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5. SUMMARY AND CONCLUSIONS

Plant parasitic nematodes are major agricultural pest, which infest almost all cultivated crop plants which is estimated to cause ~157 billion US$ economic loss annually. Clear symptoms of infection are often lacking, resulting in the inability to detect nematodes until the infestation is severe by farmers. The yield losses are often wrongly attributed to the secondary diseases of crops which have been severely weakened by nematode infection, leading to underestimation of the crop loss. Among plant parasitic nematodes, most of the damage is caused by sedentary endoparasitic genera Meloidogyne spp (Root Knot Nematode) Globodera and Heterodera spp (Cyst Nematode). The root-knot nematode (RKN), Meloidogyne incognita is an obligatory sedentary endoparasite which infects the roots of almost all cultivated plants. Following RKN infection, one or more plant root cells are re-differentiated into special feeding site for the development of reproductively competent females. These nematodes induce cellular modifications in root tissues to form giant cells by inducing plant cell mitosis without cytokinesis, leading to the formation of galls and subsequent plant death. Although chemical nematicides are the most reliable means of controlling nematodes, they are increasingly being withdrawn owing to their toxicity to humans and environment. In many countries effective nematicides have been and are continued to be de-registered. Studies using organic means, such as green manures to control nematodes have also been carried out in several laboratories but assessing their effectiveness remains difficult. Several groups have identified few of the naturally occurring resistance genes. However, experiments to transfer resistance from one crop to others have so far been unsuccessful, for reasons that remain unclear. Due to the
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limitations of current control strategies, it is imperative to look for a novel as well as safer strategy for successful nematode control.

Based on the existing knowledge, it is a known fact that RNAi can be utilized for deciphering gene function in eukaryotes. Previous studies delineated that it is possible to achieve RNAi response in nematodes, including plant parasitic nematodes. A few reports are available, using RNAi for the control of plant parasitic nematodes. However on the other hand several reports are available for chemically synthesized siRNAs and/or in vitro transcribed dsRNAs targeting nematode genes. The major hurdle in utilization of RNAi for nematode control has been due to the endoparasitic nature of the nematodes. The nematodes do not feed on the artificial diet and feeding occurs only near/after establishing infection through the plant roots. Hence in most of the available literature, the nematodes were induced to ingest dsRNA from the feeding solution by octapamine or recorcinol treatment. The potential application of in planta RNAi for nematode control lies in the availability of plant expressed dsRNA that can be readily taken up by nematodes upon feeding the plant roots. The plant parasitic nematodes develop a specialized structure inside the plant roots called feeding tube, facilitating the uptake of siRNAs.

Selection of target gene is most crucial step in deploying RNAi for nematode control. Genes involved in nematode development, especially those involved in embryogenesis, juvenile development and reproduction are of particular interest because they can possibly disrupt the life cycle of plant parasitic nematodes. Although the genes utilized in the available literature have demonstrated decrease in target transcript abundance and reduction in nematode reproduction, complete resistance for plant parasitic nematodes via host-derived RNAi has never been achieved. It is also
known from literature that not all genes are amenable for RNAi based nematode control. The factors contributing for a potent target gene for RNAi includes the nucleotide sequence utilized for silencing, copy number of the target gene (in case of multigene family conserved region might be considered), potential for achieving embryonic lethality etc. One such target is acetylcholinesterase (AChE), which is mainly involved in cholinergic neurotransmission and is also believed to be a multifunctional molecule involved in many other cellular processes including cell adhesion, apoptosis, synaptogenesis etc. This gene is also the main target of organophosphates and carbamates, two types of chemical pesticides being used extensively in agriculture and veterinary medicine against insects and nematodes. Thus AChE gene appears to be a potential target for nematode-control and could be used to develop nematode-resistant transgenic plants through host plant-mediated silencing of MiAChE gene. Different forms of AChE are encoded by multiple genes in nematodes (four) and analysis of expression pattern of these genes in C. elegans suggests that they perform non-redundant functions. In case of M. incognita, only AChE 1 and 2 has been reported. AChE-1 is believed to be involved in neurotransmission and nematode locomotion. Whereas MiAChE-2 has been proposed to have a role in contraction of pharyngeal value during feeding and is expressed through out its life cycle. Both MiAChE 1 and 2 appears to be involved in synaptic transmission. Although there is no literature available for MiAChE 3 and 4, a recent report on pinewood nematode revealed the importance of AChE 3 in protection against chemical inhibitors. In case of C. elegans, AChE 1, 2 and 3 have only 35% amino acid sequence similarity. Interestingly AChE 3 and 4 are more similar and the gene expression pattern indicates that they may perform non overlapping functions. Although AChE-4 is transcribed
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along with *AChE-3*, the extremely low levels of mature processed *AChE-4* mRNA, the failure to detect associated enzymatic activity *in vivo*, and the substitution of glutamic acid 199 by glutamine adjacent to the active site serine residue suggested that *AChE-4* is nonfunctional. Therefore, we decided to utilize the conserved region of the *MiAChE* 1 and 2. Hence in the present study, we have utilized the region which potentially targets all functional *AChE* transcripts and might result in a lethal phenotype for nematode.

