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Host-induced silencing of *Meloidogyne incognita PolA1* gene for control of root-knot nematode in transgenic tobacco and tomato

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Chapter I: General Introduction

Background to study

The Food and Agricultural Organization (FAO) of the United Nations, in 2017, projected the global population to exceed 9 billion by 2050 and 11 billion by 2100. Of the 11 billion earth inhabitants projected by the end of the century, Asia and Africa are expected to contribute over 80%. Ensuring food security to address the nutrition needs of this inexorably growing human population, particularly in Asia and Africa, is a looming major global challenge. FAO's estimation is that agriculture in 2050 must produce at least 50% more food than it did in 2012 while sub-Saharan Africa and South Asia are expected to produce over 200% of their current agricultural output to meet growing population demand. Achieving increased agricultural output in sub-Saharan Africa, according to Keating et al. (2010) and Coyne et al. (2018), will among other things require concerted efforts in the management of rising pest and disease threats.

Nematodes are among the most widespread and economically important crop pests globally (Webster, 1987). The threat posed by these pests to global agricultural output and associated food security was emphasized over three decades ago at the International *Meloidogyne* Project by Sasser and Carter (1985). Over the years, despite considerable efforts and progress made in addressing this, crop losses due to nematodes continue to be significantly substantial. Globally, annual crop losses due to nematodes were estimated in literature to range from 80 – 157 billion (Bird and Kaloshian 2003; Abad et al. 2008; Nicol et al. 2011). In sub-Saharan Africa, although there are currently no reliable statistics on economic losses due to nematodes (Coyne et al. 2018), it is reasonable to assume a much greater devastation to this already food-challenged continent given the comparatively less sophistication in agricultural practices.

Of the total number of nematodes currently known, plant-parasitic nematodes were reported to comprise approximately 15% (Wyss 1997), and root-knot nematodes (*Meloidogyne* species) considered the most economically important with a wide global distribution and host range. According to Sasser et al. (1983), *M. incognita* is the most important of the four common species of root-knot nematodes (*M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*) that cause about 95% of all root-knot nematode infestations on agricultural land. More than 1700 host plant species are said to be susceptible to the devastating effects of *M. incognita* infection (Sasser 1980). *Meloidogyne incognita*, also known as Southern root-knot nematodes are obligate sedentary endo-parasites that parasitize the root system of susceptible host plants. They disrupt water and nutrient uptake through the roots in the course of their parasitism thus affecting the whole plant and cause symptoms which range from stunting, leaf chlorosis and patchy growth with the formation of root galls (Ajjappala et al. 2012). Ultimately, *M. incognita* parasitism leads to significant reduction in yield of crops.

Management of root-knot nematode parasitism over the years has employed a number of strategies. The integrated use of chemicals, resistant crop varieties, cultural and biological practices have proven to be a very successful strategy (Fuller et al. 2008). Chemical nematicides like DCMP (2, 5-dichloro-4-methoxyphenol) and methyl bromide are effective means of nematode control. However, in recent times, concerns have been raised about these chemicals with regards to environmental toxicity and residues in food, ground water contamination and mammalian toxicity eliciting either highly stringent regulations on their use or outright ban on others (Thomason 1987). Sorribas et al. (2005) further criticized the use of chemicals for nematode control as less cost-effective and financially burdensome on

farmers. The use of cultural practices such as crop rotation as a strategy for curbing nematode infestation is limited by its inability to be applied to nematode species with cosmopolitan host ranges such as *M. incognita* (Abad et al. 2003), as well as its limited applicability to growers of specialist crops (Fuller et al. 2008).

Resistance in crops, which occur naturally or engineered through molecular techniques, is an alternative attractive approach for nematode control (Fuller et al. 2008). According to Williamson and Kumar (2006), the use of natural host resistance to suppress nematode reproduction on crops is the preferred strategy that poses the least environmental risk and more cost-effective compared to chemical control. However, this approach is also limited in its ability to control many nematode problems. For example, many natural resistance genes like tomato *Mi* gene can be overcome by virulent nematode biotypes as was reported by Castagnone-Sereno (2002) where virulent populations of *M. incognita* overwhelmed *Mi*-mediated resistance used in commercial cultivars. According to Lilley et al. (2007), the limitations of conventional control procedures provide an opportunity for plant biotechnology to introduce effective and inexpensive nematode control strategies and reduce environmental risks associated with chemical control.

Engineering nematode resistance in plants by silencing of essential genes via RNA interference (RNAi) is a strategy that has gained a lot of attention currently. RNA interference is a conserved mechanism in a wide range of eukaryotic organisms that can inactivate gene expression in a sequence-specific manner (Hirai and Kodama, 2008). It is triggered by double-stranded RNA (dsRNA), which is rapidly processed by dicer-like enzymes into RNA duplexes of 21-26 bp called small interfering RNAs (siRNAs). When combined with the RNA-induced silencing complex (RISC), siRNAs guide the recognition and, ultimately,

cleavage of their complementary mRNAs. The application of RNA interference (RNAi) to silence essential genes of plant-parasitic nematodes received attention two decades ago following the pioneering work on *Caenorhabditis elegans* by Fire et al. (1998). The strategy has since evolved as a powerful gene silencing tool for analysis of gene function in a wide variety of organisms (Hannon 2002).

