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Micro RNA-induced gene silencing strategy for the delivery of siRNAs targeting *Meloidogyne incognita* in a model plant *Nicotiana benthamiana*

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Abstract

BACKGROUND: Occurrence of multiple biotic stresses on crop plants result in drastic yield losses which may have severe impact on the food security. It is a challenge to design strategies for simultaneous management of these multiple stresses. Hence, establishment of innovative approaches that aid in their management is critical. Here, we have introgressed a micro RNA-induced gene silencing (MIGS) based combinatorial gene construct containing seven target gene sequences of cotton leaf curl disease (CLCuD), cotton leaf hopper (*Amrasca biguttula biguttula*), cotton whitefly (*Bemisia tabaci*) and root-knot nematode (*Meloidogyne incognita*).

RESULTS: Stable transgenic lines of *Nicotiana benthamiana* were generated with the T-DNA harboring *Arabidopsis* miR173 target site fused to fragments of *Sec23* and *ecdysone receptor* (*EcR*) genes of cotton leaf hopper and cotton whitefly. It also contained C2/replication associated protein (C2/Rep) and C4 (movement protein) along with *βC1* gene of betasatellite to target CLCuD, and two FMRFamide-like peptide (FLP) genes, *Mi-flp14* and *Mi-flp18* of *M. incognita*. These transgenic plants were assessed for the amenability of MIGS approach for pest control by efficacy evaluation against *M. incognita*. Results showed successful production of small interfering RNA (siRNA) through the tasiRNA (trans-acting siRNA) pathway in the transgenic plants corresponding to *Mi-flp18* gene. Furthermore, we observed reduced *Mi-flp14* and *Mi-flp18* transcripts (up to 2.37 ± 0.12-fold) in females extracted from transgenic plants. The average number of galls, total endoparasites, egg masses and number of eggs per egg mass reduced were in the range 27–62%, 39–70%, 38–65% and 34–49%, respectively. More importantly, MIGS transgenic plants showed 80% reduction in the nematode multiplication factor (MF).

CONCLUSION: This study demonstrates successful validation of the MIGS approach in the model plant, *N. benthamiana* for efficacy against *M. incognita*, as a prelude to translation to cotton.

Supporting information may be found in the online version of this article.

Keywords: *Meloidogyne incognita*; FMRFamide-like peptides; host-induced gene silencing; micro RNA-induced gene silencing; parasitism; transgenics

1 INTRODUCTION

The global scenario of climate change has resulted in the increased intensity of stresses, either alone or a combination. Despite various efforts to minimize their effect through intervention of both conventional and biotechnological approaches, successful mitigation of diverse pests and pathogens has been difficult and inadequate.[1] Hence, there is an urgent need for the exploitation of latest technologies that aid in the management of a combination of biotic stresses.[2]

RNA interference (RNAi) for knockdown of target genes has been one of the many reliable biotechnological approaches exploited in crop improvement programs.[3] It is a highly conserved biological process in which 21–24 nt small interfering RNA (siRNA)

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molecules cause degradation of homologous messenger RNAs (mRNAs), thereby reducing gene expression.[4] siRNAs can be classified as tasiRNA (trans-acting siRNA), natsiRNA (natural antisense transcript-derived siRNAs), hcsiRNA (heterochromatic siRNA), and rasiRNA (repeat-associated siRNA), etc.,[5,6] based on precursor sequences and mechanism of their biosynthesis. These molecules form cardinal aspects of host-induced gene silencing (HIGS) for the mitigation of pests and pathogens.

In this direction, micro RNA-induced gene silencing (MIGS), one of the HIGS approaches is a novel strategy based on *Arabidopsis* micro RNA 173 (miR173) that triggers production of tasiRNAs.[7,8] Addition of a 22 nt miR173 target sequence (miR173_ts) upstream of the target gene sequence(s) is sufficient to induce RNAi against the corresponding genes through the generation of tasiRNAs.[8] Since miR173 is only expressed in *Arabidopsis*, silencing of genes using MIGS in other plant species is achieved by co-expression of miR173 embedded within the MIGS construct. Though the approach is being used as a biotechnological intervention in crop improvement,[9–11] evidence for its use in management of biotic stresses is not yet demonstrated.

Cotton being an economically important crop globally, suffers from various pests and diseases that impede plant growth and productivity. Amongst them, cotton leafhopper (CLH; *Amrasca biguttula biguttula*), cotton whitefly (CWF; *Bemisia tabaci*), cotton leaf curl disease (CLCuD), and root-knot nematode (RKN; *Meloidogyne incognita*) are predominant. Though continued efforts are being made worldwide towards the control and management of these stress factors, it is important to consider their management by employing a single strategy. Targeting genes critical for the growth and survival of these pests and pathogens provide an environmentally sound strategy. In this study, we have introgressed a MIGS-based combinatorial gene construct containing target gene sequences of the respective pests and pathogens in the model plant *Nicotiana benthamiana*. Towards this, we have selected relevant genes targeting CLH, CWF, CLCuD and *M. incognita*, for their management using the MIGS approach. In the case of CLH and CWF, homologs of two genes, *Sec23*, a coatomer subunit beta (*COPβ*) and ecdysone receptor (*EcR*) were used. *Sec23* is one of the essential components of *COPII* which promotes the formation of transport vesicles from the endoplasmic reticulum (ER). It is involved in the translocation of proteins from endosomes to the cytoplasm as well as protein trafficking in the cell and is highly crucial for cell viability. *EcR* is a nuclear receptor and expressed in somatic cells of the ovary at the time of ovarian differentiation and is important during embryonic development, larval molting and metamorphosis. For CLCuD, conserved sequences of C2 gene encoding for transcriptional activator protein (TrAP), C4 gene that encodes for a silencing suppressor protein of the helper virus (family *Geminiviridae*; genus *Begomovirus*) and *βC1* gene of the associated betasatellite were targeted. For the management of *M. incognita*, FMRFamide-like peptide (FLP) genes belonging to FMRFamide-related peptides (FaRPs) that comprise the largest family of neuropeptides in nematodes were utilized. FLPs are associated with various physiological functions like host recognition, navigation, sensory perception, reproduction and parasitism.[12–15] Therefore, these neuropeptides offer a prospective alternative to be used as target-specific management of nematodes.

