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Mitigating Root Knot Nematode Propagation on Transgenic Tobacco via *in Planta* Hairpin RNA Expression of *Meloidogyne incognita*—Specific *PolA*1 Sequence

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Abstract

Root knot nematodes are top priority nematode pests that significantly constrain agricultural productivity globally especially in developing countries. However, expressing double stranded RNA (dsRNA) of essential nematode genes in susceptible plants is known to confer protection against these pests via RNA silencing. This molecular-based strategy is called host induced gene silencing (HIGS) and the selection of appropriate target nematode gene is critical to its success. In this study, therefore, we focused on root knot nematode *PolA*1, an essential single copy nuclear gene encoding the largest subunit of RNA polymerase I enzyme and evaluated its effectiveness as a target in conferring nematode resistance on *Agrobacterium*-mediated transformed tobacco plants. Transgenic tobacco expressing *Meloidogyne incognita*-specific (*MiS*) dsRNA of *PolA*1 gene showed significant reduction in nematode fecundity and multiplication compared to wild type plants in both T_0 and T_1 generations. T_0 plants showed varying degrees of agronomic vigorover WT plants possibly due to varying levels of processed siRNA. However, production of *MiS* siRNAs in the transgenic plants coupled with significant reduction of *PolA*1 transcript expression in nematodes feeding on roots of transgenic plants provided evidence of HIGS. Taken together, our results show that *PolA*1 is a potentially effective target for HIGS-mediated reduction of root knot nematode damage on transgenic tobacco. Given the homology of our target sequence among *Meloidogyne* species, this protection could be broad range against other root knot nematodes aside *M. incognita*.

Keywords

M. incognita, Nematode Resistance, *N. tabacum*, RNA Silencing, *PolA*1 Gene

1. Introduction

Plant parasitic nematodes are among the most significant constraints to sustainable agriculture and achievement of food security [1]. Root knot nematodes (*Meloidogyne spp.*), in particular, are the most economically important of the plant parasitic nematodes with a wide global distribution and broad host range [2]. Developing countries are particularly devastated by these pests through reduction in yield of key staple crops and impoverishment of resource-poor subsistence farmers. Most successful nematode management strategy over the years involved the integrated use of nematicides, resistant crop varieties and good cultural practices [3]. Concerns, however, exist over environmental and health risks associated with increased use of toxic chemical nematicides.

Engineering nematode resistance in plants through biotechnology is regarded as a multi-beneficial, less risky alternative for achieving durable, broad-spectrum resistance [4] [5]. With the complete sequencing of the *Meloidogyne incognita* genome, the past decade has proven the possibility of engineering nematode resistance in plants via molecular-based strategies like host induced gene silencing [6] [7] [8] [9]. This strategy involves the engineering of suitable host plants to express double stranded RNA (dsRNA) of an essential nematode gene *in planta.* This dsRNA then triggers the plants' conserved innate RNA silencing mechanism which rapidly processes the dsRNA into 21 - 24 bp RNA duplexes called small interfering RNAs (siRNAs). Small interfering RNAs then combine with RNA-induced silencing complex (RISC) and guide the sequence-specific recognition and degradation of complementary gene transcripts [10]. In HIGS, expression of nematode-specific dsRNA in plants generates siRNAs which are ingested by the nematodes during feeding and mediate silencing of the target nematode gene with possible conferment of plant protection [11].

HIGS strategy, therefore, has the selection of appropriate parasite target gene as a central consideration for success. Studies on HIGS have evaluated different nematode parasitism [7] and housekeeping genes [6], but there remains a crucial need to identify more effective target genes that can confer durable resistance against root knot nematodes. Among other factors to consider in choosing a candidate gene for silencing to confer durable resistance, [12] recommends a gene which the pest or pathogen cannot risk for mutation. *PolA*1 is a single-copy nuclear gene that encodes the largest subunit of the multi-subunit RNA polymerase I holoenzyme complex which synthesizes ribosomal RNA precursor, an essential component of ribosomes. Ribosomes play a crucial role in protein synthesis which is essential to proper functioning of cells and organisms. The *PolA*1 gene was found to contain a nucleotide sequence that encodes species-specific

amino acid sequence [13].

The *PolA*1 gene has a function critical to eukaryotic survival and propagation. It also has a potential advantage over multi-copy genes to confer durable resistance due to its existence in single copy per haploid genome in eukaryotes. In light of the above, this study evaluated the suitability of *M. incognita*-specific (*MiS*) sequence of the*PolA*1 gene as target for effective HIGS against *M. incognita* in transgenic tobacco expressing *MiS* dsRNA.

2. Materials and Methods

2.1. *PolA*1 Silencing Vector and Tobacco Transformation

We constructed a binary vector to express hairpin RNA of a 472 bp *MiS*target sequence of *MeloidogyneincognitaPolA*1 gene (Chukwurah *et al.* submitted, Figure 1). Briefly, *MiS* sequence was PCR-amplified from pUC57 vector using MiS5P and MiS3P primers and inserted into the entry vector, pCR8, by TA cloning. *MiS* target on pCR8 was inserted into pANDA35HK RNAi binary vector using Gateway LR Clonase II enzyme mix (Thermo Fisher Scientific). We checked both pCR8: *MiS* and pANDA35HK: *MiS* plasmids from transformed *E.*

