



Host-induced silencing of *Mi-msp-1* confers resistance to root-knot nematode *Meloidogyne incognita* in eggplant

Sonam Chaudhary · Tushar K. Dutta · Nidhi Tyagi · Tagginahalli N. Shivakumara · Pradeep K. Papolu · Kapil A. Chobhe · Uma Rao

Received: 29 January 2019 / Accepted: 2 April 2019 / Published online: 6 April 2019
© Springer Nature Switzerland AG 2019

Abstract RNA interference (RNAi)-based host-induced gene silencing (HIGS) is emerging as a novel, efficient and target-specific tool to combat phytonematode infection in crop plants. *Mi-msp-1*, an effector gene expressed in the subventral pharyngeal gland cells of *Meloidogyne incognita* plays an important role in the parasitic process. *Mi-msp-1* effector is conserved in few of the species of root-knot nematodes (RKNs) and does not share considerable homology with the other phytonematodes, thereby making it a suitable target for HIGS with minimal off-target effects. Six putative eggplant transformants harbouring a single copy RNAi transgene of *Mi-msp-1* was generated. Stable expression of the transgene was

detected in T₁, T₂ and T₃ transgenic lines for which a detrimental effect on RKN penetration, development and reproduction was documented upon challenge infection with nematode juveniles. The post-parasitic nematode stages extracted from the transgenic plants showed long-term RNAi effect in terms of targeted downregulation of *Mi-msp-1*. These findings suggest that HIGS of *Mi-msp-1* enhances nematode resistance in eggplant and protect the plant against RKN parasitism at very early stage.

Keywords dsRNA · siRNA · Parasitism · Southern hybridization · RT-qPCR

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11248-019-00126-5>) contains supplementary material, which is available to authorized users.

S. Chaudhary · T. K. Dutta · N. Tyagi ·
T. N. Shivakumara · P. K. Papolu · U. Rao (✉)
Division of Nematology, ICAR-Indian Agricultural
Research Institute, New Delhi 110012, India
e-mail: umarao@iari.res.in;
umanema@gmail.com

S. Chaudhary
School of Biotechnology, Kalinga Institute of Industrial
Technology, Bhubaneswar 751024, India

K. A. Chobhe
Division of Soil Science and Agricultural Chemistry,
New Delhi 110012, India

Introduction

Plant-parasitic nematodes (PPNs) impose a substantial economic burden on global agriculture and horticulture amounting to approximately \$173 billion annual yield losses (Elling 2013). Additionally, changes in cultivation practices for efficient water utilization and global climate change result in the emergence of PPN problems in newer crops and geographical localities (Dutta et al. 2019). PPNs are among the most difficult crop pests to manage, considering that the most damaging ones such as root-knot (RKN: *Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes reside within the host plant for the majority of their life cycle (Palomares-Rius et al. 2017). Post

hatching, second-stage juveniles (J2s) of *Meloidogyne* spp. invade the plant root and induce the formation of a hypermetabolic feeding cell (or giant cell, GC) in the vascular tissue for continuous nourishment of subsequent life stages until they attain the reproductive maturity (Mitchum et al. 2013). Cells are hypertrophied around the GCs to form galls that ultimately hinder the normal root growth and affect nutrient supply to distal plant tissues (Moens et al. 2009). The esophageal gland cells of RKN synthesize a repertoire of effector proteins that are secreted via the nematode stylet into the host cells. Effectors play essential roles in host penetration, GC establishment and maintenance, and suppression of host defence responses (Hewezi and Baum 2013).

The conventional PPN management practices heavily relied on the use of chemical nematicides, basic crop rotations and resistant crop cultivars (Dutta et al. 2019). However, many of the frontline nematicides are continually being withdrawn from market via governmental regulations owing to their non-specific and toxic effect on the environment. Considering that crop rotations are not effective to manage polyphagous RKNs, the landscape of global crop production is still under threat from PPNs due to the absence of effective, environmentally-friendly management tactics. Because of the limited availability of candidate resistance genes and sterility of certain crops impairing the progress of conventional breeding, a transgenic approach of nematode resistance appears to be a compelling alternative (Roderick et al. 2018). In view of this, a number of studies have shown that PPNs are susceptible to RNA interference (RNAi) and host-induced gene silencing (HIGS) strategy involving *in planta* generation of PPN gene-specific, double-stranded RNA (dsRNA) holds great promise (reviewed in Lilley et al. 2012; Dutta et al. 2015a). HIGS strategy has shown its utility by resisting the establishment and maintenance of PPN infection in different crop plants (Huang et al. 2006; Sindhu et al. 2009; Papolu et al. 2013; Dutta et al. 2015b; Shivakumara et al. 2017). The HIGS strategy potentially minimizes regulatory hurdles as it does not involve any novel protein/peptide expression thus reinforcing its native biosafety (Roderick et al. 2018).

The major concern in adopting HIGS strategy is the possibility of off-target effect of RNAi. Since the mechanism of RNAi occurs in a sequence-specific manner, endogenous transcripts having identity to the

siRNAs (small interfering RNAs) produced from the introduced dsRNAs may cause silencing of non-target genes in non-targeted organisms (Rosso et al. 2009; Dutta et al. 2015a). To this end, PPN effectors serve as the candidate genes for HIGS strategy because effectors lack homology with the genes of organisms that belong to other taxa (Danchin et al. 2013; Dutta et al. 2015a). Earlier, a number of *Meloidogyne incognita*-specific effector genes were identified via bioinformatics mining of RKN esophageal gland cell microaspirated products (Huang et al. 2003, 2004). Next, using HIGS strategy, several of these genes including *Mi-msp-9* (Xue et al. 2013), *Mi-msp-12* (Xie et al. 2016), *Mi-msp-16* (Huang et al. 2006; Yang et al. 2013; Dinh et al. 2014), *Mi-msp-18*, *Mi-msp-20* (Shivakumara et al. 2017) and *Mi-msp-40* (Niu et al. 2016), were shown to play decisive role in RKN parasitic success in different host plants.

The effector gene, *Mi-msp-1* (synonymised with *Mi-vap-1*; Ding et al. 2000) belongs to venom allergen-like protein (VAP, homolog of plant and animal cysteine-rich secretory proteins) family which are highly transcribed during the parasitic process of PPNs (Gao et al. 2001; Wang et al. 2007; Lozano-Torres et al. 2014; Duarte et al. 2017; Luo et al. 2018). Recently, we have demonstrated the role of *Mi-msp-1* in early parasitic stages of *M. incognita* specifically in pre- and post-parasitic J2s; *Mi-msp-1* expression was localized to the subventral esophageal glands of pre-parasitic J2. In addition, using RNAi soaking experiments we showed that *Mi-msp-1* is crucial for RKN infectivity and pathogenicity (Chaudhary et al. 2019). In the present study, we used *Mi-msp-1* as a candidate in HIGS to manage RKN infectivity in eggplant.

Materials and methods

Bioinformatics

To interrogate the MSP-1 homologs in related species, BLASTp and tBLASTn algorithms were performed against non-redundant protein sequence database in NCBI and nematode genomic and transcriptomic sequence databases at http://www6.inra.fr/meloidogyne_incognita/ and http://parasite.wormbase.org/Meloidogyne_hapla_prjna29083/ using the *Mi-msp-1* protein sequence as the query with expect value threshold of 100. The derived protein sequences were aligned using Clustal

Omega tool (<http://www.ebi.ac.uk/Tools/msa/clustalo>) with default parameters. Signal peptides for secretion were predicted using SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>). Transmembrane domains were predicted using Phobius server (<http://phobius.sbc.su.se/>). Conserved domains within the MSP-18 homologs were investigated in InterProScan (<https://www.ebi.ac.uk/interpro/>) and SMART (<http://smart.embl-heidelberg.de/>). Motif database algorithm (<http://molbiol-tools.ca/Motifs.htm>) was used to predict common motifs. Primers to amplify the targeted dsRNA of corresponding *Mi-msp-1* gene were designed using OligoAnalyzer tool (<https://eu.idtdna.com/>). Potential off-target sites in the targeted dsRNA were investigated at <http://dsCheck.RNAi.jp/>. RT-qPCR (reverse transcription quantitative PCR) primers to detect transgene expression were designed using OligoAnalyzer tool.

