performed experiments; (A) Culture of parental lines (*Splicing* and *integrase* gene specific) in petri plates (B) Transfer of small Arabidopsis plants to small pots having sterilized soilrite (C) Inflorescence of Arabidopsis plant ready for crossing (D) Emasculation and bagging of parental lines (E) The matured siliques after crossing ready for harvesting (F) Culture of resultant transgenic on clerigel and half MS media (G) Inoculation of *Meloidogyne incognita* infective juveniles at the rate of 500IJs per plant in different events

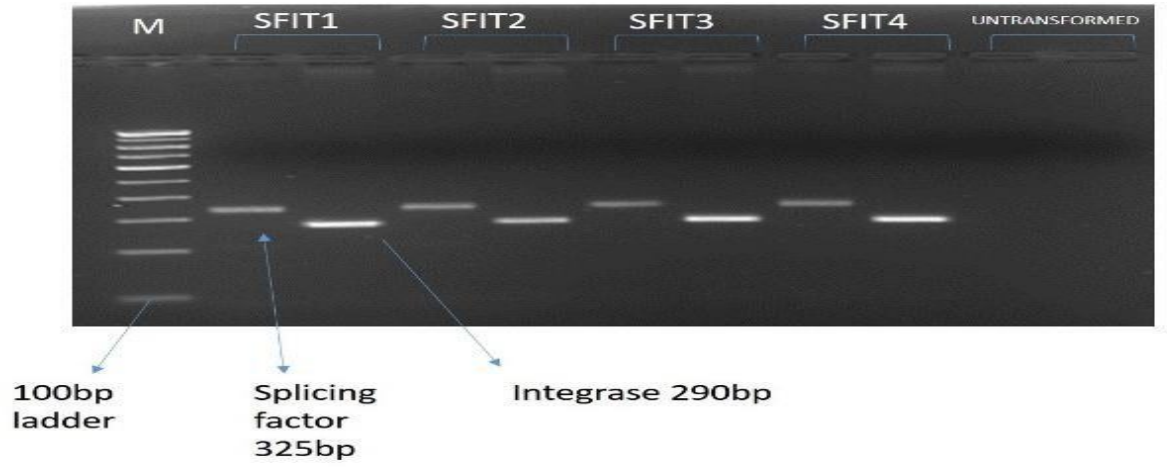

Figure 2. Confirmation of desired gene for dual gene silencing in resultant cross through PCR and gel electrophoresis. The two bands of 325 bp and 290 bp confirm the presence of gene for the silencing of *Splicing factor* and *Integrase* gene in *Meloidogyne incognita*