The advantage of MIGS approach is that it is envisaged to provide resistance to all four pathogens/pests simultaneously in a single transgenic plant. We present in this study, the proof of concept for the successful utility of this approach in the model plant *N. benthamiana* for their efficacy against *M. incognita*, as a prelude to exploitation in cotton.

2 MATERIALS AND METHODS

2.1 MIGS vector assembly

The T-DNA cassette with *Arabidopsis* miR173 under the control of *Arabidopsis* ubiquitin 10 (At4g05050) promoter (pUBQ) and octopine synthase terminator (OCSt) was amplified using SP6178 forward (atctaCTGCAGcgacgagtcagtaataaacggcgtc) and SP6179 reverse primers (gtcgaAGGCCTTCGCGAatttaggtgacactatagaatatc) with MIGS2.1 plasmid[8] as a template. The resulting polymerase chain reaction (PCR) fragment I was digested with restriction enzymes *Pst*I and *Stu*I (New England Biolabs, Ipswich, MA, USA). CaMV35S promoter and miR173 cassette from MIGS2.1 was PCR amplified using SP6180 forward (ATCTActgcagAGATTAGCCTTTTCAATTTCAGAAAG) and SP6181 reverse primers (ATTAACCTAG GttcgcttgtagagaaaaatcacAGCTCGTCCCCCGTGTTCTCTCCAA). The resulting PCR fragment II was digested with *Pst*I and *Avr*II. Further, RuBisCO small subunit terminator (rbcsT) was amplified from MIGS2.1 plasmid with SP6182 forward (atctaCCTAGGTTAATTAAGGTACCACGCGTGTCGACCCCGGGAGGCCTtgctttcgttcgtatcat cggtttc) and SP6183 reverse primers (gtagaAAGCTTcgattgatgcatgttgtcaatcaat) that included multiple cloning sites (MCS). The resulting PCR fragment III was digested with *Avr*II and *Hind*III. The aforesaid PCR fragments I, II, and III were ligated into pCAMBIA2300 vector after digestion with *Hind*III and *Eco53K*I. Subsequently, to generate MIGS construct, synthesized DNA containing gene cassette pUBQ–miR173-OCSt::35S-miR173_ts-Sec23-EcR-miR173_ts-flp14-flp18-miR173_ts-C2-C4-*β*C1–rbcsT (Bio Basic Biochemicals, Markham, Ontario, Canada) was amplified using SP6313 forward (ATCTAcctaggAAGTTTCATCAGGACTGTTATTGAAC) and SP6314 reverse primers (ATAGAcccgggATGACAAC GAGCGGAACAAACAAGG). The resulting PCR fragment was restricted with *Avr*II and *Xma*I and cloned into MIGS vector digested with the same pair of enzymes. Accession numbers and sequence length corresponding to target genes used in the MIGS construct are: XM_022351641 (*Sec23*, 379 bp), MK642616.1 (*EcR*, 337 bp), JF502372.1 (C2 and C4, 801 bp), JF502397.1 (Betasatellite associated with CLCuD, 340 bp) and AY907829 (*Mi-flp14*, 284 bp), and AY729022 (*Mi-flp18*, 407 bp). The recombinant pCAMBIA2300-MIGS vector construct was transformed into *Escherichia coli* (DH10B) cells and subsequently mobilized into *Agrobacterium tumefaciens* strain LBA4404 for transformation into *N. benthamiana*.

2.2 Generation of transgenic *N. benthamiana* plants and molecular analysis

The MIGS binary vector, pCAMBIA2300 harboring both the miR173 cassette and target genes for the control of CLH, CWF, RKN and CLCuD (pUBQ-miR173-OCSt::35S-miR173_ts-Sec23-EcR-miR173_ts-flp14-flp18-miR173_ts-C2-C4-*β*C1-rbcsT) was mobilized into the *Agrobacterium tumefaciens* strain LBA4404 (Supporting Information Fig. S1(a)). A well-established leaf disc-based regeneration method was used for *A grobacterium tumefaciens*-mediated transformation of *N. benthamiana*.[16–18] Leaf disc explants were co-cultivated with LBA4404 strain of *Agrobacterium* harboring the pCAMBIA2300-MIGS construct, followed by selection and regeneration on Murashige–Skoog (MS) medium (HIMEDIA, Mumbai, India) supplemented with 100 mg L^{-1} kanamycin (Sigma Aldrich, St Louis, MO, USA). Regenerated plants

were transferred to pots containing 200 g autoclaved soil and soilrite (3:1); maintained in a growth chamber (Labtech, Delhi, India) at 27 °C, 70% relative humidity, 14 h photoperiod for 15 days. Healthy plants were further transplanted to 6-inch diameter (dm) pots containing soil and soilrite (5:1) and moved to a glasshouse with similar conditions for plant maturation and seed development (Fig. S1(b)).

Genomic DNA was isolated from young leaves of transgenic (T_0 and T_1) and wild-type (WT) *N. benthamiana* plants using Nucleospin Plant II DNA extraction kit (Macherey-Nagel, Düren, Germany). PCR analyses were carried out using different sets of primers for the amplification of respective target/marker genes (Supporting Information Table S1). The reaction mixture contained 100 ng plant genomic DNA, 1X assay buffer (10 mmol L^{-1} Tris–HCl, 50 mmol L^{-1} KCl, 1.5 mmol L^{-1} $MgCl_2$, 0.01% gelatin), 200 µmol L^{-1} dNTP mix, 10 µmol L^{-1} each of forward and reverse primers and 1 unit of Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) in a total volume of 25 µL. PCR reaction was carried out in an automated thermal cycler (Eppendorf, Hamburg, Germany) programmed with an initial denaturation at 95 °C for 5 min followed by 30 cycles each of denaturation at 95 °C for 60 s, annealing at 58 °C for *nptII*, 60 °C for *Mi-flp14*, *Mi-flp18*, *Sec23*, *C2/Rep*, *βC1* and 65 °C for *EcR* for 1 min and extension at 72 °C for 1 min followed by a final extension of 7 min at 72 °C. The amplified products were analyzed by electrophoresis on a 1.2% (*w/v*) agarose gel (Sigma Aldrich) and documented on the Gel Doc XR+ gel documentation system (Bio-Rad, Hercules, CA, USA).