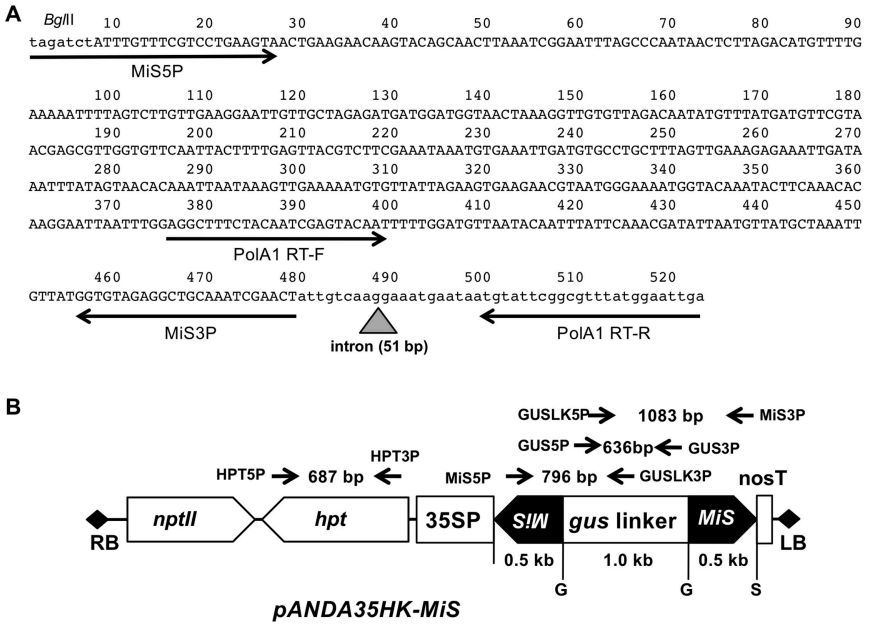

Figure 1. *MiS* target sequence and pANDA35HK RNAi binary vector. A *MiS* target sequence (472 bp, upper case) of nematode*PolA*1 gene was amplified using a pair of primer MiS5P and MiS3P. Primers PolA1 RT-F and PolA1 RT-R used in Figure 9 were located on *MiS* cDNA sequence and downstream genomic sequence (lower case) of *PolA*1 gene, respectively. *MiS* target sequence and its border sequence of *Meloidogyne incognita* was identified from accession No. CABB01001461 of Genbank database. **B** *MiS* target sequence was cloned in both sense and anti-sense orientations in pANDA35HK binary vector. 35SP: cauliflower mosaic virus 35S promoter, *nos*T: nopaline synthase gene terminator, *nptII*: kanamycin resistance gene cassette, *hpt*: hygromycin resistance gene cassette, RB: right border, LB: left border, arrows: primer positions listed in Table 1. G: *Bgl*II. S: *Sac*I.

coli by PCR and restriction analyses to confirmsequence correctness as well *MiS* integration in both sense and anti-sense orientations, respectively (Figure 2). The *PolA*1 silencing construct, pANDA35HK: *MiS*, was transformed into *Agrobacterium tumefaciens* strain EHA 105 using the freeze-thaw method.

We transformed tobacco (*Nicotianatabacum* "Petit Havana") using the *Agrobacterium*-mediated leaf disc method [14]. Transgenic tobacco calli were selected on MS basal medium supplemented with 3% sucrose, 0.1 $mg{\cdot}l^{-1}$ NAA, 1 $mg{\cdot}l^{-1}$ BA, 40 $mg{\cdot}l^{-1}$ hygromycin, 20 $mg{\cdot}l^{-1}$ meropenem and solidified with 0.8% agar. Regenerated shoots were transferred to 1/2 MS medium supplemented with 40 $mg{\cdot}l^{-1}$ hygromycin, 20 $mg{\cdot}l^{-1}$ meropenem and solidified with 0.8% agar but without growth regulators for root development.

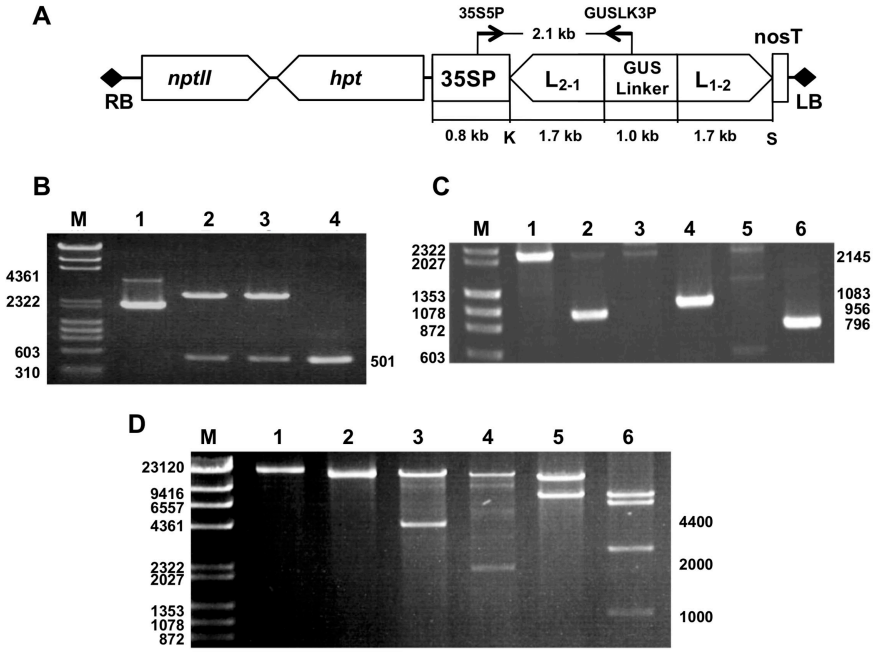

Figure 2. Molecular analyses of entry and RNAi binary vectors after cloning. A: Schematic representation of pANDA35HK binary vector. **B**: *Eco*RI cut of pCR8: *MiS* entry vector yields 2.8 kb vector backbone and 501 bp*MiS* target sequence (lanes 2 and 3). Lane 4 is PCR amplicon of *MiS* target with *MiS* specific primers. Lane 1 is uncut pCR 8: *MiS* vector. **C**: PCR analysis of pANDA35HK binary vector before and after LR recombination. Lanes 1 and 2 are expected 2.1 kb and 1.0 kb amplicons respectively from pANDA35HK and pANDA35HK: *MiS* using the primer pair: 35S5P and GUSLK3P. Lanes 3 and 4 are the expected no band and 1.1 kb amplicon from pANDA35HK and pANDA35HK: *MiS* using the primer pairs: GUSLK5P and *MiS* 3P primer pair (sense orientation). Lanes 5 and 6 are the expected no band and 0.8 kb amplicon from *pANDA35HK* and pANDA35HK: *MiS* using the primer pairs: *MiS* 3P and GUSLK3P primer pair (anti-sense orientation). **D**: Restriction enzyme analysis of pANDA35HK RNAi destination vector before and after LR recombination of target gene. Lanes 1 and 2 are undigested pANDA35HK and pANDA35HK: *MiS*, respectively. Lanes 3 and 4 are (*Sac*I + *Kpn*I)-digested pANDA35HK and pANDA35HK: *MiS* yielding vector backbones and expected 4.4 kb or 2.0 kb fragments, respectively. Lanes 5 and 6 are *Bgl*II-digested pANDA35HK and pANDA35HK: *MiS* yielding vector backbones and an expected 1.0 kb fragment with the latter and not the former. M = ΦX174 *Hae*III digest + λ-*Hin*dIII digest.

2.2. Genomic PCR Analysis of T_0 Tobacco

We extracted genomic DNA from 100 mg young leaves of putative transgenic and wild type plants using Sodium Dodecyl Sulfate (SDS) method [15]. Using different sets of primers (Table 1), we targeted regions of the binary vector corresponding to sense and anti-sense orientations of *MiS* sequence in the plants' genome (Figure 1). We also amplified a region corresponding to the hygromycin marker gene. Amplification of these regions was also carried out in the binary vector as a positive control.