Nematode culturing

A pure culture of *M. incognita* race 1 was multiplied on eggplant (*Solanum melongena* cv. Pusa Purple Long) in a greenhouse. Egg masses were extracted from the galled roots of eight-week-old plant using sterilized tweezers and were kept for hatching in a modified Baermann assembly (Southey 1986). Freshly hatched J2s were used for further experiments. For RT-qPCR studies, different post-parasitic life stages of RKN were carefully separated from the infected root using sterilized tweezers under the microscope.

Cloning of *Mi-msp-1* from *M. incognita*

Total RNA (400 ng) was extracted from pre-parasitic J2s and reverse-transcribed to cDNA using random primers (SuperScript VILO, Invitrogen) as depicted previously (Shivakumara et al. 2016). The target sequence of *Mi-msp-1* was PCR amplified from the cDNA using high-fidelity Platinum *Taq* DNA polymerase enzyme (Thermo Fisher Scientific) by following the manufacturer's instructions. Amplified fragment was cloned into pGEM-T vector (Promega) and the identity of the insert was ascertained by Sanger sequencing.

Development of *Mi-msp-1* RNAi construct for HIGS

The RNAi Gateway ready pK7GWIWG2(I) binary vector was obtained from VIB-UGent Center for Plant Systems Biology, Ghent University, Ghent, Belgium. The 515 bp sequence of *Mi-msp-1* flanked by attB1 and attB2 recombination sites were PCR amplified from the pGEM-T clone and sub-cloned into an entry vector, pDONR221 (Invitrogen) prior to cloning in the destination vector, pK7GWIWG2(I) in sense and antisense orientation connected by an intron (Shivakumara et al. 2017). Primer details are provided in Supplementary Table 1. The recombinant pK7GWIWG2(I) containing the hairpin RNA of *Mi-msp-1* (Supplementary Figure 1) was transformed into competent *Agrobacterium tumefaciens* (LBA4404) cells by electroporation and positive clones were selected via screening against antibiotic (kanamycin) resistance by following the protocol described previously (Papolu et al. 2013; Dutta et al. 2015b).

Generation of transgenic plants

Surface-sterilized (by 70% ethanol and 1% NaOCl) eggplant (cv. Pusa Purple Long) seeds were germinated on Murashige and Skoog (MS) agar (HiMedia), pH 5.8. 1 cm² cut leaf explants of 15-day-old seedling were infected with *A. tumefaciens* (LBA4404) transformants harboring the *Mi-msp-1* hairpin RNA. Pre-cultivation and co-cultivation of explants were performed as described earlier (Papolu et al. 2013; Dutta et al. 2015b; Supplementary Figure 2). Rooting was induced in the explants by supplementing the MS agar with 0.1 mg/L NAA (Sigma-Aldrich). Plants with hardened roots were transferred to the greenhouse facility at the host institute till the production of seeds of primary events (T₀). Explants co-cultured with *A. tumefaciens* harboring the empty construct (that underwent no antibiotic selection) were used as the control.

Detection of *Mi-msp-1* transgene via PCR and Southern hybridization

Genomic DNA was extracted from the harvested leaves of T₀ events using a Nucleospin plant II DNA isolation kit (Macherey–Nagel) by following the manufacturer's protocol. Putative transformants were

confirmed initially via PCR using *Taq* DNA polymerase (Thermo Fisher Scientific) with specific primer pairs (enlisted in Supplementary Table 1) followed by electrophoresis of the amplified products in 1% agarose gel. In order to investigate the integration pattern and copy number of *Mi-msp-1* transgene in PCR-positive T₀ events, Southern blot hybridization was performed. Genomic DNA (10 µg) of each event was digested with *SacI* (New England Biolabs; cuts once in the T-DNA), resolved on 0.8% agarose gel followed by transblotting to a nitrocellulose membrane (Bio-Rad). The 515 bp PCR product of *Mi-msp-1* was used as the probe. Probe labelling, hybridization and blot development was performed as previously described (Papolu et al. 2013; Dutta et al. 2015b).

Detection of *Mi-msp-1* transgene expression using RT-qPCR

In order to increase homozygosity in subsequent events, PCR-confirmed T₀ plants were self-pollinated and T₁ seeds were obtained. Surface-sterilized T₁ seeds were germinated in MS agar supplemented with kanamycin (100 mg/L) and transferred to pots containing autoclaved soil. To analyze the *Mi-msp-1* transcript abundance in T₁ plants, RT-qPCR was performed with three independent biological and three technical replicates. Total RNA was extracted from harvested leaves of PCR-confirmed T₁ plants using a Nucleospin plant II RNA isolation kit (Macherey–Nagel) followed by reverse-transcription to cDNA using a cDNA synthesis kit (Superscript VILO, Invitrogen). RT-qPCR experiment was conducted in Realplex² thermal cycler (Eppendorf) with 10 µl reaction mixture containing 1.5 ng cDNA, 750 nM of each primer and 5 µl SYBR Green PCR mastermixes (Eurogentec). The amplification reaction conditions and melt curve program were followed as previously described (Shivakumara et al. 2016). Ct (cycle threshold) values were imported from Realplex² software (Eppendorf). *Mi-msp-1* expression in different transgenic lines were calculated as average Δ ct values—the difference between the ct mean of transgene and normalizer gene, *18S rRNA* of eggplant (Papolu et al. 2016; Shivakumara et al. 2017). Primer details are documented in Supplementary Table 1.

Detection of *Mi-msp-1* integration loci in transgenic eggplant

In order to analyse the T-DNA integration sites in the transformed plants, *Mi-msp-1* integration and flanking sequences in different T₂ events (obtained via selfing of homozygous T₁ plants) were identified via genome walking (Universal genome walker 2.0, Clontech laboratories). Two µg genomic DNA (extracted from harvested leaves of T₂ plants) was used as the template in PCR reaction (catalysed by Platinum *Taq* DNA polymerase, Thermo Fisher Scientific) with T-DNA-specific primers (listed in Supplementary Table 1). PCR products were cloned and sequence verified as explained above. Obtained sequences were analysed in NCBI-BLAST and Sol Genomics Network (<http://www.solgenomics.net/>). Further, event-specific PCR was carried out to validate the specific integration sites in progeny plants. Primer details are listed in Supplementary Table 1.

Resistance evaluation of transgenic eggplant against *M. incognita*

Homozygous T₁, T₂ and T₃ lines (obtained via selfing of homozygous T₂ plants) harbouring the HIGS construct of *Mi-msp-1* were screened for resistance against RKN. The roots of a 30-day-old plant growing in 250 ml pots (containing equal proportion of soil and soilrite; Keltech Energies Ltd.) were inoculated with approximately 500 RKN J2s near the root zone. Infected plants were grown in a growth chamber at 27 ± 2 °C, 60% relative humidity and 16:8 h light:dark photoperiod. At 30 dpi (days post inoculation), plants were harvested, roots were washed carefully and different agronomic characters such as root and shoot weight were recorded along with the control plants. Nematode parasitic success was documented in terms of number of galls, egg masses, eggs/egg mass and multiplication factor (MF) as explained in Papolu et al. (2013, 2016) and Dutta et al. (2015b). Each of the transgenic lines contained at least 12 plants and the experiment was repeated twice.

Additionally, at 2 and 7 dpi, representative plants from each line were harvested and roots were stained with acid fuchsin (Shivakumara et al. 2017) to analyse the RKN early infection ability on transformed plants.

Transcription analysis of *Mi-msp-1* in RKNs extracted from transgenic plants

Total RNA was extracted from post-parasitic J2 (at 4 dpi), J3/J4 (15 dpi) and females (25 dpi) feeding on the selective T₃ plants and reverse-transcribed to cDNA as described above. RT-qPCR was performed and fold change in expression of *Mi-msp-1* gene was calculated by augmented comparative ct method and log₁₀-transformed (Shivakumara et al. 2017). *18S rRNA* of *M. incognita* was used as the normalizer gene. Primer details are given in Supplementary Table 1.