For confirmation of T-DNA integration, Southern hybridization was performed with genomic DNA isolated from the T_0 transgenic plants and WT plants according to Sambrook and Russell.[19] Genomic DNA (~10 µg) was digested with *Hind*III (New England Biolabs) by incubating at 37 °C for 16 h. The digested DNA samples were electrophoresed on a 0.8% (*w/v*) agarose gel (Sigma Aldrich) and transferred onto a Zeta-Probe GT cationized nylon membrane (Bio-Rad). The digoxigenin (DIG)-labeled *nptII* gene specific probe was synthesized following instructions for the PCR-DIG Probe Synthesis kit (Roche, Basel, Switzerland). Hybridization and detection was carried out as mentioned in the DIG Luminescent Detection Kit (Roche). Blots were developed by exposing to X-ray film (Kodak, Rochester, NY, USA) for 1 h.

Southern positive transgenic plants were further subjected to semi-quantitative reverse transcription PCR (sqRT-PCR) to estimate the accumulation of transcripts. For this, total RNA was isolated from young leaves using Nucleospin plant II RNA kit (Macherey-Nagel, Düren, Germany) and about 500 ng of total RNA was reverse transcribed to complementary DNA (cDNA) using Superscript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). sqRT-PCR was carried out using 100 ng cDNA as template, gene specific primers and conditions were the same as those used for PCR analysis with genomic DNA. The amplified fragments were resolved on a 1.2% agarose gel (*w/v*) (Sigma Aldrich).

2.3 Molecular analysis of T_1 transgenic plants

The T_0 seeds were germinated on MS medium (HIMEDIA) supplemented with 100 mg L^{-1} kanamycin (Sigma Aldrich). Shoots were further transferred to half-strength MS medium supplemented with 0.25 mg L^{-1} GA3 (Sigma Aldrich) and 100 mg L^{-1} kanamycin for further development of plantlets. Healthy plantlets with well-established roots were transferred to pots containing 200 g of autoclaved soil and sand (5:1) and maintained in a growth chamber (Labtech, Delhi, India) for acclimatization under 14 h photoperiod at 27 °C, with light intensity of ~500 µmol m^{-2} s^{-1}, 70% relative humidity for 30 days. The plants were later shifted to the glasshouse and maintained under similar conditions for complete acclimatization and maturation.

Genomic DNA was extracted from the leaves of one-month-old transgenic and WT plants and PCR analyses were carried out using different sets of primers as described earlier (Table S1). The PCR products were separated on a 1.2% (*w/v*) agarose gel (Sigma Aldrich).

PCR confirmed T_1 plants of different events were further subjected to quantitative real-time PCR (qPCR) for transcript abundance analysis. For this, total RNA was isolated and reverse-transcribed (500 ng RNA) to cDNA as described earlier. qPCR was performed using primers specific to target genes (Table S1) with three biological and three technical replicates in the RealPlex2 thermal cycler (Eppendorf). *Nicotiana tabacum 18S rRNA* (accession: HQ384692.1) was used as a reference gene for normalization of gene expression. Data interpretation was done as previously described.[20–24]

Representative samples of transgenic plants were further confirmed for the expression of siRNAs by Northern hybridization. Total RNA (small and large RNA in single fraction) was isolated from young leaves using NucleoSpin® miRNA Kit (Macherey-Nagel) and resolved on a 2.0% high resolution MetaPhor agarose gel (Sigma Aldrich), transferred onto a nitrocellulose membrane using iBlot® Gel transfer stacks according to the manufacturer's instructions (iBlot® Gel Transfer Device, Invitrogen) and later ultraviolet (UV)-crosslinked. The probe was synthesized by amplifying the *Mi-flp18* gene fragment using target gene specific primers and labeled with DIG probe synthesis system (Roche). Hybridization and detection was performed as previously described[12,21] and blots were developed by exposing to the autoradiograms (Kodak).

2.4 Bioefficacy analysis of transgenic T_1 plants against *M. incognita*

A pure culture of *M. incognita* was multiplied on the roots of eggplant (*Solanum melongena* cv. Pusa Purple Long) in the glasshouse. Fresh egg masses collected from the infected plants were hatched using the modified Baermann's assembly[25] to obtain second stage infective juveniles (J2s) for all the experiments.

Plants of *N. benthamiana* T_1 generation of different events harboring the MIGS gene construct and WT were subjected to nematode parasitism assays. For this, one-month-old plants were grown in pots (4 inch dm) containing 200 g soil and sand mix (5:1). Each plant was inoculated with about 400 J2s of *M. incognita* and maintained in a growth chamber at 27 °C, 70% relative humidity, 14 h photoperiod. Plants were harvested 35 days post inoculation (dpi) and the nematode parasitic success was determined as total number of galls, total endoparasites (J2s, J3/J4s, females), egg masses and eggs per egg mass for each plant. Additionally, to determine the effect on the reproductive potential of *M. incognita*, nematode multiplication factor (MF) [(total number of egg masses per plant × average number of eggs per egg mass) ÷ initial nematode inoculum level] was calculated for each transgenic event.[12,21] Roots were stained following the acid fuchsin-NaOCl method according to Bybd *et al.*[26] and photographed using a ZEISS SteREO Discovery V20 microscope.

Six replicates per event were used in the study and observations were made for all the replicates separately. The experiments were repeated twice under similar conditions.

2.5 Expression analyses of target gene transcripts in *M. incognita* females

Approximately 15–20 mature females were dissected out from both transgenic and WT plants separately, morphological measurements (length and width) were taken and compared with the control to assess aberration, if any. For this, five females of *M. incognita* for each of the transgenic event were compared with those extracted from WT plants for determining the size variation. Further, total RNA was isolated and reverse-transcribed (300 ng RNA) to cDNA. Transcript accumulation was assessed using qPCR for *Mi-flp14* and *Mi-flp18* genes. *Meloidogyne incognita 18S rRNA* (accession: HE667742) was used as a reference gene for the normalization of gene expression. Three biological and three technical replicates were performed. Fold-change in gene expression was calculated using $2^{-\Delta\Delta CT}$.[27]

2.6 Statistical analysis

The experimental data were checked for normality and analyzed using one-way analysis of variance (ANOVA) by completely randomized design (CRD), and statistical significance was determined at $P = 0.05$ and $P = 0.01$. Statistical comparisons were made with different treatments and subjected to Duncan Multiple Range Test (DMRT).