In the past decade, the possibility of engineering plants to produce dsRNAs that silence essential genes in attacking nematodes was demonstrated by Yadav et al. (2006) and Huang et al. (2006), targeting both house-keeping and parasitism genes respectively of *Meloidogyne incognita*. This strategy, also known as host-induced gene silencing (HIGS), is initiated when a nematode-susceptible host plant is genetically transformed with a construct that produces dsRNA of a targeted essential nematode gene. As described by Gheysen and Vanholme (2007), the dsRNA, or its siRNAs, would then be delivered from the plant to the nematode through ingestion of the plant cytoplasm. Once inside the nematode, the RNAi process would inactivate the gene targeted by the dsRNA. Inactivation of genes essential to the nematodes' reproduction and development may lead to lethal effects that limit their parasitic damage thereby protecting the host plants.

The choice of an appropriate target gene that produces a lethal effect on nematodes, when silenced, is regarded as a key consideration for the success of nematode control using HIGS strategy (Ajjappala et al. 2012). Ghang (2017) defined an appropriate candidate gene for HIGS, among other criteria, as one whose mutation cannot be risked by the nematodes. Following the successful demonstration of plant protection via HIGS and the subsequent reportage of complete genome sequence of *Meloidogyne incognita* (Abad et al. 2008) and *Meloidogyne hapla* (Opperman et al. 2008), the last decade saw numerous attempts to control

root-knot nematodes through targeting and silencing of various essential genes. Reviews published by Ajjappala et al. (2012) and Dutta et al. (2015) summarized various root-knot nematode genes targeted to include those involved in parasitism, house-keeping and development.

The *PolA1* is a house-keeping gene that encodes the largest sub-unit of the RNA polymerase I holoenzyme which catalyzes DNA-dependent synthesis of 45S rRNA precursor in the eukaryotic nucleus. It occurs as a single-copy nuclear gene per haploid genome. Synthesis of rRNA has been pointed to as a focal point for the regulation of cell metabolism and cell growth (Grummt 2003), and silencing of the *PolA1* gene in any eukaryotic organism can potentially cause deleterious effects. *PolA1* was reported by Nakamura (2010) to contain a highly conserved sequence (Ntag) that encodes a highly polymorphic amino acid sequence useful for identifying eukaryotic species.

Justification of study

Crop diseases, caused by various parasites including nematodes, induce severe economic losses that threaten agriculture particularly in developing countries. Tackling the associated food security concerns presented to a growing global human population, according to Qi et al. (2019), will require modern agriculture to develop disease-resistant varieties of crops massively. Conventional approaches used to combat root-knot nematode-induced diseases including use of agrochemicals, host resistant selection and breeding, though quite effective, all have limitations that make them insufficient to address the challenge. However, the development of biotechnological approaches, such as host-induced gene silencing, provides a novel approach to obtain disease-resistant plants against multiple pathogens without posing any significant environmental risk (Qi et al. 2019).

Since its successful demonstration (Yadav et al. 2006; Huang et al. 2006), and subsequent completion of whole genome sequence for *Meloidogyne incognita*, HIGS strategy against root-knot nematodes has been shown to be effective using different parasitism and house-keeping genes as targets. However, there is need to identify more target genes whose silencing via HIGS can confer robust resistance against root-knot nematodes. One of the criteria for identifying such target gene, according to Ghang (2017) is a gene whose mutation cannot be risked by the pathogens. The *PolA1*, following its occurrence as a single-copy per haploid genome gene and critical function in ribosome synthesis, presents a potentially viable target whose silencing can significantly affect reproduction and development and reduce damage to agricultural crops.

Aim and objectives of study

This study was therefore aimed at evaluating the efficacy of utilizing the root-knot nematode *PolA1* gene as a target of host-induced silencing for conferring nematode resistance on transgenic tobacco and tomato. Accordingly, the specific objectives of this study were:

- To produce an RNA interference silencing construct using *M. incognita*-specific sequence of *PolA1* gene cloned in both sense and anti-sense orientations
- To generate transgenic tobacco and tomato lines harboring the *M. incognita PolA1* silencing construct via *Agrobacterium tumefaciens*-mediated transformation
- To evaluate the expression of the *M. incognita PolA1* transgene into trigger double-stranded RNAs in the transgenic plants
- To compare root-knot nematode resistance parameters in *M. incognita*-infected transgenic and untransformed control lines
- To measure the relative expression of the *PolA1* target sequence in *M. incognita* adult females feeding on roots of both transgenic and non-transgenic lines

Chapter II: Mitigating root-knot nematode propagation on transgenic tobacco via *in planta* hairpin RNA expression of *Meloidogyne incognita*-specific *PolA1* sequence