Statistical analysis

Data were checked for normality and compared using one-way ANOVA followed by Tukey's HSD test in SAS statistical package. Statistical comparisons were made among different treatments or compared individually to controls, as stated in the corresponding figure legends.

Results

Mi-msp-1 target identification for generating HIGS constructs

The protein homology search of *Mi-msp-1* (Genbank Accession No. AAD01511) in the NCBI non-redundant database using BLASTp revealed 16 (*M. hispanica*), 31 (*Bursaphelenchus mucronatus*), 32 (*Heterodera glycines*, *Globodera rostochiensis*), 33 (*Ditylenchus destructor*) and 38 (*H. avenae*) percent identity with the corresponding proteins of different PPNs (query coverage ranged from 87 to 94%). *Mi-msp-1* showed 34% identity with the SCP (secretory cysteine-rich protein)-like extracellular protein of *Caenorhabditis elegans* (93% query coverage). Intriguingly, when interrogated via BLAST-based homology in *Meloidogyne* genomic resources (http://www6.inra.fr/meloidogyne_incognita/) and *M. hapla* genome in Wormbase Parasite (http://parasite.wormbase.org/Meloidogyne_hapla_prjna29083/) *Mi-msp-1* exhibited 66, 95 and 97% similarity to *M. hapla* (MhA1_Contig481), *M. arenaria* (Mar-els19144g093341) and *M. javanica* (Mjav1s01723g017848), respectively. This indicates that *Mi-msp-1* is highly conserved among some of the

RKN species. *Mi-msp-1* contains the SCP-like conserved domain at position 27–190 aa (amino acids) within which allergen V5 domain is predicted at position 155–165 aa (Fig. 1a). *Mi-msp-1* contains the predicted *N*-myristoylation (at position 52–57, 130–135, 193–198, 209–214 aa), *N*-glycosylation (85–88, 110–113 aa) and tyrosine kinase phosphorylation (56–58, 94–101 aa) motifs.

A 515 bp sequence (spanning between 128 and 642 bp of *Mi-msp-1*, Accession No. AF013289) containing the conserved SCP-like domain was identified as the target sequence for HIGS construct development (Fig. 1b). The target dsRNA sequence was queried in dsCheck database (<http://dsCheck.RNAi.jp/>; Naito et al. 2005) to identify the potential off-target sites. No exact match for processed siRNAs (19 nucleotides) of the target dsRNA in the existing database of *C. elegans*, *Drosophila melanogaster*, *Rattus norvegicus*, *Oryza sativa* and *Arabidopsis thaliana* could be found. In addition, no nucleotide match for *Mi-msp-1* dsRNA was found in eggplant genome server (<http://www.solgenomics.net/>).

Molecular characterization of transgenic eggplant harbouring HIGS construct of *Mi-msp-1*

Using leaf-disc co-culture method, *A. tumefaciens* (LBA4404) containing *Mi-msp-1* RNAi construct was transferred to eggplant (Supplementary Figure 2). The regenerated 17 events (T₀) that survived against kanamycin selection were genotyped via PCR. The presence of *Mi-msp-1*, its orientation within the T-DNA (sense or antisense), and nptII (Neomycin phosphotransferase, corresponds to kanamycin resistance) marker fragment was detected in 14 events (Supplementary Figure 3). No significant difference in the root and shoot weight of control and transgenic plants could be found (Supplementary Figure 4), indicating that neither the antibiotic resistance gene nor the RNAi construct introgression affected the growth of transgenic plants.

The PCR-confirmed 14 T₀ lines were subjected to Southern hybridization to analyze the integration pattern of *Mi-msp-1* transgene. Single copy insertion of *Mi-msp-1* was documented in line numbers 2, 3, 4, 6, 13 and 14, while double or multiple copy insertion was observed in line numbers 8, 9, 10, 11 and 12. No or very faint hybridization signal was detected in line numbers 1, 5 and 7 (Fig. 2a). Only the single copy

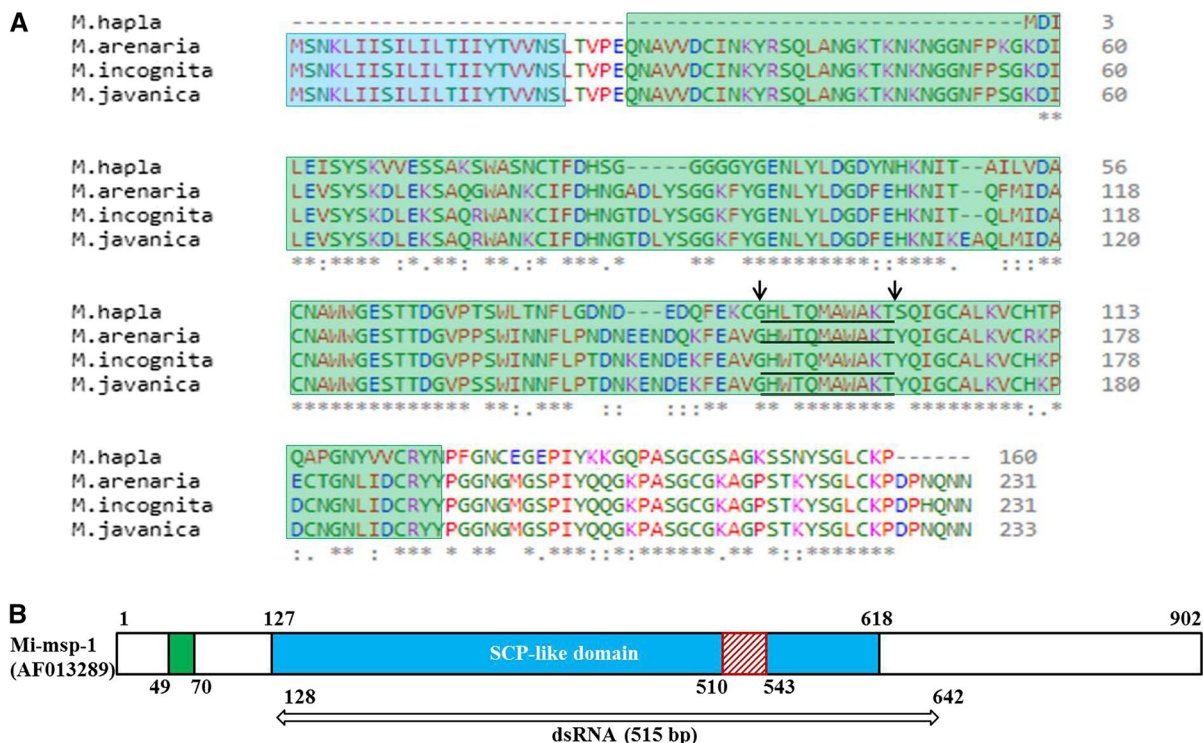


Fig. 1 *Mi-msp-1* target identification for generating dsRNA constructs for HIGS. **a** Multiple sequence alignment of MSP1 orthologs from four *Meloidogyne* species. Asterisk and colon signs indicate conserved and similar amino acids, respectively. The blue box indicates predicted signal peptides (SPs, 1-21 aa) except *M. hapla* for which no SP was predicted. The green box indicates extracellular cysteine-rich secretory protein (SCP, 27-190 aa) domain. Underlined (with arrow) regions indicate

venom allergen 5 domain (155–165 aa). **(B)** Illustration of *Mi-msp-1* (Accession No. AF013289) open reading frame (49–900 bp) and the conserved regions for which the dsRNA (indicated as two-headed arrows) was generated in this study have homology. Numbers indicate the position of nucleotides. Green and blue boxes indicate position of SP and SCP-like domain respectively. Box with diagonal pattern indicates allergen V5 domain. (Color figure online)

events harboring the RNAi construct were used for further study.