3 RESULTS

3.1 *In planta* validation of the MIGS gene construct in *N. benthamiana*

Among a large number of transformed and regenerated *N. benthamiana* plants, ten independent T_0 transformants (E5, E6, E7, E8, E9, E10, E12, E14, E15 and E16) that were phenotypically normal were selected for further analysis. The selected T_0 plants were confirmed by PCR for the presence of transgenes in the MIGS cassette using genomic DNA extracted from the leaves of these plants (Fig. 1(a)). Additionally, performance of sqRT-PCR revealed

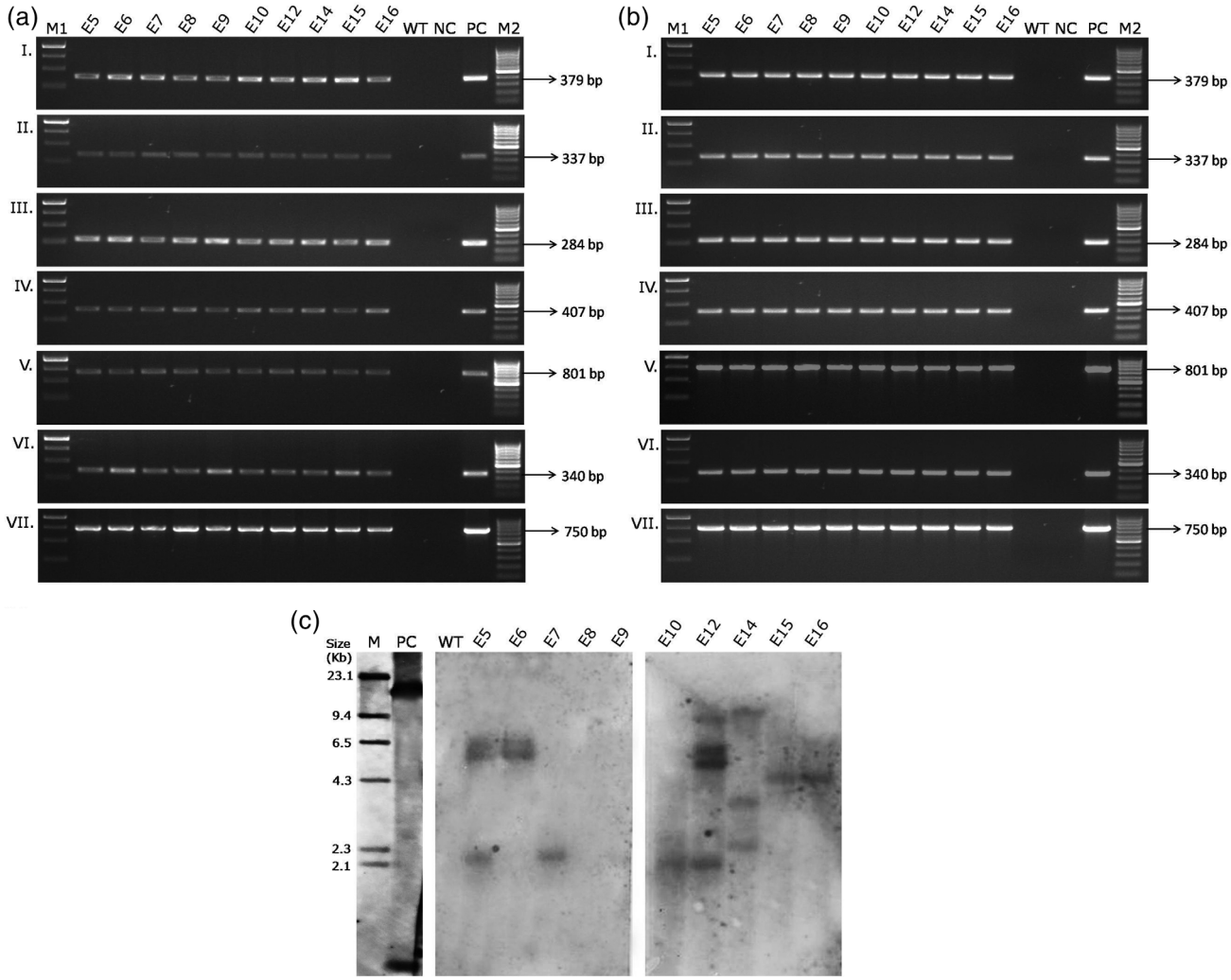

Figure 1. Molecular characterization of transgenic plants of *Nicotiana benthamiana*. (a) PCR analysis (representative images) of transgenic plants for the amplification of 379 bp *Sec23* (I), 337 bp *EcR* (II), 284 bp *Mi-flp14* (III), 407 bp *Mi-flp18* (IV), 801 bp *C2/Rep* (V), 340 bp *βC1* (VI) and 750 bp *nptII* gene (VII). Lane: M1-1 Kb marker; M2-100 bp marker; E5 to E16 – transgenic plants of different events; WT – wild-type plant; NC – negative control (water); PC – positive control of the respective genes. (b)sqRT-PCR analysis of transgenic plants for *Sec23* (I), *EcR* (II), *Mi-flp14* (III), *Mi-flp18* (IV), *C2/Rep* (V), *βC1* (VI) and *nptII* (VII). PCR fragments were resolved by electrophoresis. Lane descriptions are the same as in (a). (c) Genomic Southern analysis of transgenic plants using a DIG-labeled 750 bp *nptII* gene fragment. Lane descriptions are the same as in (a).

expression of transcripts in the transgenic plants corresponding to the target gene sequences in the MIGS construct (Fig. 1(b)). Furthermore, T-DNA integration pattern and its copy number in the PCR-positive *N. benthamiana* plants were analyzed by Southern blot hybridization. While there was an absence of a hybridization signal in the WT plant, transgenic events E7, E10, E15 and E16 showed single copy insertions and E5, E6, E12 and E14 exhibited integration of multiple copies of the T-DNA (Fig. 1(c)). These results established that the selected transgenic plants were independent integration events. Together, these results confirmed the presence of MIGS cassette in the transgenic plants and also verified the expression of the transgenes in all the transgenic events.

3.2 Characterization of transgenic *N. benthamiana* plants

The validated T_0 plants were allowed to grow and set seeds under glasshouse conditions. Ten transgenic T_1 progeny plant events (E5-2, E6-3, E7-1, E8-2, E9-2, E10-8, E12-3, E14-3, E15-2 and E16-1) were identified by screening the seeds of independent T_0 events in the presence of kanamycin. PCR analyses using primers specific to the target genes indicated T-DNA inheritance in the progeny plants (Fig. S2).