2.3. Southern Blotanalysis of T_0 Tobacco

We selected PCR positive transgenic lines and extracted genomic DNA using acetyl trimethyl ammonium bromide (CTAB) protocol [16]. We digested extracted genomic DNA (15 µg) of both transgenic and WT plants with *Sac*I enzyme (TAKARA) at 37˚C. Digestion was done overnight following which DNA fragments were separated on 0.7% agarose gel and transferred to a nylon membrane. A 472 bp *MiS* probe was labelled using PCR DIG Probe Synthesis Kit (Roche). Probe hybridization, stringency washes and chemiluminescence detection with CDP-Star were carried out following manufacturer's instructions.

Table 1. List of primers used for cloning, PCR amplification, and Southern hybridization.

Gene	Primer name	Primer sequence (5'-3')	Amplicon size (bp)
MiS	MiS5P MiS3P	*tagatct*ATTTGTTTCGTCCTGAAGTA AGTTCGATTTGCAGCCTCTACACC	472
Hpt	HPT5P HPT3P	GTGTCACGTTGCAAGACCTG CGAGTACTTCTACACAGCCA	687
MiS-sense	GUSLK5P MiS3P	TGATAGCGCGTGACAAAAACCACCCAAG AGTTCGATTTGCAGCCTCTACACC	1083
MiS-antisense	MiS3P GUSLK3P	AGTTCGATTTGCAGCCTCTACACC AAGGCCGACAGCAGCAGTTTCATCAATCA	796
GUS	GUS5P GUS3P	CATGAAGATGCGGACTTACG ATCCACGCCGTATTCGG	636
EF1a	EF1a5P EF1a3P	ACTGTGCTGTCCTGATTATTGACT GGACCAAAAGTAACAACCATACCA	471
35SP-GUSLK	35SP GUSLK3P	GATGTGATATCTCCACTGAC AAGGCCGACAGCAGCAGTTTCATCAATCA	956
RKN-2	RKN-2F RKN-2R	TCTAAGTGTTGCTGATACGGTT TCCACCGATAAGGGTAGAAT	167
PolA1 RT-PCR	PolA1RT-F PolA1RT-R	AGGCTTTCTACAATCGAGTACAAT TCAATTCCATAAACGCCGAATACA	152
EF1A-RT	EF1aRT-F EF1aRT-R	GAAAGACTTTGTTGGAAGCCCTTG GGGAACAGTTCCAATACCTCCAAT	122

2.4. Expression of *MiS* dsRNA in T_0 Tobacco

We used reverse transcription PCR (RT-PCR) to confirm expression of *MiS* dsRNA in the transgenic plants. Total RNA was extracted from 100 mg young leaves of transgenic and WT plants using the RNeasy Plant Mini Kit (Qiagen). One microgram of the purified RNA (DNAse-treated) was used as template for the synthesis of first strand cDNA using Superscript III First-Strand cDNA Synthesis Kit (Invitrogen). First strand cDNA (2 µl) from both positive and negative control reactions (without enzyme) were used as template in a 50 µl total volume for PCR amplification of a region of the GUS linker using GUS5P and GUS3P primers (**Figure 1(B)**). We also amplified elongation factor 1*α* (*EF*1*α*) using primers EF1a5P and EF1a3P (**Table 1**) as an internal control.

2.5. Northern Blot Analysis for Detection of *MiS* siRNA

We extracted small RNAs (<200 bp) from the selected transgenic and WT plants using ISOGEN II reagent (Nippon Gene). Thirty micrograms of small RNA from each sample was resolved on 17% denaturing polyacrylamide gel (acrylamide: bis 19:1) containing 7M urea. Transfer of separated small RNAs to a nylon membrane was done using a semi-dry cell (Nippon Eido) for 1 hour at 10 V/400mA. Northern hybridization was done using DIG-labelled *MiS* RNA probe (472 bp) obtained via *in vitro* transcription of the *MiS* target sequence using T7 RNA polymerase according to DIG Northern Starter Kit Version 10 (Roche) protocol. Pre-hybridization (30 min) and hybridization (overnight) were performed at 50˚C. Post-hybridization stringency washes and chemiluminescent detection of siRNA using CDP-Star were performed following protocols outlined in the DIG Northern Starter Kit (Roche) manual.

2.6. Root Knot Nematode Culture and Preparation of Inoculum

A pure culture of root knot nematode (*Melodoigyneincognita*) race 2 was maintained on a highly susceptible tomato cultivar "*Kyoryoku-beiju*" in a glass house for 2 months to enable gall formation. We prepared nematode egg suspension used asinfection inoculum by extraction from freshly uprooted galled roots. Infected roots were washed, chopped and macerated in an electric blender for 5 mins at full speed. Nematode eggs were recovered by filtration on a 25 µm sieve. Recovered eggs were reconstituted into suspension and one ml egg suspension was used to determine egg concentration by counting under a microscope.

2.7. Infection of T_0 Tobacco with *M. incognita*

One-internode stem cuttings of 3 selected transgenic (T1, T4 and T7) and WT tobacco lines were rooted in 1/2 MS medium containing 1% sucrose and 0.8% agar for two weeks. Rooted plants were acclimatized on sterile vermiculite [17], transferred to pots containing 3000 ml of sterile commercial garden soil and allowed further acclimatization in the glasshouse for 12 days. They were then individually infected with approximately 10,000 *Meloidogyne incognita* eggs of

same batch via 3 holes made around each plant root system. All tested plants were replicated 10 times and confined in the glass house for a total period of 7 weeks. Infection experiment was repeated.

After 7 weeks of nematode infection, transgenic and wild type plants were harvested, and analyzed for nematode parasitic success and key agronomic characters. Galling index (%) was scored according to [18]. Number of eggs per egg mass was evaluated by counting from ten randomly selected egg masses after roots were stained with 15 mg/L Phloxine B and egg masses treated with 1% sodium hypochlorite [19]. We used SYBR Green-based real time PCR to quantify the amount of root knot nematode juveniles in the soil by measuring nematode DNA amount per gram soil. We homogenized and bulked soil samples in which transgenic and wild type plants were grown, separately. Genomic DNA was extracted from 3 replicate soil samples from each lot using ISOIL for Beads Beating Soil DNA extraction kit (Nippon Gene). We adjusted genomic DNA concentration in all samples to 2.8 ng/μl, and used 2 μl as template in 20 μl qPCR cocktail containing 10 μl KOD SYBRqPCR Mix, 4 pmol forward primer (RKN-2F), 4 pmol reverse primer (RKN-2R), 0.4 μl 50x ROX reference dye and sterile water. Real time PCR reaction was performed in a Step One Plus Real-Time PCR system (AB Applied Biosystem). A dilution series of known concentration of *M. incognita* genomic DNA was prepared and a standard curve of the log of each known concentration in the dilution series (x-axis) was plotted against the C_t (threshold) value for that concentration (y-axis). Absolute quantification of *M. incognita* genomic DNA concentration was done by comparison with the standard curve.