T₁ progeny plants were generated by selfing the selected T₀ lines in the greenhouse. T₁ lines were genotyped through PCR. At least five plants per event were tested. Gene-specific, sense, antisense fragments, as well as *nptIII*, were detected in all the events (Supplementary Figure 5). Homozygous T₁ lines were selfed to generate T₂ plants. Accordingly, T₃ plants were generated by selfing of homozygous T₂ plants. PCR-based genotyping of T₂ and T₃ lines indicated the amplification of expected fragments in all the events tested (data not shown). Using Southern hybridization single copy integration of *Mi-msp-1* was confirmed in progeny plants of selected T₂ lines, namely 2.1, 3.3, 4.2, 6.2, 6.3, 13.1 and 14.2 (Fig. 2b). Hence, *Mi-msp-1* RNAi transgene was stably integrated and inherited in the progeny plants.

RT-qPCR assay indicated the overexpression of *Mi-msp-1* transcripts in all the selected T₁ lines. On the contrary, no *Mi-msp-1* expression was detected in the RNA isolated from control plants. Therefore, *Mi-msp-1* expression data are simply presented as Δ ct relative to the ct of *18S rRNA*. As ct value is inversely proportional to quantitative expression value, significantly ($P < 0.05$) greater expression of *Mi-msp-1* was recorded in line numbers 6.2, 13.1 and 14.2, compared to line numbers 2.1, 3.3 and 4.2 (Fig. 3). Similar results were obtained with T₂ and T₃ lines as well (data not shown).

Assessment of *Mi-msp-1* integration loci in transgenic eggplant

In order to characterize the T-DNA insertion sites in selective T₂ events, event numbers 6.2 and 14.2 (these

Fig. 2 Southern blot for **a** T₀ and **b** T₂ eggplant transformants harboring the HIGS construct of *Mi-msp-1*. Genomic DNA extracted from untransformed/empty vector control (UC) plants did not show any hybridization signal. Positive control (PC) indicates the probe used for hybridization was specific to *Mi-msp-1* gene. M: Lambda *Hind*III digest marker. Lanes 1–14: DNA samples from different T₀ events

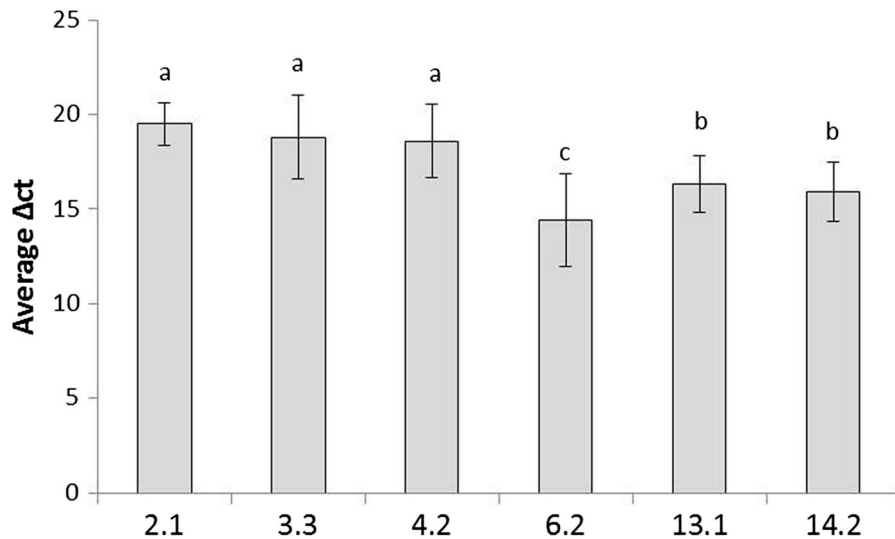
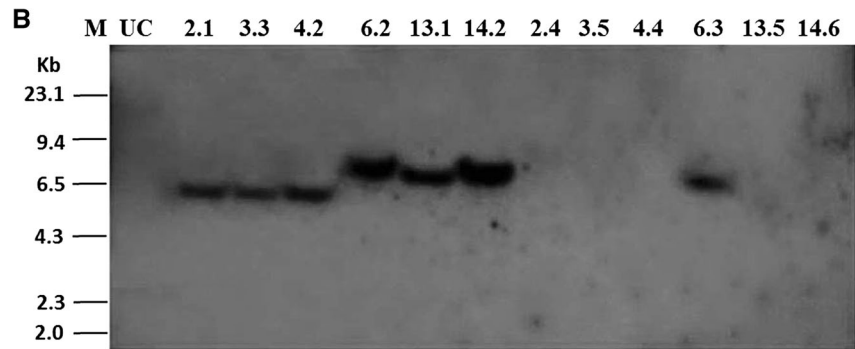
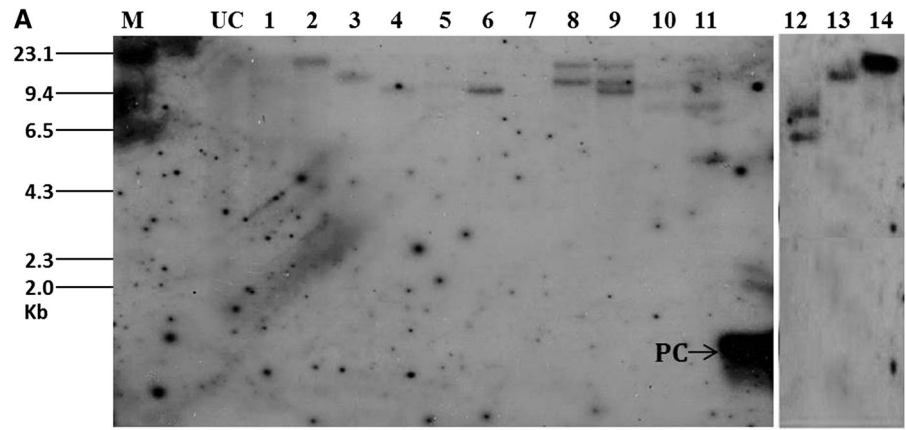


Fig. 3 Detection of *Mi-msp-1* expression in T₁ eggplant transformants by RT-qPCR. Relative transcript levels of *Mi-msp-1* are expressed as Δct values which denote the difference in ct mean of transgene and the reference gene (*18S rRNA* of eggplant). Higher Δct values indicate the lower expression of

transgene in the corresponding event. Each bar represents mean \pm SEM derived from three independent biological and three technical replicates. Bars with different letters are statistically different at $P < 0.05$, Tukey’s HSD test

lines exhibited greatest expression of *Mi-msp-1* transgene RT-qPCR assays) were evaluated via genome walking. Fragments flanking the T-DNA were detected in the progeny plants of events 6.2 and 14.2 using event-specific primers. The absence of these fragments in other selected events suggested the independent integration of T-DNA in events 6.2 and 14.2 (Supplementary Figure 6). Sequences flanking the T-DNA region showed homology with the genome sequence of eggplant (<http://www.solgenomics.net/>). The sequence of the T-DNA flanking PCR product corresponding to events 6.2 and 14.2 showed 100% (Sequence ID: Sme2.5_00538.1) and 98.34% (Sequence ID: Sme2.5_04649.1) identity with the *S. melongena* draft genome sequence, respectively.

