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RNAi effect of the sex and larval development genes on root-knot nematode development and multiplication

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Abstract
Crop production is affected by abiotic and biotic stresses. Plant parasitic nematodes (PPN), apart from bacteria, fungus and viruses are major biotic stresses affecting agricultural crop production. Plant parasitic nematodes cause annual crop loss of about 173 billion US$ worldwide. Among the plant parasitic nematodes, sedentary endoparasites, namely root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes cause maximum damage to the crops and of the two, root knot nematodes are responsible for the larger share of damage because of their polyphagous feeding ability. Several pest management techniques have been used to manage PPNs but agrochemicals are most successful in reducing nematode populations but use of agrochemicals cause toxicity and pollution of the environment and is not a preferable. Most acceptable and environment friendly approach of nematode management is the use of natural resistance as no special practice or additional cost is needed for cultivation. Natural resistance sources among the cultivable species of crop plants are very few and as a result researchers look forward to use DNA recombinant technology to engineer resistance. Host delivered RNAi of genes crucial for RKN development and parasitism are good targets for resistance development. The RNAi approach is specific to the nematode and has no protein product thereby safe for the environment. In this study two *M. incognita* genes were targeted, Tra-1 and Bcat-1, responsible for its sex and larval development, dsRNA constructs of the two genes were individually incorporated in Arabidopsis using Agrobacterium based transformation technique. The study results indicate a significant reduction of 50% and 52% reduction in the number of galls; 42.22% and 53.33% reduction in no. of females; 44.44% and 57% reduction in no. of egg masses; 46.12% and 54.26% reduction in no. of eggs per egg mass, in Tra-1 and Bcat-1 events respectively in comparison to the non-transformed Arabidopsis control plants. It can be concluded from the results that RNAi approach can be used to manage root-knot nematodes. Nanotechnology based foliar application technology of dsRNA have been already generated for virus management. Further development may see a similar technology for nematode management also.

Keywords: RNAi, sex, root-knot, multiplication, nematode

Introduction
Nematodes are multicellular organisms belonging to the Ecdysozoa group. Animals belonging to this group characteristically shed cuticle by the process called ecdysis. They are known to be parasites of animals (invertebrates and vertebrates 44 percent of the described species), parasites of plants (15 percent of the described species) and free living forms feeding on bacteria, fungi and protozoans (40 percent of the described species). More than 4300 nematode species have been documented to parasitize plants (Decraemer and Hunt, 2006) [2].
Plant parasitic nematodes (PPNs) pose a grave threat to the commercial crops worldwide (Ali *et al.,* 2017) [1]. These microscopic organisms are known to feed on varied plant parts, from root to seed. Most PPNs are soil borne and hence prefer plant roots to feed on. The sedentary endoparasitic nematodes constitute the most elite group of the plant-parasitic nematodes. These nematodes enter the host roots and affect the host physiology to produce a permanent feeding site by transforming a host cell so as to draw cell sap as food source for completing their lifecycles. Root-knot nematode genera is the most economically damaging sedentary endoparasites of the agriculturally important crops. The annual economic loss caused by PPNs across the world is estimated at about 173 billion US dollars (Elling *et al*. 2013) [3] but this figure would definitely be more as in several parts of the world the farming community is still not aware of nematode diseases.
The RKN, *Meloidogyne* species are the most common and wide spread sedentary endoparasitic parasites with a host range of about 2000 plant species. They account for about 5% of the total global crop loss (Sasser and Carter, 1985) [4]. In India, southern root-knot nematode, *M. incognita* alone causes losses up to 27.21% (Jain *et al*., 2007) [5].