Introduction

Plant parasitic nematodes are among the most significant constraints to sustainable agriculture and achievement of food security (Abd-Elgawad et al. 2015). 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 (Ajjappala et al. 2012). 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 (Fuller et al. 2008). 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 (Atkinson 1995; Thomas and Cottage 2006). 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 (Yadav et al. 2006; Huang et al. 2006; Dutta et al. 2015; Shivakumara et al. 2017). This strategy involves the *in planta* expression of double stranded RNA (dsRNA) of an essential nematode gene to trigger innate RNA interference (RNAi) machinery. RNAi silencing is a cellular mechanism triggered by dsRNA which is rapidly processed into 21-24 bp RNA duplexes called small interfering RNAs (siRNAs) which then combine with RNA-induced silencing complex (RISC) and guide the sequence-specific recognition and degradation of complementary mRNAs (Ntui et al. 2015). In HIGS, expression of nematode-specific dsRNA

in plants generates siRNAs which are ingested by the nematodes during feeding and mediates silencing of the target gene in nematodes (Tamilarasan and Rajam 2013).

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 (Huang et al. 2006) and housekeeping genes (Dutta et al. 2015), 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, Ghang (2017) recommends a gene which the pest or pathogen cannot risk for mutation. *PolA1* 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. The *PolA1* gene contain a nucleotide sequence that encodes species-specific amino acid sequence (Nakamura 2010).

The *PolA1* 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 (*MiPA*) sequence of the *PolA1* gene as target for effective HIGS against *M. incognita* in transgenic tobacco expressing *MiPA* dsRNA.

Materials and methods

Construction of *PolA1* silencing vector

A 472 bp *M. incognita*-specific (*MiPA*) coding sequence of nematode *PolA1* gene was selected as target from the genomic sequence CABB01001461 (NCBI). This target sequence did not show perfect match of more than 20 bp stretch to whole genomic sequence of tobacco through NCBI blast (data not shown). *MiPA* sequence was synthesized and cloned into pUC57 vector. *MiPA* target sequence was then PCR-amplified by Taq polymerase using MiPA-5P and MiPA-3P primers (Table 1), purified and inserted into the entry vector, pCR8, by TA cloning. A unique *Bgl*II restriction site was added to the 5'-end of MiPA-5P (Table 1) to aid in downstream vector analyses. pCR8::*MiPA* plasmid was then mobilized into DH5α competent *E. coli* cells by freeze thaw method. Transformed *E. coli* cells were selected on Luria-Bertani (LB) medium containing 100 mg l^{-1} spectinomycin. Selected colonies were grown overnight in liquid LB medium containing 100 mg l^{-1} spectinomycin and plasmids extracted using Fast Gene Plasmid Mini Kit (Nippon Genetics).

Colonies carrying correct target *MiPA* inserts were confirmed by restriction digestion and PCR before sequencing. A pure culture of selected positive colony was made by streaking on solid LB medium containing 100 mg l^{-1} spectinomycin. The colony was subsequently grown overnight in liquid LB medium containing 100 mg l^{-1} spectinomycin and pCR8::*MiPA* plasmids were extracted. The extracted pCR8::*MiPA* plasmids were inserted into pANDA35HK RNAi binary vector (https://bsw3.naist.jp/simamoto/pANDA/real/pANDA35HK_map.htm; Figure 2-1) using Gateway LR Clonase II enzyme mix (Thermo Fisher Scientific).

Table 1

List of oligonucleotide primers used for cloning, PCR amplification, probe labelling and qRT-PCR

Gene	Primer name	Primer sequence (5′ – 3′)	Product length (bp)
MiPA	MiPA5P MiPA3P	*tagatct*ATTTGTTTCGTCCTGAAGTA AGTTCGATTTGCAGCCTCTACACC	472
HPT	HPT5P HPT3P	GTGTCACGTTGCAAGACCTG CGAGTACTTCTACACAGCCA	687
MiPA-sense	GUSLK5P MiPA3P	TGATAGCGCGTGACAAAAACCACCCAAG AGTTCGATTTGCAGCCTCTACACC	1083
MiPA-antisense	MiPA3P 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	PolA1 RT-F PolA1 RT-R	AGGCTTTCTACAATCGAGTACAAT TCAATTCCATAAACGCCGAATACA	152
EF1A-RT	EF1a RT-F EF1a RT-R	GAAAGACTTTGTTGGAAGCCCTTG GGGAACAGTTCCAATACCTCCAAT	122

*_tagatct_ – Unique *Bgl*II restriction enzyme site incorporated into the forward primer sequence