Evaluation of eggplant transformants for resistance against *M. incognita*

Six independent T₁ events (2.1, 3.3, 4.2, 6.2, 13.1 and 14.2) harbouring the *Mi-msp-1* RNAi construct were tested for resistance against RKN to assess the HIGS effect of the target gene. At 30 dpi, the average numbers of galls per plant was significantly ($P < 0.01$) reduced by 33.78–46.92% in transgenic lines compared to control plants harbouring the empty vector. Accordingly, the number of egg masses was reduced by 41.05–51.16% in all RNAi lines compared to control plants (Fig. 4; Supplementary Table 2). As adult RKN females produces its progeny in a single egg mass, the number of egg masses implies the similar number of reproducing females. Therefore, HIGS of *Mi-msp-1* resulted in the reduced galling of roots presumably because attenuated development of RKN J2 to females in transgenic lines. In corroboration, RKN fecundity in terms of number of eggs per egg mass was markedly ($P < 0.01$) decreased by 36.08–41.20% in RNAi lines than control. Consequently, the calculated multiplication factor (MF, indicative of nematode reproductive fitness and parasitic success on a given host plant) of RKN was substantially ($P < 0.01$) reduced by 62.37–70.62% in transgenic lines in comparison to the control plants (Fig. 4; Supplementary Table 2). Within the T₁ events no significant ($P > 0.01$) difference in RKN parasitism was documented. However, within the T₂ and T₃ lines, events 14.2 and 13.1 showed significantly ($P < 0.01$) greatest and least reduction in MF, respectively (Supplementary Figure 7). Overall, the

level of resistance in T₁ lines was comparable in both T₂ and T₃ lines (Supplementary Table 2; Supplementary Figure 7). This suggests the no resistance breakdown in progeny plants of *Mi-msp-1* RNAi lines.

In addition, perturbed infectivity of RKN due to HIGS effect of *Mi-msp-1* in early parasitic stages of nematodes was documented. Significantly ($P < 0.01$) less penetrating J2 (at 2 dpi) and spike-tail J3/J4 stages (at 7 dpi) were observed in different T₁ lines (2.1, 3.3, 4.2, 6.2, 13.1 and 14.2) compared to control plants (Fig. 5). This documented the HIGS effect of *Mi-msp-1* in early parasitic stages of RKN.

Expression analysis of *Mi-msp-1* in nematodes extracted from transgenic eggplant

In order to analyse the long-term effect of HIGS on suppressing the target transcripts in feeding worms, RT-qPCR was performed in post-parasitic J2, J3/J4 and young females of *M. incognita* extracted from the roots of different T₃ lines (2.1, 3.3, 4.2, 6.2, 13.1 and 14.2). The mRNA levels of *Mi-msp-1* in post-parasitic J2s were significantly ($P < 0.01$) downregulated by 2.5 ± 0.35 to 5.5 ± 0.5 fold in specimens exposed to transgenic lines, compared to worms extracted from non-transgenic control plants. Similarly, *Mi-msp-1* transcripts were significantly ($P < 0.01$) attenuated by 1.1 ± 0.3 to 3.5 ± 0.7 fold in J3/J4 stages in transgenic lines compared to control. Consequently, a significant ($P < 0.01$) suppression of *Mi-msp-1* by 1 ± 0.35 to 2.5 ± 0.55 fold was recorded in T₃ lines compared to control (Fig. 6). Our results suggest that dsRNA/siRNA molecules corresponding to *Mi-msp-1* gene were ingested by *M. incognita* during the early parasitic process.

Discussion

RNAi is a nematode management approach that can replace the highly toxic and less specific chemical-based PPN management strategies (Roderick et al. 2018). The present work identifies the potential of a RKN-specific parasitism gene, *Mi-msp-1*, in conferring nematode resistance in eggplant using HIGS approach. Nevertheless, a possibility of off-target effect on a wide range of organisms in the rhizosphere due to the higher degree of sequence conservation among the target transcripts cannot be avoided while