The second stage juvenile is the infective stage of the RKN. It penetrates in to the meristem region of the plant roots and transforms the host cell into a feeding cell called the giant cell. The external manifestation of the disease is the formation of knots or galls at the site of infection. Nematode secreted proteins (effectors) produced in the three oesophageal glands are thought to be involved in the RKN juvenile root penetration, formation and maintenance of the giant cells. RKN infected plants exhibit reduced vigour, yield, and show symptoms of water and nutrient stress. Managing root-knot nematode infestation is very challenging. Fallowing, soil solarisation, flooding, crop rotation have been attempted but are of low impact. (Roberts, 1995; Williamson and Kumar, 2006) [6, 7]. Use of RKN resistant cultivars for cultivation is a good choice but there are very few R genes available for cultivated crops (Roberts, 1995; Williamson and Kumar, 2006) [6, 7]. Biological control agents have been identified for RKN management but their effectiveness, long term establishment and sustainability in field under natural conditions has been a problem, establishment of the bio-control agents are the main problems with nematode bio-control agents. Chemical based management practices are most potent to control RKN parasitism but are harmful to the environment and human applicators as well. As a result most of the chemicals used for nematode management have been placed on the banned list and even those available now may soon go off the shelf because of high toxicity to the environment.

Sustainable agriculture practices insist on green or environment friendly management approaches. Nematode resistance is undoubtedly the best alternative for fighting nematode attack as it suppresses nematode development and reproduction, is low cost, sustainable and doesn't require any special cultivation practices (Hussey and Janssen, 2002) [8]. Breaking of host resistance by nematode populations, the suppleness and consistency of natural host resistance are some concerns of utilizing resistance as a management strategy for nematode management (Williamson, 1998; Atkinson *et al.,* 2003; Williamson and Kumar, 2006) [9, 10, 7]. Considering that the current management options are insufficient, biotechnological strategies need to be utilized to engineer resistance against nematodes. Specific gene-silencing by RNAi technology offers a great potential for disease management.

Host delivered RNA interference (RNAi) has shown good potential for the RKN management. RNAi has been successfully used to silence the expression of genes having role in nematode parasitism, survival, development, metabolism and gamete formation. Using the reverse engineering approach, RNAi technology has be ensued to identify the gene function or mechanisms of the cellular processes associated with the genes. RNAi gene silencing is accurate and has also been shown to occur in cells or tissues which are far away from the site of dsRNA entry (Rosso *et al*., 2009; Tamilarasan and Rajam, 2013; Banerjee *et al*., 2017) [11, 12, 13].

Materials and Methods

The technique of single egg mass inoculation was used to multiply and maintain root-knot nematode (RKN), *Meloidogyne incognita* culture on brinjal plants.

Genes involved in embryo development and nematode larval development in *Caenorhabditis elegans* were identified through in silico analysis. Worm Base was used to get the spliced CDS section of *C. elegans* genes, which were then used to find homologues in *M. incognita* genome sequences. Worm Base Parasite (http://parasite.wormbase.org/ftp.html) and *Meloidogyne* Genomic Resources, INRA (http://www6.inra.fr/meloidogyne_incognita).Two characterized genes tra1 and bcat-1 were selected for developing dsRNA-RNAi constructs for subsequent transformation into Arabidopsis; to study the effect of host generated RNAi on the larval development of *M. incognita*. the primers were made for the selected genes. for development dsRNAconstructs of Tra-1 and Bcat-1 Gateway cloning was done using the vector pDONR. By using the freeze and thaw procedure, specific *Tra-1* dsRNA and *Bcat-1*dsRNA constructs were mobilised into *Agrobacterium tumefaciens s*train GV3101 and used to create *Arabidospsis* transgenics using the floral-dip method (Bent *et al.,* 2006). The transgenic lines were screened through hygromycin selection and PCR using gene-specific primers for the Tra-1 and Bcat-1 genes was used to screen Arabidopsis transgenic (T1) plants for the presence of T-DNA inserts subsequently bioassay studies were done with the parameters considering no. of females per plants, no. of eggs per egg mass, no. of egg masses per plants. All of the findings were based on at least five separate experiments. Experiment was in completely randomized design (CRD)in our experiment. The data represented the average (mean) of all the experiments with standard error. ANOVA and Student's t-tests were used to examine the level of significance at $p \leq 0.05$ and/or $p \leq 0.01$ for statistically significant differences between the means of replicates of samples.