Expression of the transgenes in T_1 generation plants was confirmed by qPCR using primers specific to different target genes (*Sec23*, *EcR*, *Mi-flp14*, *Mi-flp18*, *C2/Rep*). The results indicated significant expression of various transgenes in the progeny plants of different transgenic events. Among them, event E10-8 had the highest expression level of *Mi-flp14*, *Mi-flp18* and *EcR*. However, events E9-2, E6-3 and E14-3 accumulated the least in terms of average ΔCT values (Fig. 2). In contrast, events E6-3 and E8-2 showed the highest expression of *Sec23* and *C2/Rep* genes respectively. The ΔCT values (difference in the CT mean) of the transgene and the normalizer gene, *18S rRNA* were used to describe the relative expression of target genes in the different transgenic events. Greater ΔCT values indicate lower quantitative expression of transgenes and *vice versa*. None of the target gene transcripts were detected in the WT plants.

3.3 Transgenic MIGS plants produce siRNA corresponding to *Mi-flp18* gene of *M. incognita*

We assessed for the production of siRNAs corresponding to *Mi-flp18* gene in the MIGS construct. Northern blot analysis showed that plants E5-2, E8-2, E9-2, E14-3 and E16-1 (Fig. 3(a)) and E6-3 and E12-3 (Fig. 3(b)) accumulated siRNAs corresponding to *Mi-flp18*. These results indicated that the transgenic plants could potentially be effective against *M. incognita*.

3.4 Reduced expression of *Mi-flp14* and *Mi-flp18* genes in the nematodes extracted from transgenic plants

We performed qPCR with adult females of *M. incognita* that were isolated from the transgenics and WT plants. The expression of *Mi-flp14* and *Mi-flp18* was down-regulated significantly ($P < 0.05$) in *M. incognita* females extracted from the transgenic plants compared to those from WT plants (Fig. 3(c)). Greatest reduction was observed in females extracted from E10-8 transgenic event with 2.37 ± 0.12-fold in case of *Mi-flp14* and 1.2 ± 0.14-fold in case of *Mi-flp18* (Fig. 3(c)). We observed slight up-regulation of *Mi-flp14* and down-regulation of *Mi-flp18* in females extracted from E9-2 transgenic event.

3.5 MIGS transgenic plants confer partial resistance against *M. incognita* infection

Nematode-challenged transgenic and WT *N. benthamiana* plants were harvested after 35 days and various parameters such as total number of galls, total endoparasites, egg masses, and eggs per egg masses were recorded to determine the effect of transgenes on nematode infection, development and reproduction. We observed reduced root galling and an increased growth of roots in the transgenics compared to WT plants (Fig. 4(a)). The results

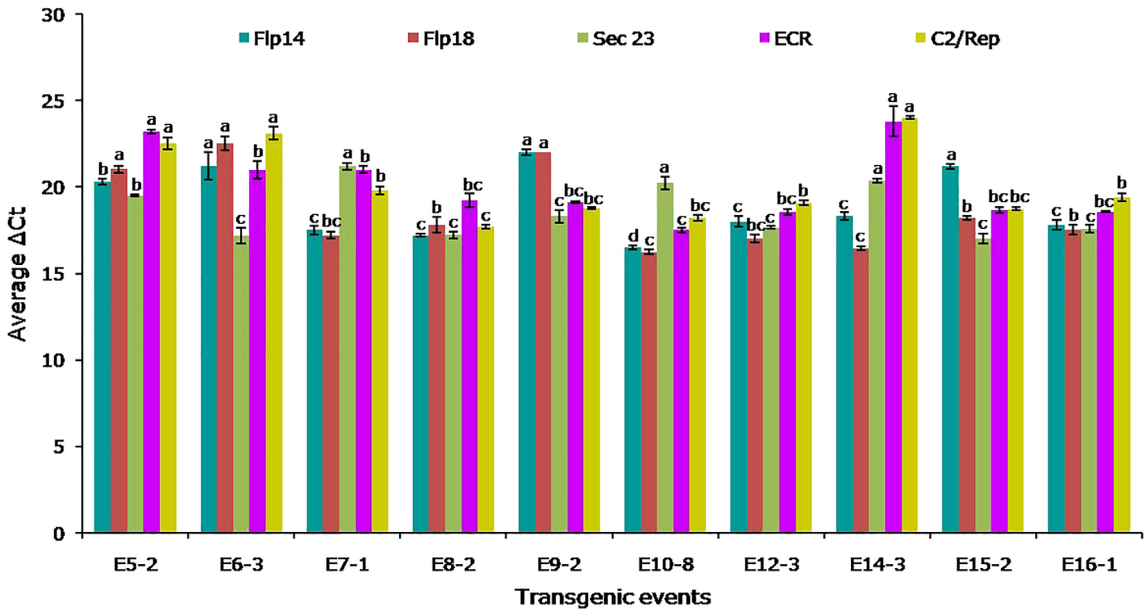

Figure 2. Expression analysis of transgenic plants for transcript accumulation of target genes in the MIGS construct. qPCR analyses of ten T_1 generation *Nicotiana benthamiana* transgenic events (E5-2, E6-3, E7-1, E8-2, E9-2, E10-8, E12-3, E14-3, E15-2 and E16-1) showing varied levels of transcript accumulation for target genes, *Mi-flp14*, *Mi-flp18*, *Sec23*, *EcR*, and *C2/Rep*. Relative transcript levels of target transgenes are expressed as ΔCT values which denote the difference in CT mean of transgene and the reference gene (*18S rRNA* gene of *N. tabacum*). Higher values represent the lower expression of the transgene and *vice versa*. Each bar represents mean ± standard error of the mean derived from three independent biological and three technical replicates. Bars with different letters are statistically different at $P < 0.05$. None of the target gene transcripts were detected in the wild-type (WT) plants.