Based on off-target analysis using BLASTN, the region considered for developing RNAi construct was highly specific to nematode *MiAChE* and was found to have no considerable homology with available sequences of plants, animals, beneficial insects and even with other nematode genes. The m-fold analysis predicted the ability of the RNAi construct to form a stable hairpin structure.

The partial cDNA of *MiAChE* sequence to be targeted by RNAi silencing was amplified from the total RNA of infective stage juveniles of *M. incognita* by Reverse Transcriptase PCR and cloned in pGEM-T easy vector along with appropriately added restriction sites. Further, the *MiAChE* was cloned in RNAi binary vector (pMVRhp) in sense and antisense orientation, separated by chalcone synthase intron. The expression of the hairpin construct was driven by the constitutive 35S promoter. *Agrobacterium tumefaciens* LBA4404 strain harboring *MiAChEhp* construct was then used for generating tobacco and tomato RNAi transformants. Around 20 tobacco and tomato RNAi transformants expressing dsRNA of *MiAChE* were developed and analyzed for the presence of introduced transgene by PCR. Subsequently, integration of the transgene was also analyzed by Southern hybridization. Single to multiple copy events were obtained in different RNAi lines examined. The expression of the dsRNA in the
selected RNAi lines was confirmed by the presence of \textit{MiAChE} transcripts by semi-quantitative RT-PCR. Further, the accumulation of siRNAs corresponding to \textit{MiAChE} was validated by Northern blotting. The tomato and tobacco RNAi lines displayed normal phenotype similar to that of the controls.

Transgene segregation analysis was carried out by germinating the seeds of RNAi lines on kanamycin-amended medium. Few of the RNAi lines followed Mendelian pattern of segregation (3:1), while some of the seeds (tobacco RNAi lines 18, 21, 29 and tomato RNAi lines 9 and 17) were not able to germinate on kanamycin-amended medium (excluded from the study).

Nematode resistance assays were performed in T\textsubscript{1} tobacco and tomato RNAi lines, which exhibited an enhanced level of resistance to nematode infection. Although infection occurs in the RNAi lines, the size of the galls formed were considerably smaller than that of control. In certain lines, no visible galls were observed even after 8 weeks post infection, though staining of the roots indicated the presence of immature/smaller females inside the roots. Based on these observations, we conclude that the silencing of \textit{MiAChE} altered the developing nematode structure and decreased the nematode reproduction, hence hampering the parasitism. Also we observed drastic reduction in the number of egg masses and altered anomalies in the nematode development in the RNAi lines, indicating the potential role of \textit{AChE} in nematode reproduction. The root characteristics analysis revealed that the RNAi lines were phenotypically normal to that of control plants. The target transcript reduction in the nematode feeding on the RNAi lines clearly indicated the utility of RNAi for nematode control.
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The present study has successfully demonstrated the utilization of RNAi for the control of plant parasitic nematodes. Targeted silencing of the conserved region of nematode AChE through host derived RNAi resulted in reduced fecundity. Thus this study has also demonstrated the critical role of AChE in nematode development and reproduction. The enhanced resistance to nematode infection displayed by different RNAi lines strongly suggests their utilization in nematode control. Further knowledge gained from this study in association with the existing literature could be instrumental in delineating the role of AChE in nematode development. Based on literature and our study results it can be proposed that engineering crops for nematode resistance through host delivered RNAi appears to be quite promising strategy.