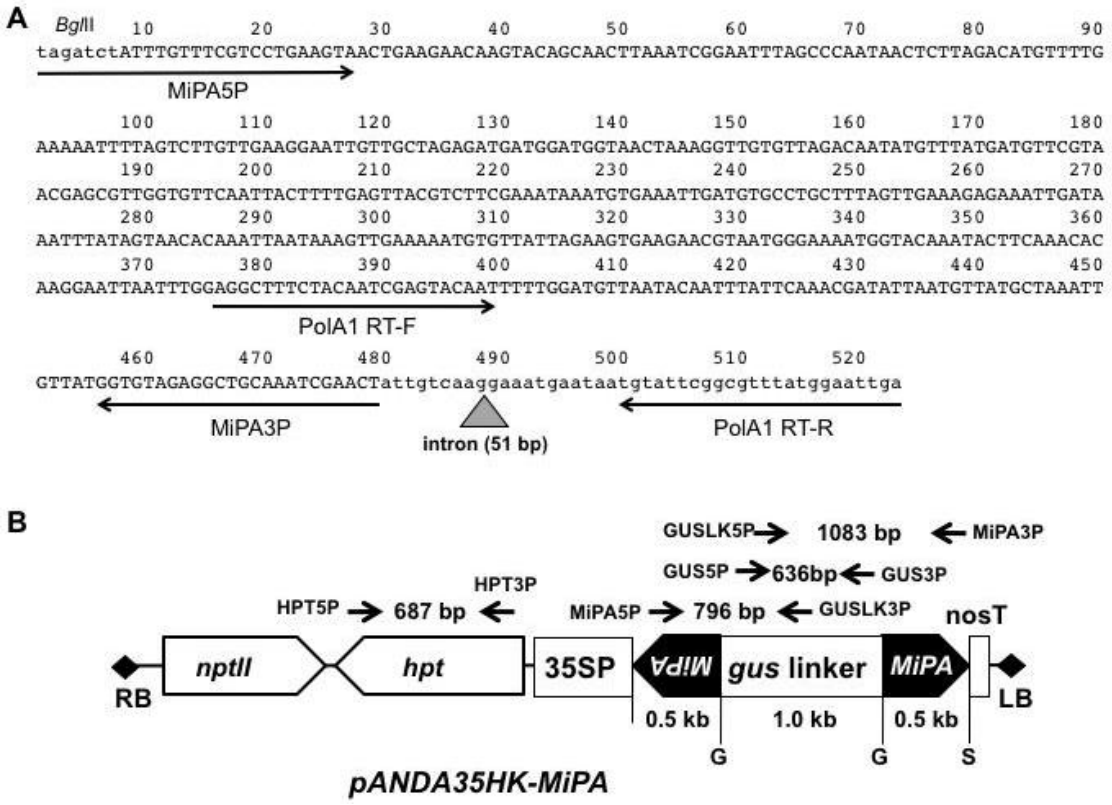


Figure 2-1. *MiPA* target sequence and pANDA35HK RNAi binary vector. A *MiPA* target sequence (472 bp, upper case) of nematode *PolA1* gene was amplified using a pair of primer MiPA5P and MiPA3P. Primers PolA1 RT-F and PolA1 RT-R used in Figure 2-10a were located on *MiPA* cDNA sequence and downstream genomic sequence (lower case) of *PolA1* gene, respectively. *MiPA* target sequence and its border sequence of *Meloidogyne incognita* was identified from accession no. CABB01001461 of Genbank database. **B** *MiPA* 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.

pANDA35HK::*MiPA* vector was transformed into DH5α *E. coli* cells using the freeze thaw method. Recombinant *E. coli* were selected on LB plates containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} hygromycin. pANDA35HK::*MiPA* plasmids from transformed *E. coli* were checked by PCR and restriction analyses (Figure 2-2) to confirm *MiPA* integration in both sense and anti-sense orientations. Positive colonies were selected and purified by streaking on LB medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} hygromycin. pANDA35HK::*MiPA* plasmids were extracted from selected *E. coli* cells grown overnight in liquid LB medium containing 50 mg l^{-1} kanamycin and finally mobilized into *Agrobacterium tumefaciens* strain EHA 105 using the freeze-thaw method. Polymerase chain reaction was used to confirm agro colonies carrying correct inserts.

***Agrobacterium*-mediated transformation of tobacco**

Tobacco (*Nicotiana tabacum* 'Petit Havana') was transformed using the *Agrobacterium*-mediated leaf disc method (Kong et al. 2014). Healthy leaves were excised from one-month-old *in vitro* grown tobacco plants and sectioned into 5-7 mm^2 leaf discs. Leaf explants were then immersed completely into *Agrobacterium tumefaciens* (EHA 105) infection suspension (1:10 ratio overnight grown agrobacterium suspension and MS basal medium) for 1 minute with gentle agitation. Infected leaf explants were subsequently blotted on sterile laboratory wipe to remove excess agrobacterium suspension before being co-cultivated on MS medium supplemented with 3% sucrose and 0.8% agar for 3 days in the dark with the adaxial leaf surface upwards.