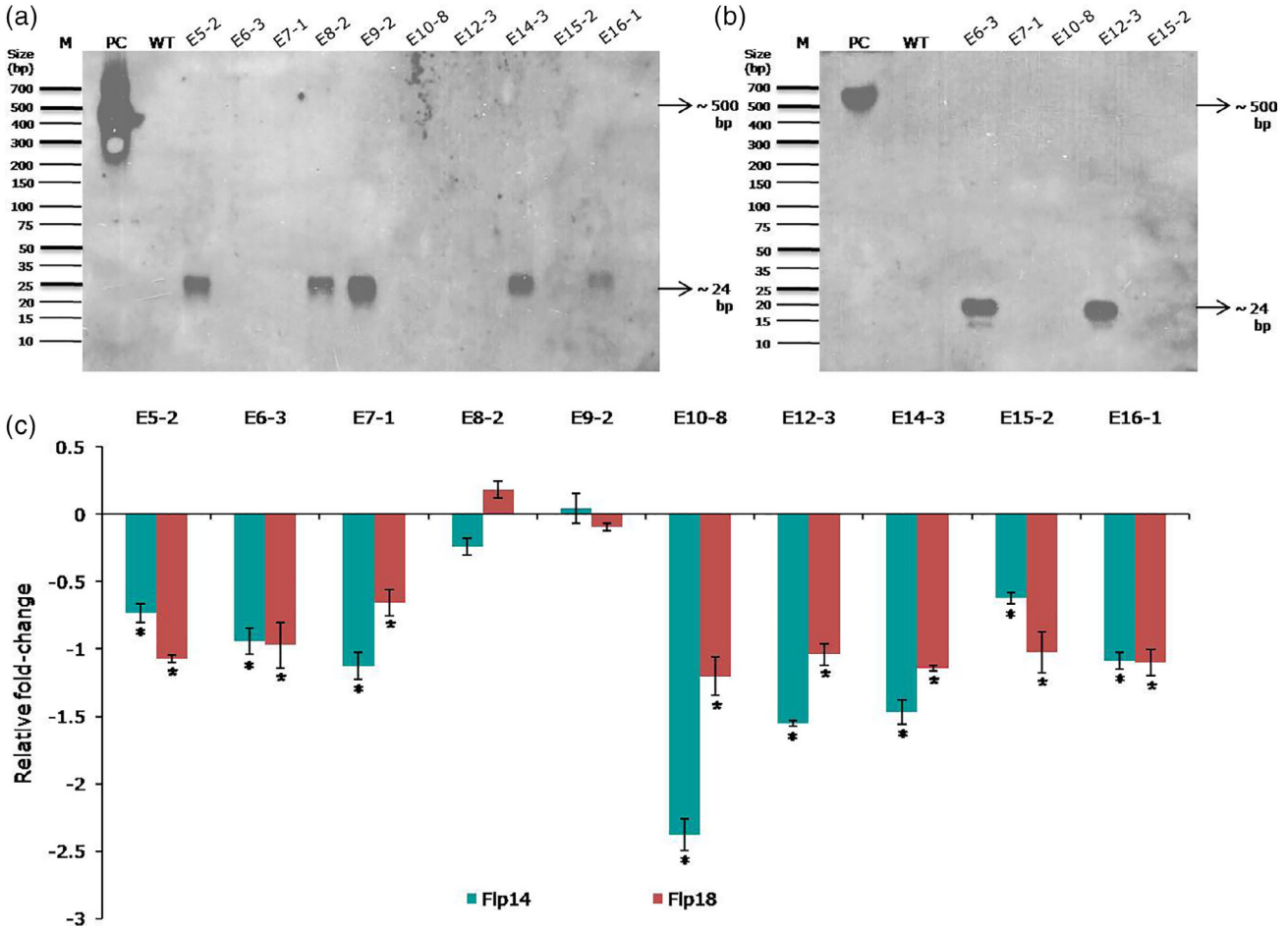

Figure 3. Northern blot analyses for the detection of siRNA corresponding to *Mi-flp18* in T_1 transgenic plants. (a) Small RNAs extracted from various T_1 transgenic plants and wild-type (WT) plants were separated by gel electrophoresis and probed with a DIG-labeled *Mi-flp18* gene fragment. Lane: M – representative size marker; PC – positive control (*Mi-flp18* gene specific DIG-labeled probe (~500 bp); WT – wild type plant; E5-2 to E16-1 – transgenic plants of different events. (b) Same as (a) except lane E6-3 to 15-2 corresponds to E6-3, E7-1, E10-8, E12-3, E15-2 transgenic plants (re-probing). (c) Relative fold change in the expression of target genes *Mi-flp14* and *Mi-flp18* in *Meloidogyne incognita* females collected from transgenic plants. *Meloidogyne incognita 18 s rRNA* was used as a reference gene and fold change was calculated using $2^{-\Delta\Delta CT}$ method. Each bar represents mean ± standard error of the mean derived from three independent biological and three technical replicates, and asterisks indicate significant difference at $P < 0.05$.

demonstrated average galling to be in the range of 63 to 120 in various T_1 plants compared to about 164 in WT plants (Fig. 4(b)) that corresponds to a reduction of 27% to 62% in average galling in the transgenic events. Consistent with this, we observed significant reduction in the total endoparasites which were in the range of 64 to 131 in T_1 plants compared to about 215 in WT plants (Fig. 4(b)). As a result, 39–70% reduction was observed in total endoparasites within the roots of transgenic plant. Similarly, the average number of egg masses was in the range of 53 to 95 in the T_1 plants while it was 152 in the WT plants (Fig. 4(b)) which resulted in a 38–65% decrease in the number of egg masses. The fecundity or the reproduction ability of the females was also affected as the number of eggs per egg mass were found to be in the range of 267 to 344 in T_1 plants while WT plants showed about 521 eggs per egg mass (Fig. 4(b)), indicating a reduction of about 34–49%. Finally, the derived MF was in the range of 40.3 to 81.7 in transgenic T_1 plants and 198 in WT plants (Fig. 4 (b)) demonstrating a reduction of about 80% in the transgenic plants. Additionally, measurements were made for five adult females for each transgenic event and compared with those from WT plants. The comparison of the size of *M. incognita* females showed that the adult females extracted from most of the transgenic plants were smaller in size (length: 692.5 ± 37.33 µm; width: 483.4 ± 28.66 µm) compared to those from WT plants (length: 895.33 ± 43.50 µm; width: 646.66 ± 54.55 µm) (Fig. 5).