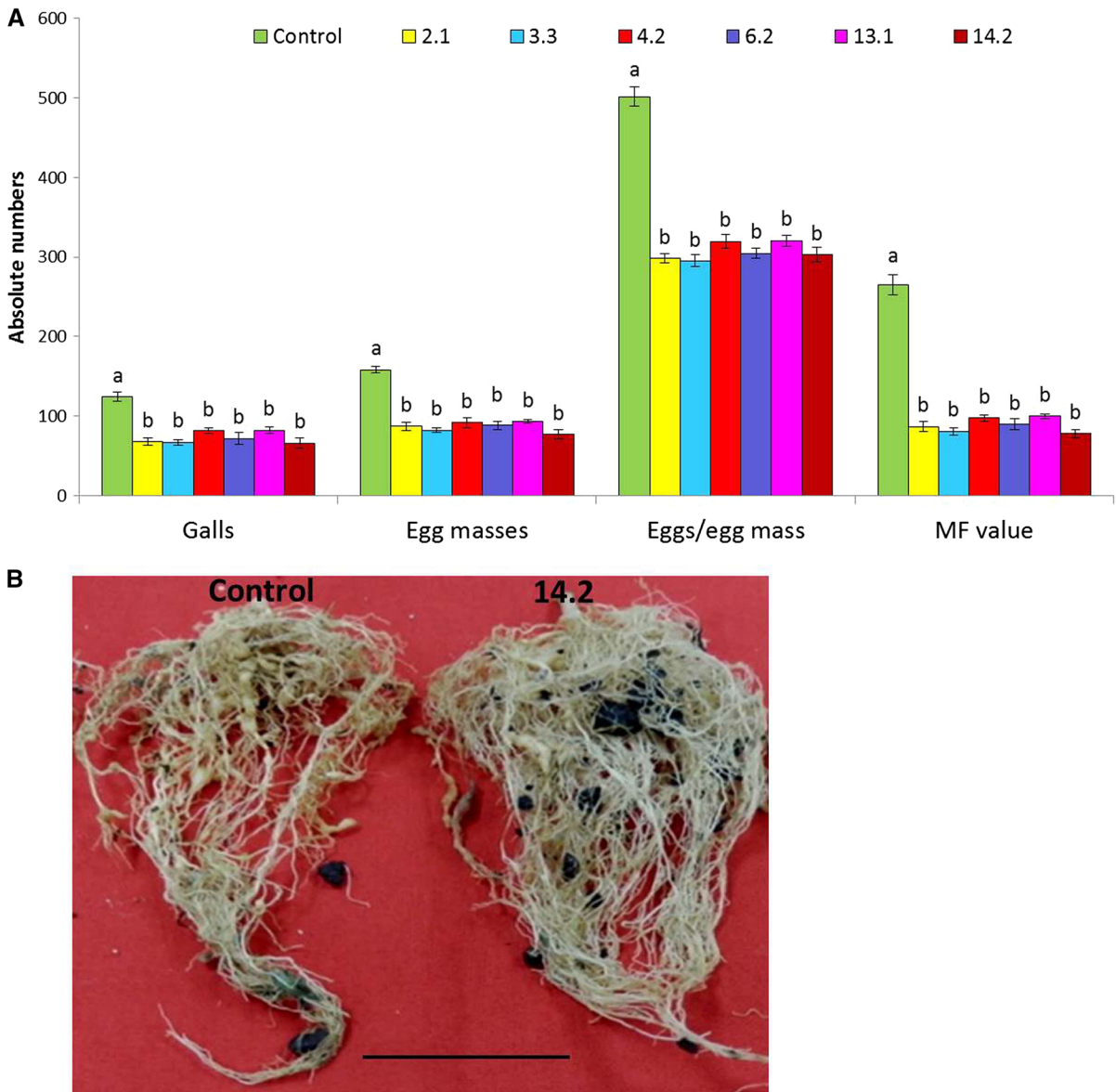


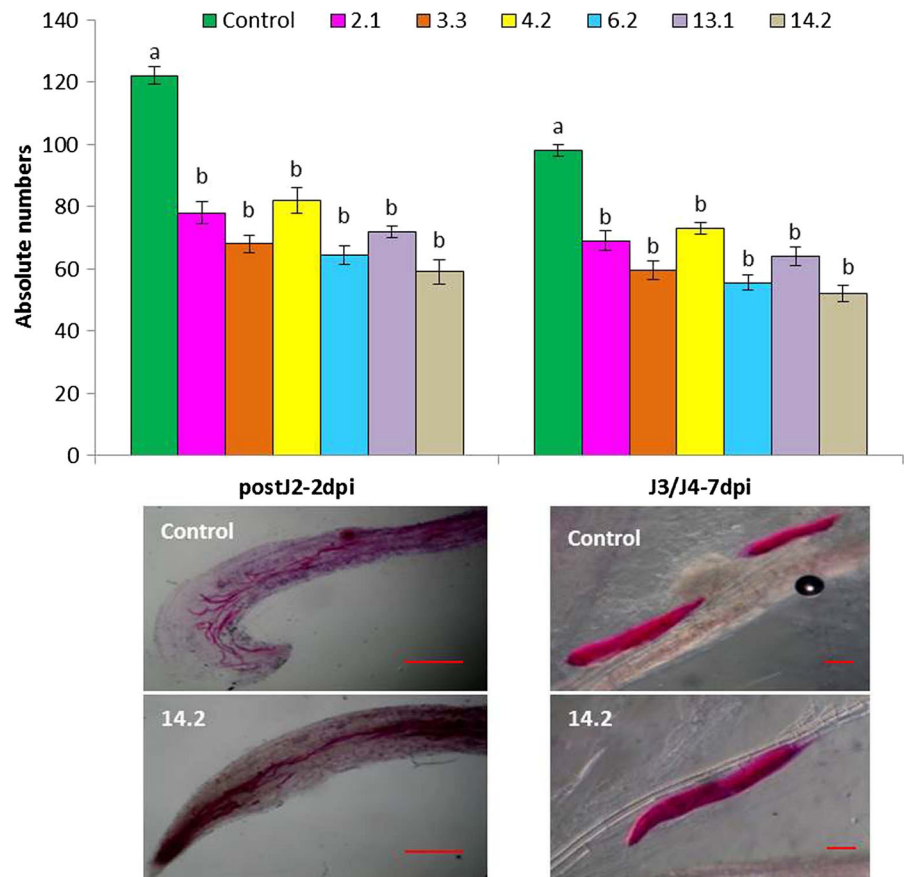
Fig. 4 Effect of HIGS of *Mi-msp-1* on development and reproduction of *M. incognita* in eggplant **a** Absolute numbers of galls induced, egg masses, eggs per egg mass and the respective MF (multiplication factor) of *M. incognita* in different T₁ events (2.1, 3.3, 4.2, 6.2, 13.1 and 14.2) and control plants harboring empty vector are shown at 30 dpi. Each bar

represents the mean \pm SE of $n = 12$, and bars with different letters (within each parameter) indicate a significant difference at $P < 0.01$, Tukey’s HSD test. **b** Photograph shows that intensity of galling was higher in control plants than T₁ event number 14.2 which had comparatively higher root mass. Scale bar = 5 cm

adopting this technique if the design of dsRNA molecule is improper. In view of this, a 515 bp sequence of *Mi-msp-1* (containing the SCP-like domain that codes for VAP proteins) was identified as the dsRNA sequence for HIGS construct development in our study. Earlier, using this sequence *M. incognita* parasitic ability was considerably

suppressed in tomato and adzuki bean via in vitro and in vivo RNAi approach (Chaudhary et al. 2019). Incidentally, *Mi-msp-1* protein exhibited higher degree of sequence homology to corresponding proteins of *M. hapla* (66%), *M. arenaria* (95%) and *M. javanica* (97%), but showed negligible sequence conservation in other PPNs and *C. elegans*. This

Fig. 5 Penetration and development of *M. incognita* in eggplant T₁ lines (2.1, 3.3, 4.2, 6.2, 13.1 and 14.2) harboring *Mi-msp-1* HIGS construct and control plants containing empty construct. Nematodes were stained with acid fuchsin at 2 (as post-parasitic J2) and 7 (as spike-tail stages) dpi. Each bar represents the mean \pm SE of nematode numbers in each plant ($n = 12$), bars with different letters indicate a significant difference at $P < 0.01$, Tukey's HSD test. Lower panel shows the stained nematodes in control and transgenic lines. Scale bar = 500 μ m



exemplifies its potential as RNAi target to manage RKNs. It is to be noted that the effector genes are the excellent candidate for HIGS in view of the biosafety concerns since effectors lack significant homology to nematodes other than PPNs (Danchin et al. 2013; Dutta et al. 2015a). In addition, the transformed dsRNA sequences had no 19 nucleotide matches in the genomes of *Arabidopsis thaliana*, *Oryza sativa*, *Drosophila melanogaster*, *Rattus norvegicus* and *C. elegans* that represent five different organisms in dsCheck server (Naito et al. 2005) which employs off-target search algorithm to analyse the identical nucleotide matches for siRNAs processed from the target dsRNAs. *Mi-msp-1* dsRNA sequences also had no nucleotide matches in the published genome of eggplant, which was selected as the host for this study. This indicates the rational design of dsRNA molecule in order to reduce the likely risk.

Using *Agrobacterium*-mediated transformation transgenic eggplants that express *Mi-msp-1* dsRNA were generated. Since the efficacy of HIGS is

compromised by copy number effects of the RNAi transgene (multi-copy T-DNA integration at the loci near to hypermethylated region of the host genome may lead to transcriptional gene silencing of RNAi cassettes; Kerschen et al. 2004; Majumdar et al. 2017), only the single-copy events harbouring the *Mi-msp-1* dsRNA (confirmed via Southern hybridization) were used for subsequent studies. Selected six homozygous T₀ lines were selfed to generate T₁ lines and by continuous selfing of subsequent homozygous lines T₃ plants were generated. The stable integration and inheritance of the RNAi transgene was ascertained in the majority of these transformants. However, the expression of the RNAi transgene (quantified via RT-qPCR) was variable among the different T₁/T₂/T₃ events, suggesting that the *Mi-msp-1* transgene was randomly integrated at various transcriptionally active sites in the eggplant genome. In order to validate this hypothesis, T-DNA integration sites were characterized in selective T₂ events, 6.2 and 14.2, via genome walking. As the event-specific PCR failed to detect the

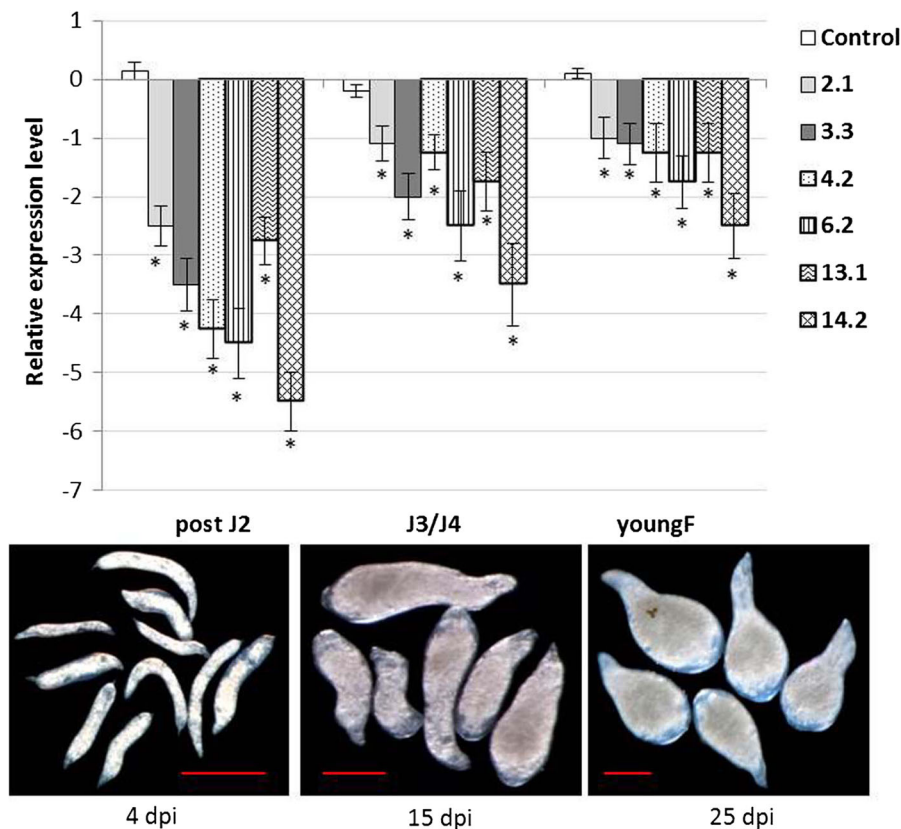


Fig. 6 Effect of HIGS on the transcript abundance of *Mi-msp-1* in *M. incognita* post-parasitic stages extracted from the roots of eggplant T₃ events (2.1, 3.3, 4.2, 6.2, 13.1 and 14.2). Using *18S rRNA* gene of *M. incognita* as reference, expression of *Mi-msp-1* was quantified via augmentative comparative ct method and fold change values were log₁₀-transformed. Bars represent mean expression level \pm SE of three biological and three technical