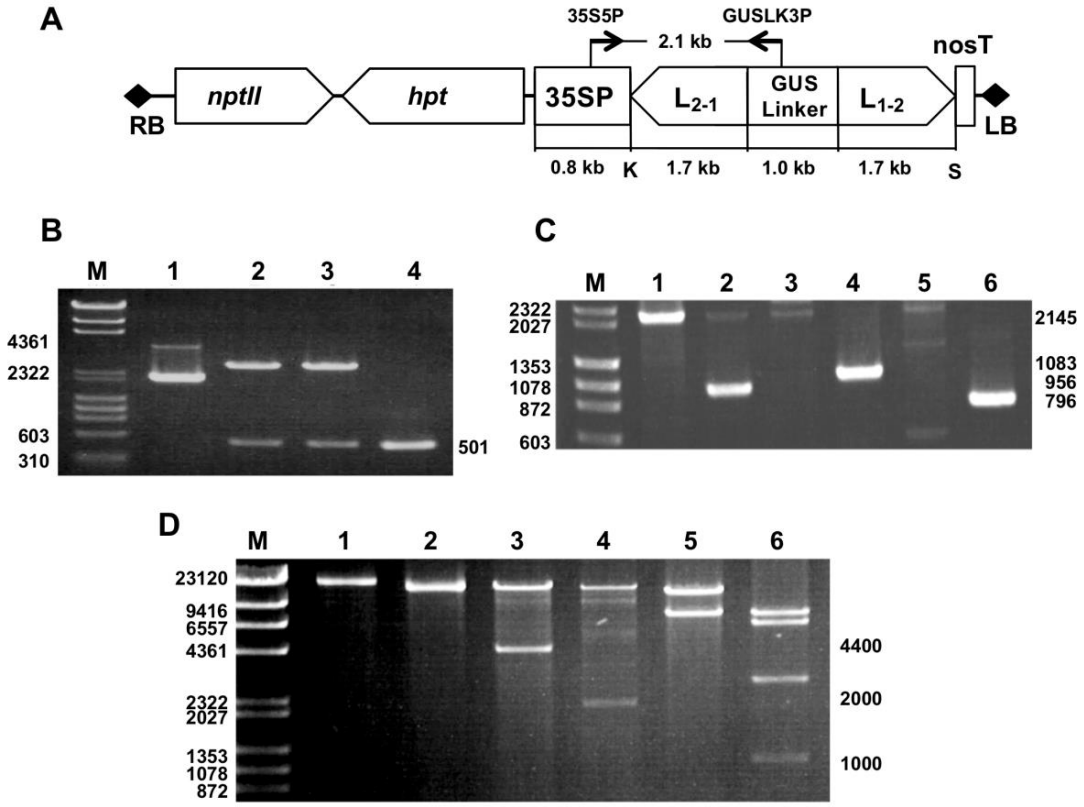


Figure 2-2. Molecular analyses of entry and RNAi binary vectors after cloning. a: Schematic representation of pANDA35HK binary vector. b: *Eco*RI cut of pCR8::*MiPA* entry vector yields 2.8 kb vector backbone and 501 bp *MiPA* target sequence (lanes 2 and 3). Lane 4 is PCR amplicon of *MiPA* target with *MiPA* specific primers. Lane 1 is uncut pCR 8::*MiPA* 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::*MiPA* 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::*MiPA* using the primer pairs: GUSLK5P and *MiPA* 3P primer pair (sense orientation). Lanes 5 and 6 are the expected no band and 0.8 kb amplicon from *pANDA35HK* and pANDA35HK::*MiPA* using the primer pairs: *MiPA* 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::*MiPA*, respectively. Lanes 3 and 4 are (*Sac*I + *Kpn*I)-digested pANDA35HK and pANDA35HK::*MiPA* 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::*MiPA* 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.

Agrobacterium elimination, tissue differentiation and selection of transgenic cells were achieved in one step by sub-culturing the explants from co-cultivation medium to MS basal medium supplemented with 3% sucrose, 0.1 mg l^{-1} NAA, 1.0 mg l^{-1} BA, 40 mg l^{-1} hygromycin, 20 mg l^{-1} meropenem and solidified with 0.8% agar. The explants were sub-cultured on fresh selection medium every two weeks until multiple shoots were formed. Vigorous resistant shoots of at least 2 cm were excised and transferred to rooting medium containing half strength MS medium supplemented with 40 mg l^{-1} hygromycin, 20 mg l^{-1} meropenem and solidified with 0.8% agar but without growth regulators.

Genomic PCR analysis of primary transgenic (T_0) tobacco

Genomic DNA was extracted from 100 mg young leaves of putative transgenic and wild type tobacco plants using Sodium Dodecyl Sulfate (SDS) method (Ahmed et al. 2009). Using different sets of primers (Table 1), regions of the binary vector corresponding to sense and anti-sense orientations of *MiPA* sequence in the plants' genomes (Figure 2-1) were targeted and amplified with Taq polymerase. A region corresponding to the hygromycin marker gene was also amplified. Amplification of these regions was also carried out using the binary vector as a positive control. PCR cocktail was constituted to a total volume of 25 µl as follows: Distilled water (18.3 µl), 10x Ex Taq Buffer (2.5 µl), dNTP mix (2.0 µl), forward primer (0.5 µl), reverse primer (0.5 µl), genomic DNA template (1.0 µl containing 100 ng), Taq polymerase enzyme (0.2 µl).