Result and Discussion

After 30 days post inoculation, plants were carefully uprooted and washed. Roots of the transformed and non transformed *Arabidopsis* plants were cleaned and root galls were counted under stereomicroscope. Significant reduction in number of root galls was observed when transformed plants were compared with non-transformed (control) plants. Average number of galls in *Tra-1*events was 20 galls and was 50% less when compared with non-transformed inoculated Arabidopsis plant. Similarlythe*Bcat-1*events, had19 galls exhibiting52% reduction in no. of galls when compared to non-transformed *Arabidopsis* plants which had 40 galls. Therefore, data shows there was significant reduction in no. of galls after using (dsRNAi) construct.

Significant reduction in no. of females was observed in both Bcat-1 andTra-1 transformed plants when compared with non-transformed plants. In transformed there was significant reduction in number of females per plants when compared with non-transformed plants. In average number of females, there was 42.22 percent reduction in Tra-1tranfomed plants as compared to wild type plants. Similarly in Bcat-1transformed plants there was 53.33 reductions in the no. females when compared with control (wild type) plants. This indicated the significant reduction in number of females in both gene transformed plants

We observed that silencing the *Tra-1* gene resulted in a 46.12 percent reduction in eggs per egg mass, while silencing the *Bcat-1* gene resulted in a 54.26 percent reduction, when compared to non-transformed plants, both genes resulted in a significant reduction of eggs per egg mass; however, due to their independent nature, there was no significant difference

between genes.
Numbers of egg masses produced plants of each replication were counted under stereo zoom microscope after 45 days of inoculation. Average number of egg masses was calculated and compared with non-transformed Arabidopsis plants. It was found that, egg mass production in *Tra-1* gene transformed plants was reduced by 44.44 percent when compared with control non-transformed plants. Plants transformed with the *Bcat-1* gene could reduce egg masse formation when compared with non-transformed control plants.

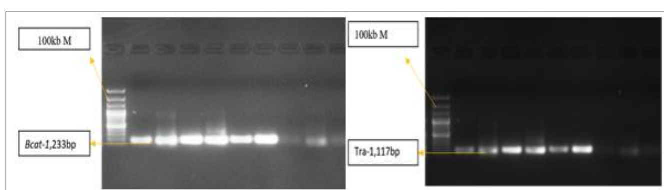

Fig 1: Confirmation of Tra-1 and Bcat-1gene by colony PCR

Fig 2: Floral dip method for transformation

Fig 3: Comparative degree of galling in different treatments

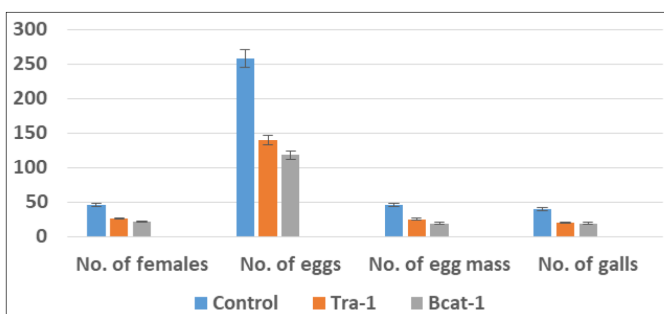

Fig 4: Comparative analysis effect of different treatments on no. of females, no. of galls, no. of egg masses and no. of eggs

Conclusion

The results showed 50% and 52% reduction in the number of galls; 42.22% and 53.33% reduction in number of females; 44.44% and 57% reduction in number of egg masses; 46.12% and 54.26% reduction in the number of eggs per egg mass, in the Arabidopsis events harbouring *Tra-1* and *Bcat-1* dsRNA respectively in comparison with the non-transformed RKN inoculated Arabidopsis plants (Figure 5). Varied range of reduction in nematode galls, eggs, eggs per egg mass have been reported by authors studying RNAi of different genes on the RKN.

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