replicates. Asterisks indicate significant differential expression ($P < 0.01$) in comparison with the worms extracted from non-transgenic control plants. In lower panel photomicrographs show different nematode stages, i.e. post-parasitic J2 (post J2), J3/J4 and young females (youngF) extracted at 4, 15 and 25 dpi, respectively. Scale bar = 500 μ m

expected fragments (containing eggplant sequence adjacent to T-DNA) in events other than 6.2 and 14.2, the independent introgression of T-DNA (containing *Mi-msp-1* dsRNA cassette) in different T₂ events was confirmed.

Resistance screening studies indicated a pronounced decline in *M. incognita* development and reproduction in all the T₁ events compared to non-transgenic control plants with reductions of 33.78–46.92, 41.05–51.16, 36.08–41.20 and 62.37–70.62% in terms of gall number, egg masses, eggs per egg mass and multiplication factor, respectively. Further, the consistent HIGS effect was observed in T₂ and T₃ events suggesting no resistance breakdown in progeny plants. The partial nematode resistance documented in our study is in agreement

with the HIGS studies of other effector genes of PPNS (Huang et al. 2006; Dutta et al. 2015a; Niu et al. 2016; Xie et al. 2016; Shivakumara et al. 2017). In order to achieve superior nematode resistance, a combinatorial HIGS was attempted using fusion constructs of cysteine and serine protease gene of *M. incognita* in tomato (Antonino de Souza Júnior et al. 2013). Although, the nematode resistance level could not be increased in terms of efficacy of dual construct compared to single gene cassette in that study. This may be because the higher level of dsRNA delivery saturates the dsRNA processing (by *RNaseIII/Dicer* enzyme) ability of both hosts and nematodes. The failure to amplify the silencing signal by *RdRp* (RNA-dependent RNA polymerase that generates secondary siRNAs from primary ones; Pak et al. 2012) due to

higher supply of exogenous RNAs can also be another reason in this case. Conversely, crossing of two *Arabidopsis* RNAi lines expressing housekeeping genes enhanced HIGS efficacy against *M. incognita* (Charlton et al. 2010). Given the transient nature of RNAi effect (Rosso et al. 2009) it is practically impossible to achieve complete nematode resistance. As RKNs complete three generations in an annual crop, a 60% reduction in RKN multiplication per generation is sufficient to minimize nematode population below the economic threshold (Fuller et al. 2008; Shivakumara et al. 2017). Notably, T₂ and T₃ events conferred 46.68–73.22 and 44.47–72.92% *M. incognita* resistance, respectively, in terms of reduced multiplication factor in our study. Additionally, RKN penetration and development (to J3/J4) was retarded in transgenic eggplant compared to control plants, suggesting that HIGS of *Mi-msp-1* had negatively altered the early-stage parasitic behavior of *M. incognita*.

In order to evaluate the long-term HIGS effect of *Mi-msp-1* on RKN development, different post-parasitic nematode stages were extracted from T₃ eggplant and subjected to RT-qPCR analysis. A significant reduction in *Mi-msp-1* expression was documented in post-parasitic J2, J3/J4 and young females extracted from all the T₃ transformants compared to nematodes isolated from non-transgenic control plants. It is assumed that the *Mi-msp-1* HIGS effect in *M. incognita* is systemic and is propagated to the entire nematode body upon uptake of dsRNA/siRNA molecule via the stylet orifice. Moreover, RNAi effect was perpetuated across the developmental stages of nematodes till maturity and speculatively *M. incognita* offspring with defunct *Mi-msp-1* would be produced. The heritable nature of RNAi is well studied in *C. elegans* (Grishok et al. 2000), *M. javanica* (Gleason et al. 2008) and *M. chitwoodi* (Dinh et al. 2014). It remains unclear in the present study whether RKNs have ingested dsRNAs directly and processed them into siRNAs (via Dicer) or they ingested the host-processed siRNAs directly. Either or both is possible as RKNs can efficiently ingest large biomolecules via stylet orifice (Li et al. 2007; Zhang et al. 2012).

HIGS is emerging as a powerful strategy for enhancing disease resistance trait in plants to various pests and pathogens (Majumdar et al. 2017). Of late, a few of the RNAi-based genetically modified crops are on course for commercialization, e.g. Pioneer's PlenishR high oleic acid soybean by DuPont and

SmartStax Pro to control western corn earworm by Monsanto. This strategy is more advantageous than classical transgenic approaches as HIGS is not dependent on host's requirement to produce foreign proteins (Atkinson et al. 2012) that eventually may become allergenic/toxic. HIGS also ensures continuous delivery of dsRNA/siRNA molecules generated by the host plants for uptake by biotrophic pathogens including PPNs. However, compared to other pathogens the status of RNAi-based control of PPNs is still in its infancy (Roderick et al. 2018). In this context, HIGS of *Mi-msp-1* holds great potential for management of *M. incognita* (this parthenogenetic species has broadest host range and is more devastating compared to its sexual relatives).

Acknowledgements Ph.D. Student SC acknowledges her co-guide Dr. Vishakha Raina, School of Biotechnology, KIIT, Bhubaneswar, India. Current investigation was funded by Department of Biotechnology, Government of India (Grant No. BT/PR5908/AGR/36/727/2012).

Author contributions SC performed all the experiments. TKD wrote the MS and analysed of the data. NT, TNS, PKP and KAC helped in performing experiments. UR conceived the experiment and edited the MS. All the authors read and approved the final MS.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Atkinson HJ, Lilley CJ, Urwin PE (2012) Strategies for transgenic nematode control in developed and developing world crops. *Curr Opin Biotechnol* 23:251–256
- Charlton WL, Harel HYM, Bakhetia M, Hibbard JK, Atkinson HJ, McPherson MJ (2010) Additive effects of plant expressed double-stranded RNAs on root-knot nematode development. *Int J Parasitol* 40:855–864
- Chaudhary S, Dutta TK, Shivakumara TN, Rao U (2019) RNAi of an esophageal gland specific *Mi-msp-1* gene alters the early stage infection behaviour of root-knot nematode, *Meloidogyne Incognita*. *J Gen Plant Pathol*. <https://doi.org/10.1007/s10327-019-00837-x>
- Danchin EGJ, Arguel MJ, Campan-Fournier A, Perfus-Barbeoch L, Magliano M, Rosso M-N et al (2013) Identification of novel target genes for safer and more specific control of root-knot nematodes from a pan-genome mining. *Plos Pathogen* 9:e1003745