2.8. Generation of T_1 Tobacco Plants and Nematode Infection

We acclimatized T_0 plants (T1and T7) and transferred them to the green house for T_0 seed production. We germinated T_0 seeds of lines T1 and T7 in 0.5x MS medium supplemented with 1% sucrose and 100 mg/L kanamycin to generate their T_1 progeny plants. We confirmed via PCR the presence of *MiS* sense and anti-sense regions as well as hygromycin gene in the genomes of the T_1 plants.

T_1 plants showing presence of all three amplicons were transferred to 300 ml autoclaved garden soil and acclimatized under high humid conditions in growth room for 10 days. The plants were further acclimatized in the green house for 11 days after which they were individually infected with approximately 500 freshly extracted eggs of *M. incognita*. Nematode-infected plants were grown in confinement for 49 days and analyzed for nematode parasitic success. Nematode parasitic parameters evaluated include number of nematode galls on roots, number of nematode egg masses, number of nematodes eggs per mass and nematode multiplication. We counted nematode galls by root inspection with the aid of magnifying lens. Nematode multiplication was evaluated by multiplying the number of egg masses per gram root by number of eggs per egg mass and dividing by initial amount of egg inoculum used for infection. Infection experi-

ment was repeated twice.

2.9. *PolA*1 Target Gene Expression in Adult Feeding Female Nematodes

We extracted adult female nematodes feeding on roots of WT and T_1 transgenic tobacco plants under a stereo microscope (Olympus SZX9) and stored them in −80˚C after flash freezing in liquid Nitrogen. Total RNA was extracted from the samples using ISOGEN (Nippon Gene), and 300 ng from each was converted to cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). Quantitative real time PCR (qRT-PCR)was then performed to amplify a 152 bp target region of the *PolA*1 gene (**Figure 10(A)**) using SYBR Green technology in StepOnePlus™ thermal cycler (Applied Biosystems).

We prepared PCR cocktail for each reaction by mixing 10 µl KOD SYBR qPCR Mix (TOYOBO), 0.2 µM each of forward and reverse primers (PolA RT-F and PolA RT-R), 0.4 µl 50x ROX reference dye, 1 µl cDNA (10x dilution) and distilled water to a total of 20 µl. Amplification reaction was carried out at a hot start of 98˚C for 2 min, followed by 40 cycles of 98˚C for 10 s, 55˚C for 10 s and 68˚C for 30 s in a 96-well µltra Amp PCR plate (Sorenson Bioscience). We assessed specificity of the amplification by melt curve analysis at 60˚C - 95˚C after 40 cycles.

Three biological and three technical replicates were used with each sample. We used mean C_t values (normalized against internal reference gene) to calculate the fold change in *PolA*1 expression in the nematodes using the $2^{-\Delta\Delta CT}$ method. Root knot nematode elongation factor was used as the internal reference gene. We expressed *PolA*1 transcript abundance in nematodes extracted from transgenic roots as a percentage relative to the transcript level in nematodes extracted from wild type plants.

2.10. Statistical Analyses

All experimental units were laid in a completely randomized design (CRD) in the green house, and data generated after nematode infection were analyzed by a one-way analysis of variance (ANOVA) using SigmaPlot 14.0 software (SYSTAT). Significantly different means were separated using the Duncan's Multiple Range Test (DMRT).

3. Results

3.1. Genomic PCR Analyses of T_0 Transgenic Tobacco

Following *agrobacterium* transformation, selection and regeneration of tobacco plantlets, we generated and genotyped 13 primary transgenic lines. We used 2 sets of primers GUSLK5P/MiS3P and MiS3P/GUSLK3P (**Figure 1(B)**) to amplify 1083 bp and 796 bpgenomic regions corresponding to the sense and anti-sense orientations of *MiS* target. We also amplified a 687 bp genomic region corresponding to hygromycin marker gene (*hpt*) using primers HPT5P and

HPT3P (Figure 1(B)). Sense, anti-sense and *hpt* amplicons for 13 transgenic lines are shown in Figure 3(A). Plasmid DNA was amplified as a positive control while untransformed plants (WT) showed no amplification.

3.2. Southern Analysis of T_0 Transgenic Tobacco

We subjected nine PCR-positive transgenic lines to Southern hybridization to analyze their *MiS*T-DNA integration patterns. Using probes specific to *MiS* target, single, double and triple copy T-DNA insertions were observed with the different lines (Figure 3(B)). Wild type plants showed no hybridization signal with the probe.

3.3. Expression of *MiS* dsRNA in T_0 Tobacco

We derived complementary DNA (cDNA) from selected T_0 plants (T1, T4 and T7) and conducted reverse-transcription PCR (RT-PCR) analysis to confirm expression of *MiS* dsRNA in the transgenic lines. Using primers GUS5P and GUS3P (Figure 1(B)), we amplified a 636 bp fragment corresponding to the GUS linker region between sense and anti-sense orientations of the *MiS* target sequence in the selected lines (Figure 3(C)). Wild type plants showed no amplification for the GUS linker region. Elongation factor (*EF*1*a*) used as internal