4 DISCUSSION

Multiple pests and pathogens are known to infect plants and cause heavy economic damage posing a threat to food security. Cotton in particular is affected by a large number of pests and pathogens, that include leaf curl viruses, root knot nematodes and leaf hoppers.[28–30] Managing a plethora of such pests and pathogens by employing single or multiple strategies is difficult to accomplish. Towards this, several biotechnological interventions are being employed that include RNA-based technologies such as RNAi and artificial miRNAs.[31–33] Gene silencing or RNAi is one of the most important mechanisms of gene regulation in plants, which is mediated by 21–24 nt long RNA molecules, also known as siRNA.[3] The tasiRNAs are recently identified class of small RNAs, which are derived from the *TAS* gene-encoded transcripts after being acted upon by a 22 nt sized miRNA.[8] The miR173 directs the cleavage of *TAS1* and *TAS2* leading to the generation of tasiRNAs from the

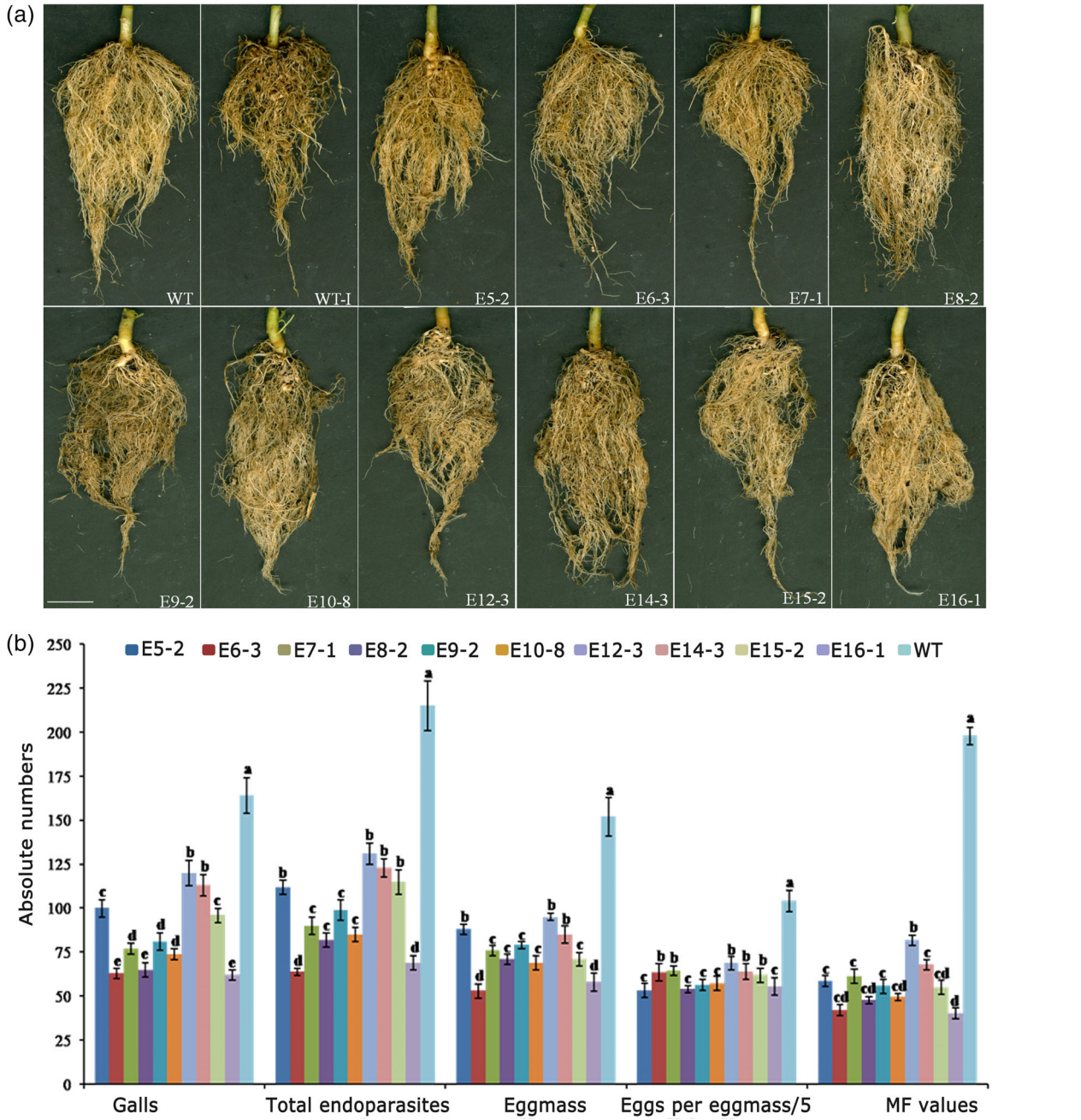

Figure 4. Bioefficacy analyses of T_1 generation transgenic plants against *Meloidogyne incognita*. (a) Variation in the galling intensity (representative images) in the roots of T_1 plants expressing the MIGS construct, 35 dpi *vis-à-vis* wild type. WT – wild-type plant (healthy); WT-I – wild type plants with *M. incognita* infection; E5-2, E6-3, E7-1, E8-2, E9-2, E10-8, E12-3, E14-3, E15-2 and E16-1 – transgenic plants challenged with *M. incognita*. Scale bar = 5 cm. (b) Number of galls, total endoparasites, egg mass, eggs per egg mass, and the multiplication factor (MF) of *M. incognita* in different transgenic events: E5-2, E7-1, E8-2, E10-8, E6-3, E9-2, E12-3, E14-3, E15-2 and E16-1 and in WT plants at 35 dpi. Each bar represents the mean ± standard error of the mean of $n = 6$.

sequences located downstream of miR173 recognition site. The cleavage mediated by miR173 is sufficient to initiate transitivity as targeting of a given gene by miR173 results in the production of secondary siRNAs originating from the target nucleotide sequence.[8]

This strategy leads to the emergence of a gene regulation technique termed as 'micro RNA-induced gene silencing' (MIGS), that is essentially based on the unique feature of the miR173 to trigger the generation of secondary siRNAs (tasiRNAs) from its target sequences.[8] RNAi has its own limitations of accommodating large number of gene targets, which eventually would result in poor expression of the double-stranded RNA (dsRNA). However, MIGS can be employed to simultaneously silence multiple genes by fusing multiple MIGS modules (miR173 target site plus the sequence of interest) to generate a single MIGS construct, which subsequently can be then cloned into a binary vector of choice for plant transformation. This fusion MIGS construct is capable of simultaneously silencing different genes with same efficiency.[8]