- de Souza Júnior JDA, Coelho RR, Lourenço IT, da Rocha Fragoso R, Viana AAB, de Macedo LLP et al (2013) Knocking-down *Meloidogyne incognita* proteases by plant-delivered dsRNA has negative pleiotropic effect on nematode vigor. *PLoS One* 8:e85364
- Ding X, Shields J, Allen R, Hussey R (2000) Molecular cloning and characterisation of a venom allergen AG5-like cDNA from *Meloidogyne incognita*. *Int J Parasitol* 30:77–81
- Dinh PT, Zhang L, Brown CR, Elling AA (2014) Plant-mediated RNA interference of effector gene Mc16D10L confers resistance against *Meloidogyne chitwoodi* in diverse genetic backgrounds of potato and reduces pathogenicity of nematode offspring. *Nematology* 16:669–682
- Duarte A, Maleita C, Egas C, Abrantes I, Curtiss R (2017) Significant effects of RNAi silencing of the venom allergen-like protein (Mhi-vap-1) of the root-knot nematode *Meloidogyne hispanica* in the early events of infection. *Plant Pathol* 66:1329–1337
- Dutta TK, Banakar P, Rao U (2015a) The status of RNAi-based transgenic research in plant nematology. *Front Microbiol* 5:760
- Dutta TK, Papolu PK, Banakar P, Choudhary D, Sirohi A, Rao U (2015b) Tomato transgenic plants expressing hairpin construct of a nematode protease gene conferred enhanced resistance to root-knot nematodes. *Front Microbiol* 6:260
- Dutta TK, Khan MR, Phani V (2019) Plant-parasitic nematode management via biofumigation using brassica and non-brassica plants: current status and future prospects. *Curr Plant Biol*. <https://doi.org/10.1016/j.cpb.2019.02.001>
- Elling AA (2013) Major emerging problems with minor *Meloidogyne* species. *Phytopathol* 103:1092–1102
- Fuller VL, Lilley CJ, Urwin PE (2008) Nematode resistance. *New Phytol* 180:27–44
- Gao B, Allen R, Maier T, Davis EL, Baum TJ, Hussey RS (2001) Molecular characterisation and expression of two venom allergen-like protein genes in *Heterodera glycines*. *Int J Parasitol* 31:1617–1625
- Gleason CA, Liu QL, Williamson VM (2008) Silencing a candidate nematode effector gene corresponding to the tomato resistance gene *Mi-1* leads to acquisition of virulence. *Mol Plant-Microbe Interact* 21:576–585
- Grishok A, Tabara H, Mello CC (2000) Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* 287:2494–2497
- Hewezi T, Baum TJ (2013) Manipulation of plant cells by cyst and root-knot nematode effectors. *Mol Plant-Microbe Interact* 26:9–16
- Huang G, Gao B, Maier T, Allen R, Davis EL, Baum TJ 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
- Huang G, Dong R, Maier T, Allen R, Davis EL, Baum TJ et al (2004) Use of solid-phase subtractive hybridization for the identification of parasitism gene candidates from the root-knot nematode *Meloidogyne incognita*. *Mol Plant Pathol* 5:217–222
- 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. *Proc Natl Acad Sci USA* 103:14302–14306
- Kerschen A, Napoli CA, Jorgensen RA, Müller AE (2004) Effectiveness of RNA interference in transgenic plants. *FEBS Lett* 566:223–228
- Li XQ, Wei JZ, Tan A, Aroian RV (2007) Resistance to root-knot nematode in tomato roots expressing a nematocidal *Bacillus thuringiensis* crystal protein. *Plant Biotechnol J* 5:455–464
- Lilley CJ, Davies LJ, Urwin PE (2012) RNA interference in plant parasitic nematodes: a summary of the current status. *Parasitology* 139:630–640
- Lozano-Torres JL, Wilbers RHP, Warmerdam S, Finkers-Tomczak A, Diaz-Granados A, van Schaik CC et al (2014) Apoplastically secreted allergen-like proteins of cyst nematodes modulate the activation of basal plant innate immunity by cell surface receptors. *Plos Pathogen* 10:e1004569
- Luo S, Liu S, Kong L, Peng H, Huang W, Jian H et al (2018) Two venom allergen-like proteins, HaVAP 1 and HaVAP 2, are involved in the parasitism of *Heterodera avenae*. *Mol Plant Pathol*. <https://doi.org/10.1111/mpp.12768>
- Majumdar R, Rajasekaran K, Cary JW (2017) RNA interference (RNAi) as a potential tool for control of mycotoxin contamination in crop plants: concepts and considerations. *Front Plant Sci* 8:200
- Mitchum MG, Hussey RS, Baum TJ, Wang X, Elling AA, Wubben M, Davis EL (2013) Nematode effector proteins: an emerging paradigm of parasitism. *New Phytol* 199:879–894
- Moens M, Perry RN, Starr JL (2009) *Meloidogyne* species—a diverse group of novel and important plant parasites. In: Perry RN, Moens M, Starr JL (eds) *Root-knot nematodes*, UK. CAB International Publishers, Wallingford, pp 1–17
- Naito Y, Yamada T, Matsumiya T, Ui-Tei K, Saigo K, Morishita S (2005) dsCheck: highly sensitive off-target search software for double-stranded RNA-mediated RNA interference. *Nucleic Acids Res* 33:W589–W591
- Niu J, Liu P, Liu Q, Chen C, Guo Q, Yin J et al (2016) Msp40 effector of root-knot nematode manipulates plant immunity to facilitate parasitism. *Sci Rep* 6:19443
- Pak J, Maniar JM, Mello CC, Fire A (2012) Protection from feed-forward amplification in an amplified RNAi mechanism. *Cell* 151:885–899
- Palomares-Rius JE, Escobar C, Cabrera J, Vovlas A, Castillo P (2017) Anatomical alterations in plant tissues induced by plant-parasitic nematodes. *Front Plant Sci* 8:1987
- Papolu PK, Gantasala NP, Kamaraju D, Banakar P, Sreevathsa R, Rao U (2013) 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
- Papolu PK, Dutta TK, Tyagi N, Urwin PE, Lilley CJ, Rao U (2016) Expression of a cystatin transgene in eggplant provides resistance to root-knot nematode, *Meloidogyne incognita*. *Front Plant Sci* 7:1122
- Roderick H, Urwin PE, Atkinson HJ (2018) Rational design of biosafe crop resistance to a range of nematodes using RNA interference. *Plant Biotechnol J* 16:520–529
- Rosso M-N, Jones JT, Abad P (2009) RNAi and functional genomics in plant parasitic nematodes. *Ann Rev Phytopathol* 47:207–232

- Shivakumara TN, Papolu PK, Dutta TK, Kamaraju D, Chaudhary S, Rao U (2016) RNAi-induced silencing of an effector confers transcriptional oscillation in another group of effectors in the root-knot nematode, *Meloidogyne incognita*. *Nematology* 18:857–870
- Shivakumara TN, Chaudhary S, Kamaraju D, Dutta TK, Papolu PK, Banakar P et al (2017) Host-induced silencing of two pharyngeal gland genes conferred transcriptional alteration of cell wall-modifying enzymes of *Meloidogyne incognita* vis-à-vis perturbed nematode infectivity in eggplant. *Front Plant Sci* 8:473
- Sindhu AS, Maier TR, Mitchum MG, Hussey RS, Davis EL, Baum T (2009) Effective and specific *in planta* RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success. *J Exp Bot* 60:315–324
- Southey JF (1986) Laboratory methods for work with plant and soil nematodes. Ministry of Agriculture, Fisheries, and Food Reference Book, London, p 402
- Wang X, Li H, Hu Y, Fu P, Xu J (2007) Molecular cloning and analysis of a new venom allergen-like protein gene from the root-knot nematode *Meloidogyne incognita*. *Exp Parasitol* 117:133–140
- Xie J, Li S, Mo C, Wang G, Xiao X, Xiao Y (2016) A novel *Meloidogyne incognita* effector Misp12 suppresses plant defense response at latter stages of nematode parasitism. *Front Plant Sci* 7:964
- Xue B, Hamamouch N, Li C, Huang G, Hussey RS, Baum TJ, Davis EL (2013) The 8D05 parasitism gene of *Meloidogyne incognita* is required for successful infection of host roots. *Phytopathol* 103:175–181
- Yang Y, Jittayasothorn Y, Chronis D, Wang X, Cousins P, Zhong G-Y (2013) Molecular characteristics and efficacy of 16D10 siRNAs in inhibiting root-knot nematode infection in transgenic grape hairy roots. *PLoS One* 8:e69463
- Zhang F, Peng D, Ye X, Yu Z, Hu Z, Ruan L et al (2012) In vitro uptake of 140 kDa *Bacillus thuringiensis* nematocidal crystal proteins by the second stage juvenile of *Meloidogyne hapla*. *PLoS One* 7:e38534

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.