Southern blot analysis of T_0 tobacco

Genomic DNA was extracted from selected PCR positive transgenic tobacco lines using a cetyl trimethyl ammonium bromide (CTAB) protocol (Rogers and Bendichl 1985). The extracted genomic DNA (15 µg) of both transgenic and WT plants were digested overnight at 37ºC with *Sac*I enzyme (TAKARA) which makes a single cut within the T-DNA. Plasmid DNA was also digested with *Sac*I and used as a positive control. Post-digestion DNA fragments were separated on 0.7% agarose gel at 50V for 4 hours, transferred to a nylon membrane (Immobilon R-Ny$^+$; Millipore Corporation, USA) overnight by capillary transfer method and fixed using a UV transilluminator for 3 min. A 472 bp *MiPA* 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 (Roche Diagnostics, Mannheim, Germany) instructions.

Expression of *MiPA* dsRNA in T_0 tobacco

Reverse transcription PCR (RT-PCR) was used to confirm expression of *MiPA* 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) and treated with RNase-free recombinant DNase I enzyme (TAKARA) to eliminate any contaminating genomic DNA. One microgram (1 µg) of the purified RNA was then used as template for the synthesis of first strand cDNA using Superscript III First-Strand cDNA Synthesis Kit (Invitrogen). Negative control reactions were also set up which contained all the reagents minus reverse transcriptase enzyme to check for genomic DNA contamination. 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 2-1). We also amplified elongation factor 1α (*EF1α*) using primers EF1a5P and EF1a3P (Table 1) as an internal control.

Northern blot analysis for detection of *MiPA* siRNA

Small RNAs (< 200 bp) were extracted 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 10V/400mA. Northern hybridization was done using DIG-labelled *MiPA* RNA probe (472 bp) obtained via *in vitro* transcription of the *MiPA* target sequence using T7 RNA polymerase according to DIG Northern Starter Kit Version 10 (Roche). Pre-hybridization (30 min) and hybridization (overnight) were performed at 50ºC. 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.

Root-knot nematode culture and preparation of inoculum

A pure culture of root-knot nematode (*Melodoigyne incognita*) race 2 was maintained on a highly susceptible tomato cultivar '*Kyoryoku-beiju*' in a glass house for 2 months to enable gall formation. Nematode egg suspension used as infection inoculum was prepared by extraction from freshly uprooted galled roots. Infected roots were washed, dried with paper towel, chopped into 1-2 cm segments and macerated in an electric blender for 5 mins at full speed. The suspension was subsequently filtered twice through three sieves – 250 µm, 176

µm and 25 µm and nematode eggs were recovered on the 25 µm sieve. Recovered eggs were washed briefly with sterile water and reconstituted into suspension. One ml egg suspension was then placed on a counting slide and the average number of nematode eggs determined under a microscope.

Acclimatization of T_0 tobacco and infection 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. The rooted plants were acclimatized on sterile vermiculite (Appendix 1a) following the protocol of Cruz-Mendivil et al. (2011). The plants were gently pulled out of culture vessels and their roots freed from agar by gentle washing. They were subsequently transferred to pots containing sterile vermiculite and covered with transparent polythene material to maintain a high humid environment. Covered pots were kept under controlled conditions in the growth room (25ºC, 65% relative humidity, 16:8 hour photoperiod, 40 µmol m^{-2} s^{-1} irradiance). Nutrients were supplied to the plants through ferti-irrigation with 20 ml of sterile half strength MS medium every 3 days for 14 days. The polythene covers were perforated every two days and completely removed at the end of the acclimatization period.

The plants were then transferred to pots containing 3000 ml of sterile commercial garden soil and allowed further acclimatization in the glasshouse for 12 days in readiness for nematode infection (Appendix 1b). Each acclimatized transgenic and WT plants was 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 conducted twice.

Evaluation of *M. incognita* parasitic parameters

Seven weeks post-nematode infection (Appendix 2), transgenic and wild type plants were sampled and analyzed for nematode parasitic success and key agronomic characters. Nematode parasitic parameters evaluated include;

i. Galling index (%) – This was scored on the basis of 11-point scaling system according to Bridge and Page (1980), and calculated as a percentage using the formula of Zech (1971). Galling index = (10A + 9B + 8C + 7D + 6E + 5F + 4G + 3H + 2I + J) / 10N × 100

ii. Number of *M. incognita* eggs per egg mass - Roots were first immersed in a solution of 15 mg/L Phloxine B stain for 20 minutes to increase visibility of the egg masses. Ten egg masses were manually isolated at random from each root system using fine tweezers and shaken vigorously in a solution of 1% sodium hypochlorite (NaOCl) for 3 minutes to release individual eggs from the egg masses. The egg suspension was then sieved through 76 µm and 25 µm sieve, rinsed under gentle flowing tap water before collecting the released eggs in 50 ml sterile distilled water. Egg count was done by counting 1 ml of agitated egg suspension under light microscope (10x). Each count was done twice for each sample and average taken to represent the sample. Mean number of eggs per egg mass was then calculated for all samples.