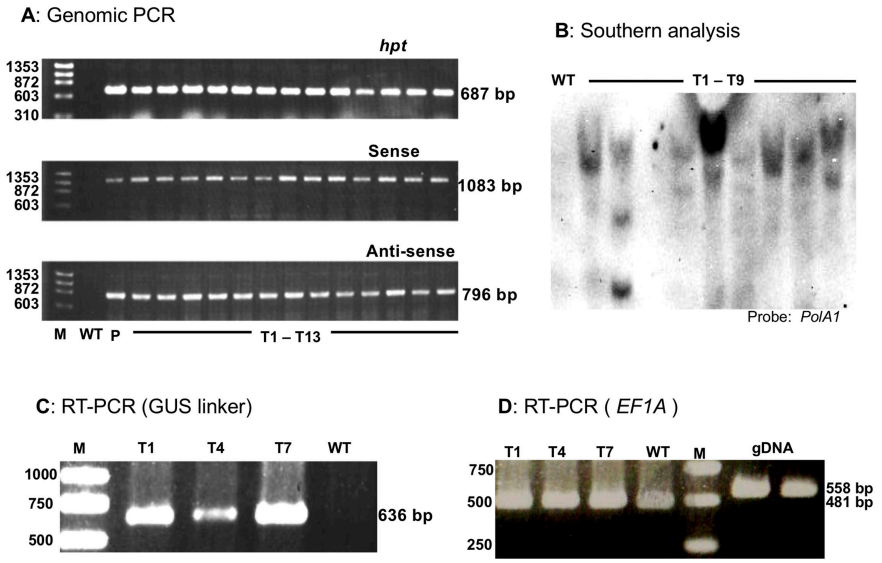

Figure 3. Molecular analyses of T_0 transgenic tobacco. **A**: PCR amplification of *hpt* marker gene, sense orientation and anti-sense orientation of nematode *MiS* target fragment. Plasmid control (P) showed similar band size while the wild type plants (WT) showed absence of target amplicons. M: ΦX174 *Hae*III digest marker. **B**: Southern blot analysis. Probe used for hybridization was specific to *MiS* target sequence. Wild type plants (WT) showed no hybridization signal. **C**: RT-PCR analysis detected GUS linker fragment of RNAi construct and is indicative of expression of the trigger dsRNA. Wild type plants (WT) showed no expression of *PolA*1 hairpin RNA. **D**: RT-PCR of elongation factor (*EF*1*a*), used as control, was amplified from cDNA (481 bp) of selected transgenic and wild type tobacco lines and from 2 genomic DNA controls (558 bp). M: DL 2000 DNA Marker (TAKARA).

control reference gene was amplified using primers EF1a5P and EF1a3P (Table 1). Transgenic and wild type plants both showed a 481 bp amplicon of *EF*1*a*. We also amplified 2 genomic DNA controls containing a 77 bp intron with the same primers and obtained a higher 558 bp amplicon (Figure 3(D)).

3.4. Northern Blot Analysis for *MiS* siRNA in T_0 Tobacco

DIG-labelled *MiS*RNA probe hybridized to sequence-specific siRNAsin the transgenic plants that expressed Mi*SdsRNA* (Figure 4). We did not detect any hybridization signal with WT plants. Ethidium-bromide staining of the polyacrylamide gel after electrophoresis showed equivalent loading of small RNAs across all samples.

3.5. Bio-Efficacy of T_0 Transgenic Tobacco against *M. incognita*

An inspection of the test plants' roots showed wild type plants with reduced and necrotic root system having larger multiple fused galls compared to the transgenic lines (Figure 5(A)). Nematode galling index was equally highest on roots of wild type tobacco plants (70.0%). The transgenic lines showed reduced root galling indices particularly T1 and T7 (54.3% and 58.6% respectively. Galling index on T4 was 62.7% (Figure 5(B)). Nematode eggs per mass was highest on roots of WT plants (613) but significantly reduced ($p < 0.05$) by 26.4% in transgenic line T1. Nematodes eggs on lines T7 and T4 were reduced by 17.3% and 2.5% respectively but not significantly (Figure 5(C)). Quantification of nematode DNA by real time PCR showed that soil pooled from wild type plants contained significantly more ($p < 0.05$) nematode DNA per gram (617.6 ng/g soil) than those of transgenic plants T1 (235.0 ng/g), T7 (149.8 ng/g) and T4 (303.4 ng/g) (Figure 5(D)). This represented a 61.9%, 75.7% and 50.9% reduction respectively.

The transgenic plants were generally more vigorous post-infection compared to WT plants (Figure 6(A)). Number of leaves determined for all treatment groups before nematode infection was comparable ($p > 0.05$). All plants had an average of 13 healthy leaves (Figure 6(B)). After 7 weeks of nematode infection,

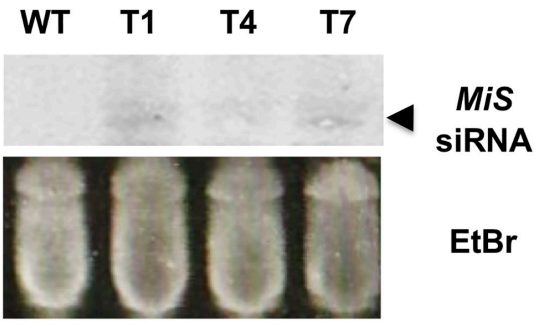

Figure 4. Northern analysis for detection of *MiS* siRNAs. *MiS* siRNA was detected with DIG-labelled *MiS* RNA probe in the transgenic lines. WT plants showed no hybridization signal. EtBr shows equivalent loading of small RNAs across samples.

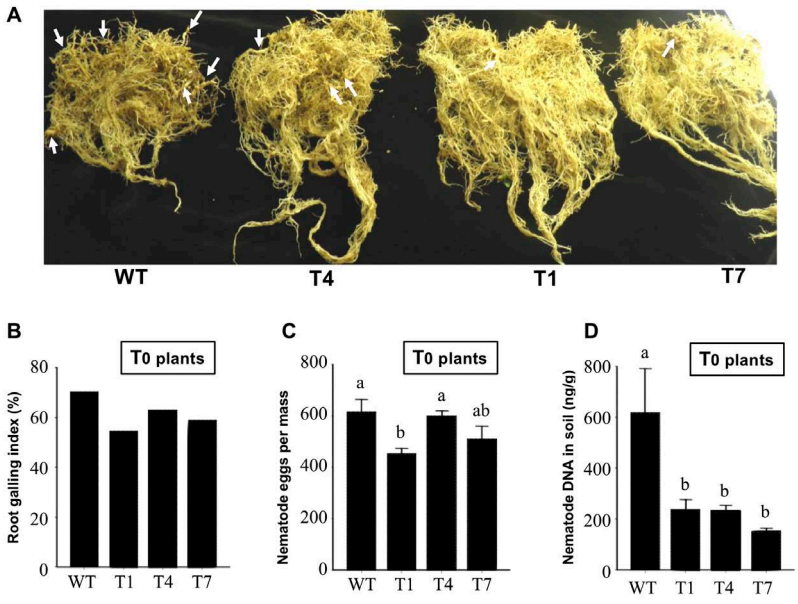

Figure 5. Nematode infection analyses in T_0 transgenic tobacco. **A**: WT roots showed more damage (necrosis) and reduced length compared to transgenic lines' roots. **B**: Root galling indices were generally reduced in transgenic lines compared to WT. **C**: Nematode fecundity, measured by number of eggs per egg mass, was significantly reduced ($p < 0.05$) in transgenic lines compared to WT plants. **D**: qPCR analysis of nematode DNA concentration in soil after infection showed reduced ($p < 0.05$) presence of *M. incognita* in soils with transgenic lines than WT.