In this study, we describe the generation of transgenic *N. benthamiana* plants with MIGS construct expressing various

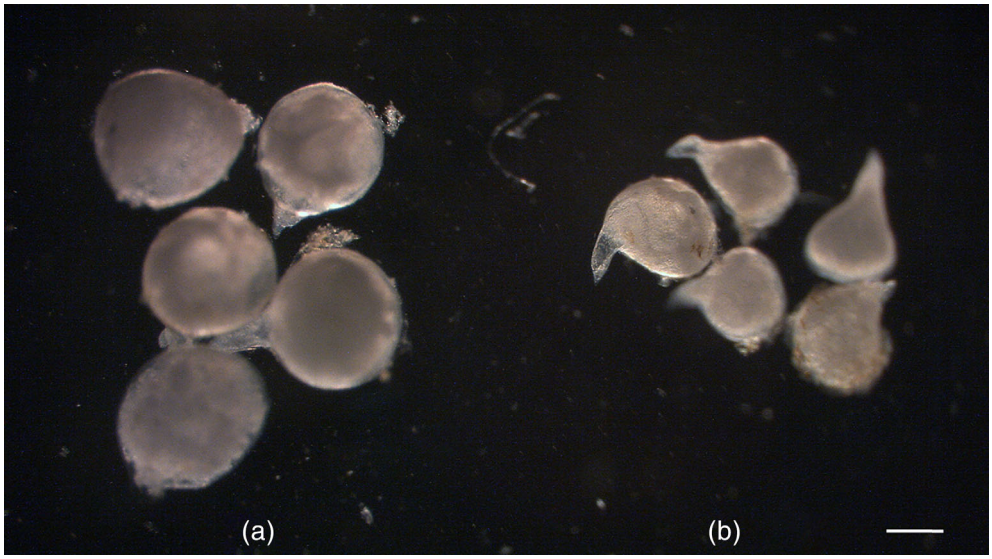

Figure 5. Effect of MIGS-mediated targeting of *Mi-flp14* and *Mi-flp18* on the development and reproduction of *Meloidogyne incognita*. Comparison of mature *M. incognita* females extracted from transgenic plants compared to wild-type (WT) plants. Measurements were made for five adult females for each transgenic event and compared with those from WT plants. Adult females from WT plants were larger (length: 895.33 ± 43.50 μm; width: 646.66 ± 54.55 μm) (a), compared to those from transgenic plants (length: 692.5 ± 37.33 μm; width: 483.4 ± 28.66 μm) (b), (representative images). Scale bar = 500 μm.

target sequences against multiple pests/pathogens, specifically of cotton. The major hypothesis was to assess the amenability of the approach in the stable integration of the MIGS cassette and expression of all the target gene sequences contained in the T-DNA. Further, due to non-infective nature of CLH and CLCuD on *N. benthamiana*, we demonstrate the efficacy of these MIGS-based transgenic plants in controlling the proliferation of *M. incognita* infection.

FLPs represent the largest family of neuropeptides known in invertebrates. Neuropeptides play many vital roles in the regulation of physiological and behavior processes in nematodes.[12–14] Therefore, these neuropeptides signaling system has been proposed to be the potential target for the management of nematodes.[34–37] In this regard, we have previously demonstrated that the host-delivered RNAi of two FLPs, *Mi-flp14* and *Mi-flp18* leads to decline in reproductive ability and parasitism of *M. incognita*.[12] This provided leads in support of the selection of these two FLPs for the amenability of the MIGS approach in control of pests and pathogens.

Progeny plants of ten independent transgenic events with proof of T-DNA integration and expression were subjected to efficacy analyses against deliberate challenging of *M. incognita*. In support of the MIGS strategy, we observed reduced average root galling and an increased growth of roots in most of the transgenic events compared to WT plants. Altogether, root galling up to 62% and nematode MF up to 80% was declined significantly in the transgenic events. Further, stringent bioefficacy analyses demonstrated that the strategy was effective in not only generating siRNAs corresponding to *Mi-flp18* gene and down-regulation of expression of *Mi-flp14* and *Mi-flp18* genes but also in the reduction of nematode parasitism. This effective reduction in the MF demonstrated in the present study was also observed earlier during the silencing of genes individually by host-delivered RNAi approach in transgenic tobacco.[12] Therefore, FLP genes can be considered as effective targets for the control of the nematode *M. incognita*, irrespective of the approaches used to produce siRNAs against FLP genes.

Targeting FLPs for perturbed parasitism in nematodes have been established in various studies. Information accrued from the literature[13] has demonstrated reduced penetration and parasitism along with unusual phenotypes in nematodes, through RNA-based silencing of FLPs in *Globodera pallida*. Similarly, inhibition of nematode ovijection and physiological anomalies in *Ascaris suum* after FLPs were silenced by RNAi has also been established.[38] Further, reduction in average galling and ability to reproduce was observed upon silencing of FLPs in *M. graminicola*-rice interaction.[39,40]

The study also showcases the successful utility of combinatorial targeting of genes as proven in the nematode bioefficacy analysis. Control of nematode parasitism by simultaneous silencing of many genes has been demonstrated in *Heterodera schachtii*[41] and also in *M. incognita*.[42] These studies established that simultaneous and/or combinatorial gene knockdown not only strengthens resistance to nematode infestation, but also extends the spectrum of resistance against diverse nematode species.[43]

The findings described in this article are a proof of concept for the successful validation of the MIGS strategy for the delivery of siRNAs in plants as well as its efficacy against *M. incognita*. Likewise, gene silencing targeted to specific genes involved in various metabolic pathways, processes of either biotic or abiotic stress alone or in combination can also be achieved using the MIGS strategy.

AUTHOR CONTRIBUTIONS

U.R., B.L.P., R.S., S.D.-K.: conceptualization; U.R., B.L.P., R.S.: supervision and manuscript preparation; U.R., B.L.P., R.S., S.D.-K.: editing. A.B., B.L.P.: transgenic plant development and their confirmation. A.H.: molecular characterization of transgenic plants, nematode bioassays, data analysis and manuscript preparation. K.K.: carried out Southern blot analysis. S.D.-K.: design and development of the MIGS construct. B.P., U.R., B.L.P., R.S.: fund acquisition, coordination and facilitation of scientific interactions. All authors read and approved the final article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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