iii. Estimation of *M. incognita* juvenile population in soil – Real time PCR was used to quantify the amount of root knot nematode juveniles by measuring nematode DNA amount per gram soil using SYBR Green technology. Soil samples in which transgenic and wild type plants were grown were homogenized and bulked separately. Genomic DNA was extracted from 3

replicate soil samples from each lot using ISOIL for Beads Beating Soil DNA extraction kit (Nippon Gene). Genomic DNA concentration was adjusted to 2.8 ng/µl in all samples and 2 µl was used as template in 20 µl qPCR cocktail containing 10 µl KOD SYBR qPCR 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) plotted against the C_t (threshold) value for that concentration (y-axis) was generated. Absolute quantification of *M. incognita* genomic DNA concentration in all soil samples was done by comparison with the standard curve.

Key agronomic characters evaluated include leaf and root fresh and dry weights, plant height and girth, percentage of healthy and chlorotic leaves. All dry biomass weight was measured by drying leaves and roots to a constant weight in an oven at 60ºC.

Generation of T_1 tobacco plants and nematode infection

T_0 tobacco plants (T1 and T7) were transferred to the green house and allowed to produce T_0 seeds. T_0 seeds of lines T1 and T7 were surface sterilized and germinated in half strength MS medium supplemented with 1% sucrose and 100 mg/L kanamycin to generate their T_1 progeny plants. PCR amplification was used to confirm the presence of *MiPA* sense and anti-sense regions as well as hygromycin gene in the genomes of the T_1 plants. 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 (Appendix 3) 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 egg mass and nematode multiplication. Nematode galls were counted by root inspection with the aid of magnifying lens. Nematode multiplication was evaluated by multiplying the number of egg masses per root by number of eggs per egg mass and dividing by initial amount of egg inoculum used for infection. Infection experiment was replicated twice.

Expression of *PolA1* gene in adult feeding female nematodes

Adult female nematodes feeding on roots of WT and T_1 transgenic tobacco plants were extracted under a stereo microscope (Olympus SZX9) and stored in -80ºC following 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 *PolA1* gene (Figure 2-10) using SYBR Green technology in StepOnePlus™ thermal cycler (Applied Biosystems).

PCR cocktail for each reaction was prepared 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 98ºC for 2 min, followed by 40 cycles of 98ºC for 10s, 55ºC for 10s and 68ºC for 30s in a 96-well µltra Amp PCR plate (Sorenson Bioscience). We assessed specificity of the amplification by melt curve analysis at 60-95ºC 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 *PolA1* expression in the nematodes using the $2^{-\Delta\Delta CT}$ method. Root knot nematode elongation factor was used as the internal reference gene. *PolA1* transcript abundance in nematodes extracted from transgenic roots as expressed as a percentage relative to the transcript level in nematodes extracted from wild type plants.

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).

Results

Genomic PCR analyses of T_0 transgenic tobacco

Following *Agrobacterium* transformation, selection and regeneration of tobacco plantlets, 13 primary transgenic lines were generated and genotyped. Two sets of primers GUSLK-5P/MiPA-3P and MiPA-3P/GUSLK-3P (Figure 2-1) were used to amplify 1083 bp and 796 bp genomic regions corresponding to the sense and anti-sense orientations of *MiPA* target. A 687 bp genomic region corresponding to hygromycin marker gene (*hpt*) was also amplified using primers HPT-5P and HPT-3P. Sense, anti-sense and *hpt* amplicons for 13 transgenic lines are shown in Figure 2-3a. Plasmid DNA was amplified as a positive control while untransformed plants (WT) showed no amplification.

Southern analysis of T_0 transgenic tobacco

Nine PCR-positive transgenic lines were subjected to Southern hybridization to analyze their *MiPA* T-DNA integration patterns. Using probes specific to *MiPA* target, single, double and triple copy T-DNA insertions were observed with the different lines (Figure 2-3b). Wild type plants showed no hybridization signal with the probe.