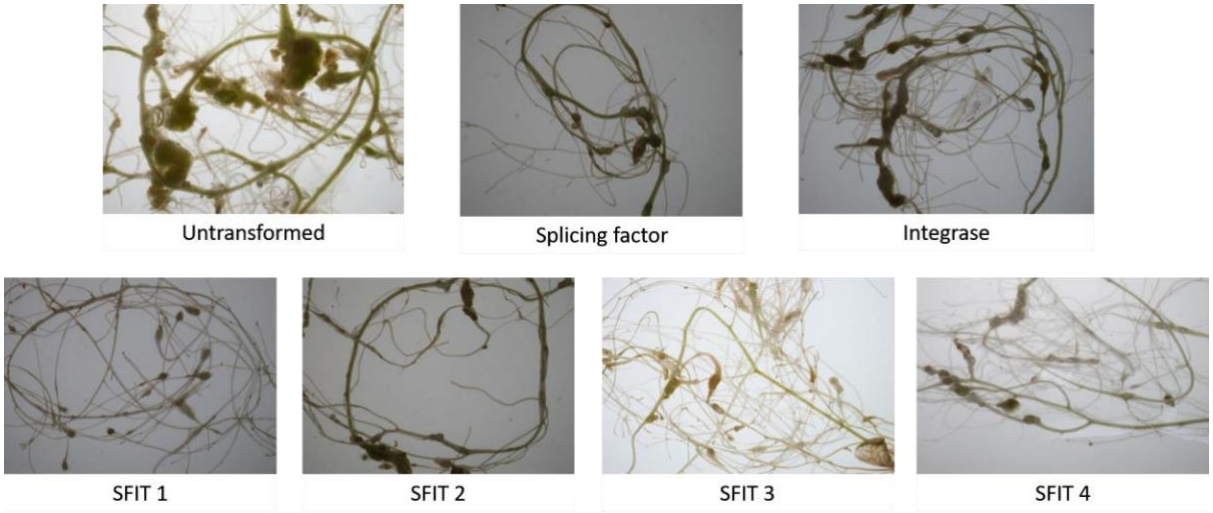

Figure 3. Galling intensity observed in treatments under study. Untransformed represent control. SPL represent individual silencing of splicing gene. INT represent individual silencing of integrase gene. SFIT1, SFI2, SFIT3 and SFIT4 represents lines silencing both splicing and integrase gene obtained after crossing.

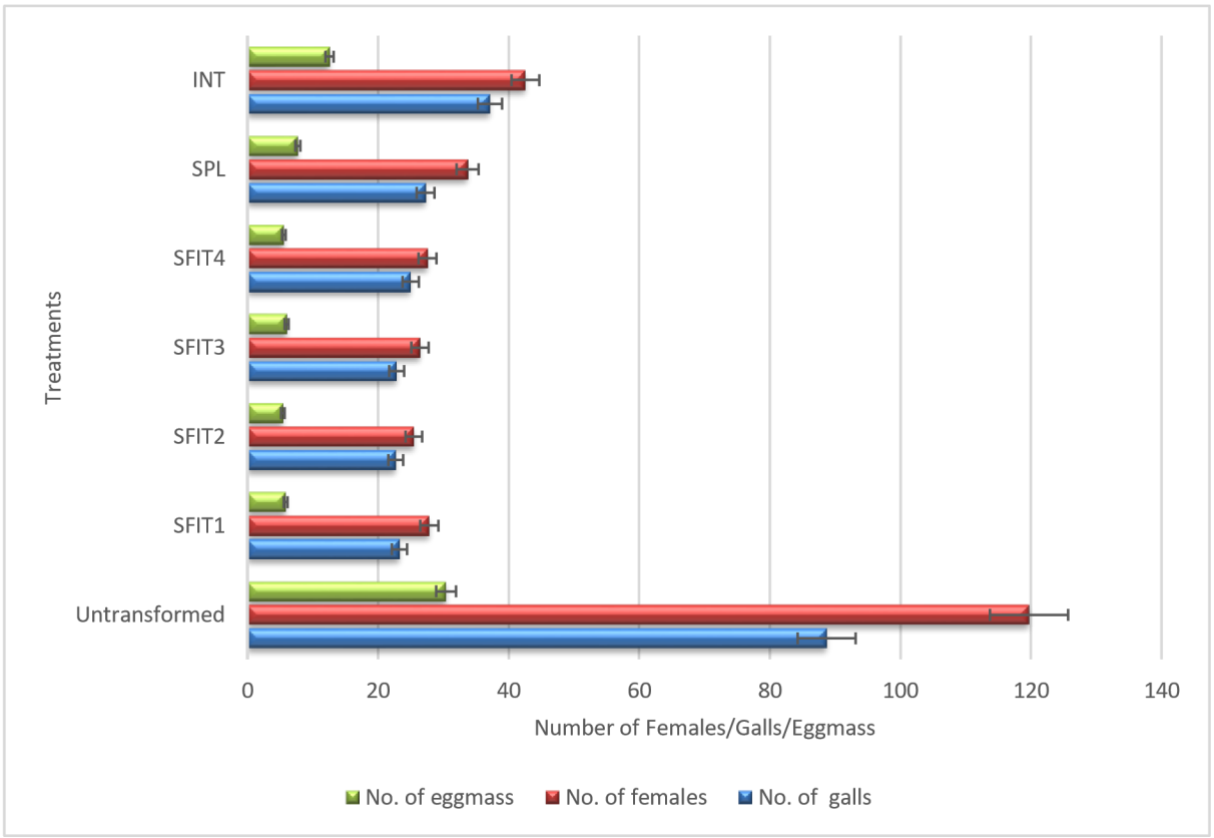

Figure 4. Efficacy of Host induced gene silencing on parasitic capability of *Meloidogyne incognita* in different treatments

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