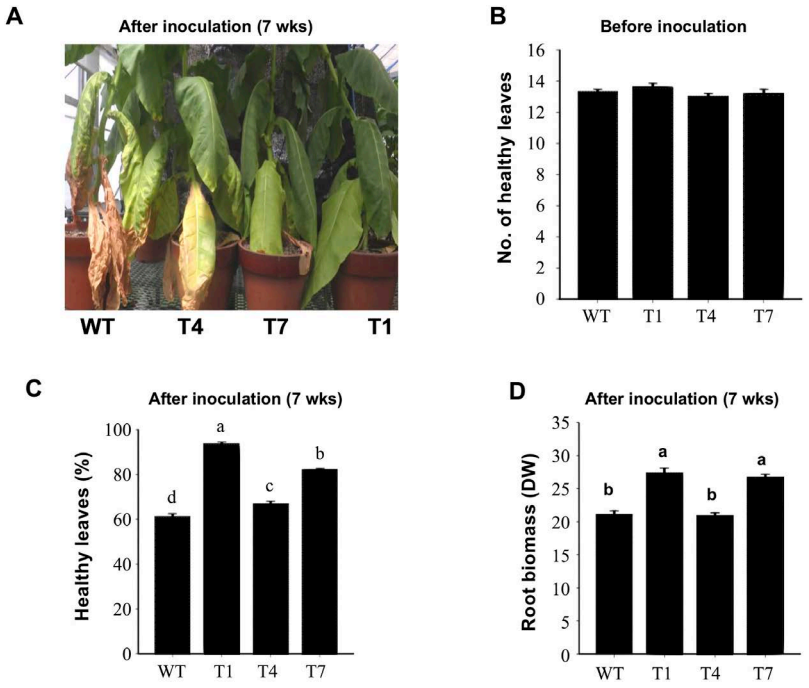

Figure 6. Agronomic assessment of T_0 tobacco after 7 weeks of nematode infection. **A**: Transgenic lines were comparatively more vigorousthan WT. **B**: Number of healthy leaves before infection with nematodes was comparable ($p > 0.05$) in all plants. **C**: Transgenic lines had significantly higher ($p < 0.05$) percentage of green standing leaves after infection **D**: Root biomass was significantly higher ($p < 0.05$) in transgenic lines than WT.

however, mean percentage of green, standing leaves reduced significantly ($p < 0.001$) in WT plants by 32.5% and 20.7% respectively compared to T1 and T7 (Figure 6(C)). Fresh root biomass was also significantly reduced ($p < 0.05$) in the WT plants by 23.1% and 21.4% compared to lines T1 and T7 respectively, but comparable to T4 (Figure 6(D)).

Disease condition was more outstanding on WT leaves compared to transgenic plants (Figure 7(A)). We measured the distance from base of each plant up the stem to the last diseased leaf. This parameter, termed disease progression, was significantly reduced ($p < 0.001$) in transgenic lines T1 and T7 by about 80.0% compared to WT plants. Compared to T4, disease progression was significantly reduced by 12.0% (Figure 7(B)). Fresh leaf biomass was significantly reduced ($p < 0.001$) in WT plants compared to those of T1 and T7 by 22.0% and 14.2% respectively. Compared to T4, WT plants showed 6.9% reduction in fresh leaf biomass (Figure 7(C)). In the same vein, dry leaf biomass in WT plants was reduced significantly by 19.9% and 14.2% compared to T1 and T7 respectively, but comparable to T4 (Figure 7(D)).

3.6. Molecular Characterization of T_1 Tobacco Lines

We extracted genomic DNA from 100 mg young leaves of T_1 tobacco progeny plants generated from transgenic lines T1 and T7. We excluded T4 from further

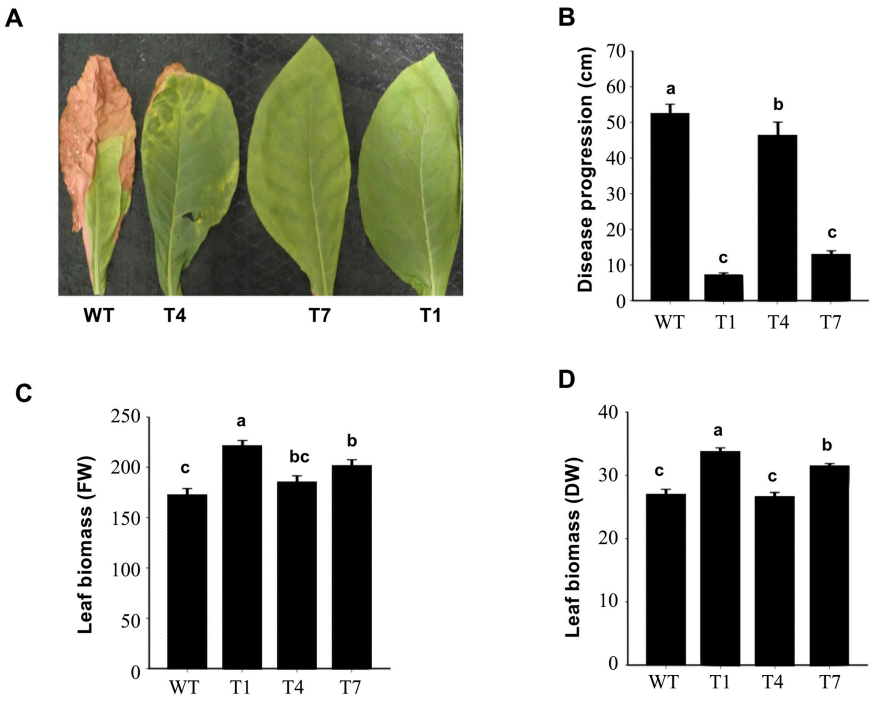

Figure 7. Disease progression in T_0 tobacco lines after 7 weeks of nematode infection. **A**: WT leaves showed severe leaf disease conditions compared to those of transgenic lines. **B**: Disease progression measured as distance from base of each plant to the last diseased leaf was significantly higher ($p < 0.05$) on WT plants than transgenic lines. Fresh leaf biomass **(C)** and dry leaf biomass (**D**) was significantly higher ($p < 0.05$) in the transgenic lines than WT.

analyses owing to its comparatively weaker T_0 phenotype. PCR amplification was carried out with same primer sets used with T_0 plants. We obtained correct amplicons for hygromycin gene (687 bp) as well as *MiS* target in both sense (1083 bp) and anti-sense orientations (796 bp) with the transgenic lines. Wild type plants showed no amplification signal (**Figure 8(A)**).

3.7. Bio-Efficacy of T_1 Tobacco against *M. incognita*

Nematode galls per gram root were significantly reduced ($p < 0.05$) in T_1 plants of transgenic lines T7 and T1 by 25.5% and 20.6% respectively compared to WT plants. T7 and T1 were comparable ($p > 0.05$) in mean number of galls on their roots (**Figure 8(B)**). Nematode egg masses were also significantly reduced ($p < 0.05$) by 13.8% and 11.4% on transgenic lines T1 and T7 respectively, compared to WT plants (**Figure 8(C)** and **Figure 8(A)**). The transgenic plants had significantly reduced ($p < 0.05$) number of nematode eggs permass. Compared to WT plants, nematode eggs per mass on T1 and T7 were reduced by 20.9% and 17.7% respectively (**Figure 8(D)**). Nematode multiplication on transgenic lines T1 and T7 was also significantly reduced ($p < 0.05$) by 30.7% and 26.9% respectively compared to WT plants (**Figure 8(E)**).

Agronomic traits evaluated in the test plants included shoot weights, fresh root weights and root lengths. All T_1 test plants were comparable ($p > 0.05$) in these characters (**Figures 9(B)-(D)**).

3.8. Relative Expression of *PolA*1 in Adult Feeding Female Nematodes

We used qRT-PCR analyses to compare *PolA*1 transcript expression in adult female nematodes feeding on roots of transgenic and wild type plants. Relative to expression in nematodes feeding on roots of WT plants, *PolA*1expression was significantly reduced ($p < 0.05$) by 34.3% and 31.5% respectively in nematodes feeding on transgenic lines T1 and T7 (**Figure 10(B)**). Analysis of melt curves for reference and *PolA*1 after qRT-PCR showed single peaks that indicate specific target amplification.

4. Discussion

HIGS strategy against parasitic nematodes employs an *in planta* approach in which susceptible host plants are engineered to express dsRNA of essential nematode genes and deliver resultant siRNAs to the feeding pests. These small regulatory RNAs achieve silencing of targeted endogenous gene transcripts in the nematodes and confer protection on host plants [20]. In this study, we targeted *M. incognita PolA*1 gene for HIGS considering that it plays a crucial role in protein synthesis and its effective silencing in eukaryotes (including nematodes) could lead to deleterious effects. We constructed pANDA35HK: *MiS* plant expression vector to integrate *MiS* target sequence in sense and anti-sense orientations (**Figure 1(B)**) such that upon expression, would produce *MiS*dsRNA.

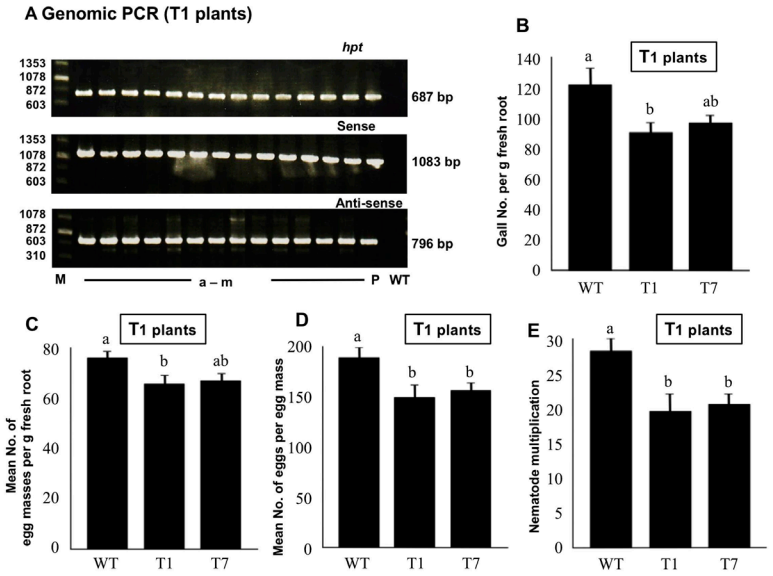

Figure 8. Molecular and nematode infection analyses in T_1 transgenic tobacco. A: PCR amplification of *MiS* sequence in sense and anti-sense orientations as well as *hpt* gene in genome of T_1 tobacco plants showed correct 1083 bp, 796 bp and 687 bp amplicons respectively. **B**: Gall number per g fresh root was not significantly different ($p > 0.05$) in transgenic lines and WT. **C**: Mean number of egg masses per g fresh root was significantly reduced ($p < 0.05$) in transgenic lines compared to WT plants. **D**: Mean number of eggs per egg mass was not significantly different ($p > 0.05$) across the tested lines. **E**: Nematode multiplication was more ($p < 0.05$) on WT roots compared to those of transgenic lines. Bar means with different alphabets are significantly different ($p < 0.05$). Infection experiment was conducted twice and similar results were obtained.

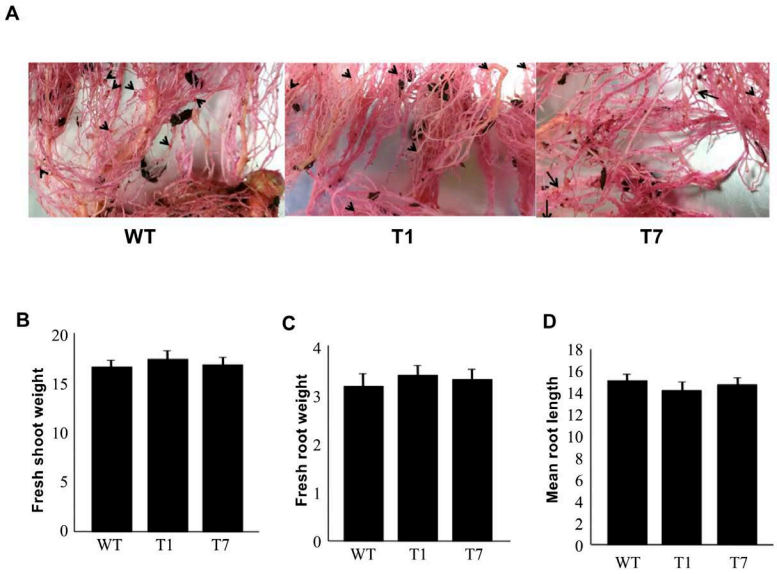

Figure 9. Egg mass staining and agronomic characters of T_1 tobacco. (A): Phloxine B-stained roots of transgenic and WT tobacco plants showing pink egg masses. WT roots had increased number of nematode egg masses (pink arrows) compared to transgenic tobacco roots. Transgenic and WT plants did not differ significantly ($p > 0.05$) in (**B**): Fresh shoot weight (**C**) Fresh root weight and (**D**) Mean root length, after 35 days of nematode infection.

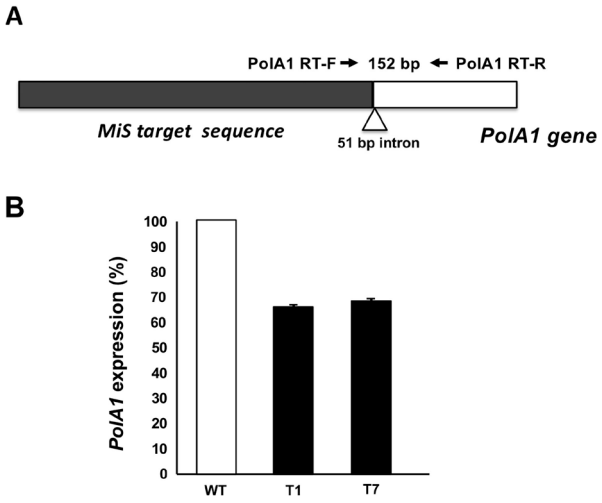

Figure 10. *PolA*1 transcript expression in adult nematodes feeding on WT and transgenic plants' roots. A: Short fragment (152 bp) of *PolA*1 transcript was amplified by RT-PCR using primers PolA1 RT-F and PolA1 RT-R, shown in Figure 1. **B**: Reduced *PolA*1 transcript expression in adult female nematodes isolated from T1 and T7 roots relative to those from WT roots. *Meloidogyne incognita* elongation factor was used as internal control gene. Relative gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method and expressed as % relative to WT (100%).

We confirmed successful transformation of tobacco with this silencing construct via PCR and Southern blot analyses (Figure 3(A) and Figure 3(B)). We also showed evidence of expression of *MiS* dsRNA via RT-PCR amplification of the GUS linker between the target sequences in the transgenic lines (Figure 3(C)). Production of dsRNAs triggers eukaryotic cellular RNA silencing machinery [21], thus *MiS* dsRNAs can activate RNAi events in the transgenic tobacco. Detection of *MiS* siRNAs in the transformed plants (Figure 4) is a confirmation that *MiS* dsRNAs produced by the transgenic plants were processed by dicer into siRNAs. *Inplanta*-produced siRNAs act as mediators of gene silencing via facilitation of the degradation of complementary genes' transcripts in invading parasites [8] [22].

Nematode bioassay results showed that all tested plants (transgenic and WT) had nematode galls on their roots and none of the transgenic lines exhibited complete resistance to root knot infection. However, reduced gall indices on T_0 and T_1 transgenic lines (Figure 5(A), Figure 8(B)) is an evidence of superior root reaction to nematode infection among the transgenic lines, which according to [19] depicts a relative indication of resistance. Our T_0 and T_1 transgenic tobacco showed reduced nematode egg masses (Figure 9(A)), and eggs per mass compared to wild type plants (Figure 5(C), Figure 8(C), Figure 8(D)). This finding indicates a higher level of resistance due to reduced reproductive ability of nematodes on the transgenic lines. Reduction in the amount of root knot nematode DNA in soil on which T_0 transgenic plants were grown compared to wild type (Figure 5(D)) as well as reduced parasite multiplication on T_1 plants (Figure 8(E)) are complementary data that provide evidence of restrained para-

site propagation. This is proof of some resistance in plants expressing nematode *MiS* dsRNA. Suppressed nematode reproduction and development is a phenotype that is consistent with previous successful HIGS studies using different target genes like *MSP* [23] [24] [25] [26].

All T_0 transgenic lines except T4 showed superiority in agronomic vigour and susceptibility to disease conditions compared to WT plants (**Figures 6(A)-(D)**, **Figures 7(A)-(D)**). This indicates their relative resistance to a disease complex initiated by nematode infection and compounded by other secondary pathogens. Nematodes are known to have both direct and indirect effects on tobacco plants particularly. Indirect effects include the ability of nematodes to increase the susceptibility of the plant to other diseases such as brown spot and blank shank diseases [27] [28]. The weaker phenotype of T4 lines may have to do with reduced amount of processed *MiS* siRNAs (**Figure 4**), which in turn reduced plant protection against nematode-initiated disease complex. Small interfering RNAs are known to mediate the RNA silencing machinery which down-regulates target gene transcripts in the parasites resulting in attenuated development and reduced host parasitism [29]. Reduced silencing efficiency due to relatively low siRNA production may be attributable in part to inefficient processing of *PolA*1 dsRNA to siRNAs by dicer enzymes. Moderate silencing of *NtFAD3* gene in tobacco was attributed to inefficient dsRNA processing by dicer [30].

T_1 tobacco plants, however, were comparable to WT in agronomic traits evaluated after 35 days of nematode infection (**Figures 9(B)-(D)**). We attribute this result to the low amount of inoculum (500 eggs) used to induce infection since higher inoculum can usually induce greater pest pressure and disease condition in the host plants [19]. Unlike T_0 plants infection where 10,000 eggs were used, we used 20 times lesser eggs with T1 plants due to their relatively young age at infection. This concentration seems inadequate to produce distinguishable agronomic data in the plants at 35 days post infection. Longer exposure to nematodes may be required for optimum nematode multiplication and plant stress induction.

Feeding nematodes isolated from roots of transgenic tobacco plants showed significant reduction of *MiS* transcripts relative to those on WT (**Figure 10(B)**). Down-regulation of target gene transcripts in parasites has been characterized as an indicator of effective host induced RNAi in transgenic plants [29]. Effective silencing of *PolA*1 gene in the feeding nematodes provides evidence of uptake of small regulatory RNAs from the transgenic plants. It further corroborates the enhanced suppression of nematode multiplication in the transgenic plants showing that *PolA*1 plays a crucial reproductive and developmental function in these pests.

According to [12], a good candidate gene for HIGS to achieve durable resistance must be one for which the host pest or pathogen cannot risk its mutation. *PolA*1 satisfies this requirement due to its single copy existence and essentiality in eukaryotic survival. Hence, although some previously evaluated candidate

genes [8] [9] [31] showed greater potency for nematode control than was obtained in this study with *PolA*1, the potential durability of *PolA*1-conferred resistance will be a crucial benefit for crop production. The high homology of our target sequence among *Meloidogyne* species is an additional benefit that can extend durable resistance across all root knot species in the *Meloidogyne* genus.

5. Conclusion

In this study, we have shown that transgenic tobacco plants expressing *MiS*dsRNA of root knot nematode *PolA*1 gene processed regulatory siRNAs and knocked down *PolA*1 transcript expression in feeding nematodes. T1 and T7 transgenic lines showed reduced nematode damage and improved agronomic vigor possibly due to host induced *PolA*1 silencing in the nematode pests. Targeting *PolA*1 gene for silencing in pathogens may thus be an effective strategy for molecular-based plant protection. However, it is important to aim at improving the resistance obtained in this study by using other RNAi vectors containing intron linkers for increased siRNA processing and improved silencing efficiency.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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