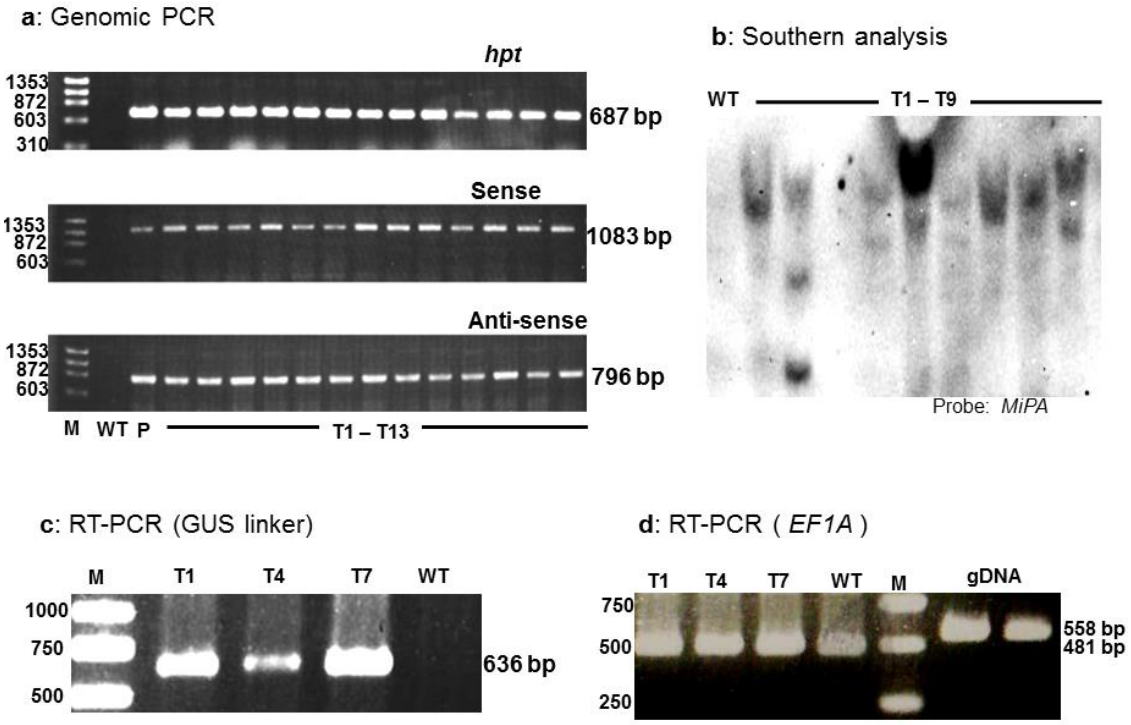


Figure 2-3. Molecular analyses of T_0 transgenic tobacco. **A** PCR amplification of *hpt* marker gene, sense orientation and anti-sense orientation of nematode *MiPA* target fragment. Plasmid control (P) showed similar band size while the wild type plants (WT) showed absence of target amplicons. M: ΦX174 *Hae*III digest marker. **B** Southern blot analysis. Probe used for hybridization was specific to *MiPA* 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 *PolA1* hairpin RNA. **D** RT-PCR of elongation factor (*EF1a*), 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 2,000 DNA Marker (TAKARA).

Expression of *MiPA* dsRNA in T_0 tobacco

Complementary DNA (cDNA) derived from selected T_0 plants (T1, T4 and T7) were used in a reverse-transcription PCR (RT-PCR) analysis to confirm expression of *MiPA* dsRNA in the transgenic lines. Using primers GUS5P and GUS3P (Figure 2-1), a 636 bp fragment corresponding to the GUS linker region between sense and anti-sense orientations of the *MiPA* target sequence in the selected lines was amplified (Figure 2-3c). Wild type plants showed no amplification for the GUS linker region. Elongation factor (*EF1a*) used as internal control reference gene was amplified using primers EF1a5P and EF1a3P (Table 1). Transgenic and wild type plants both showed a 481bp amplicon of *EF1a*. Two genomic DNA controls containing a 77 bp intron were also amplified with the same primers and obtained a higher 558bp amplicon (Figure 2-3d).

Northern blot analysis for *MiPA* siRNA in T_0 tobacco

DIG-labelled *MiPA* RNA probe hybridized to sequence-specific siRNAs in transgenic plants that expressed *MiPA* dsRNA. No hybridization signal was detected with WT plants. Ethidium-bromide staining of the polyacrylamide gel after electrophoresis showed equivalent loading of small RNAs across samples (Figure 2-4).

Bio-efficacy of T_0 transgenic tobacco against *M. incognita*

An inspection of the test plants' roots showed wild type plants with reduced necrotic root system and having larger multiple fused galls compared to the transgenic lines (Figure 2-5a). 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 2-5b).

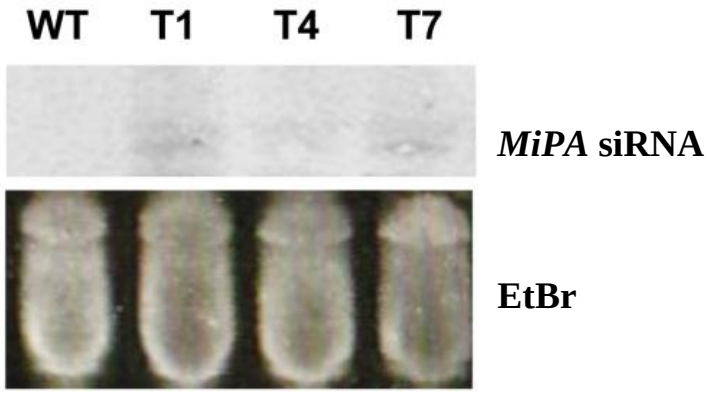


Figure 2-4. Northern analysis for detection of *MiPA* siRNAs. *MiPA* siRNA was detected with DIG-labelled *MiPA* RNA probe in the transgenic lines. WT plants showed no hybridization signal. EtBr shows equivalent loading of small RNAs across samples

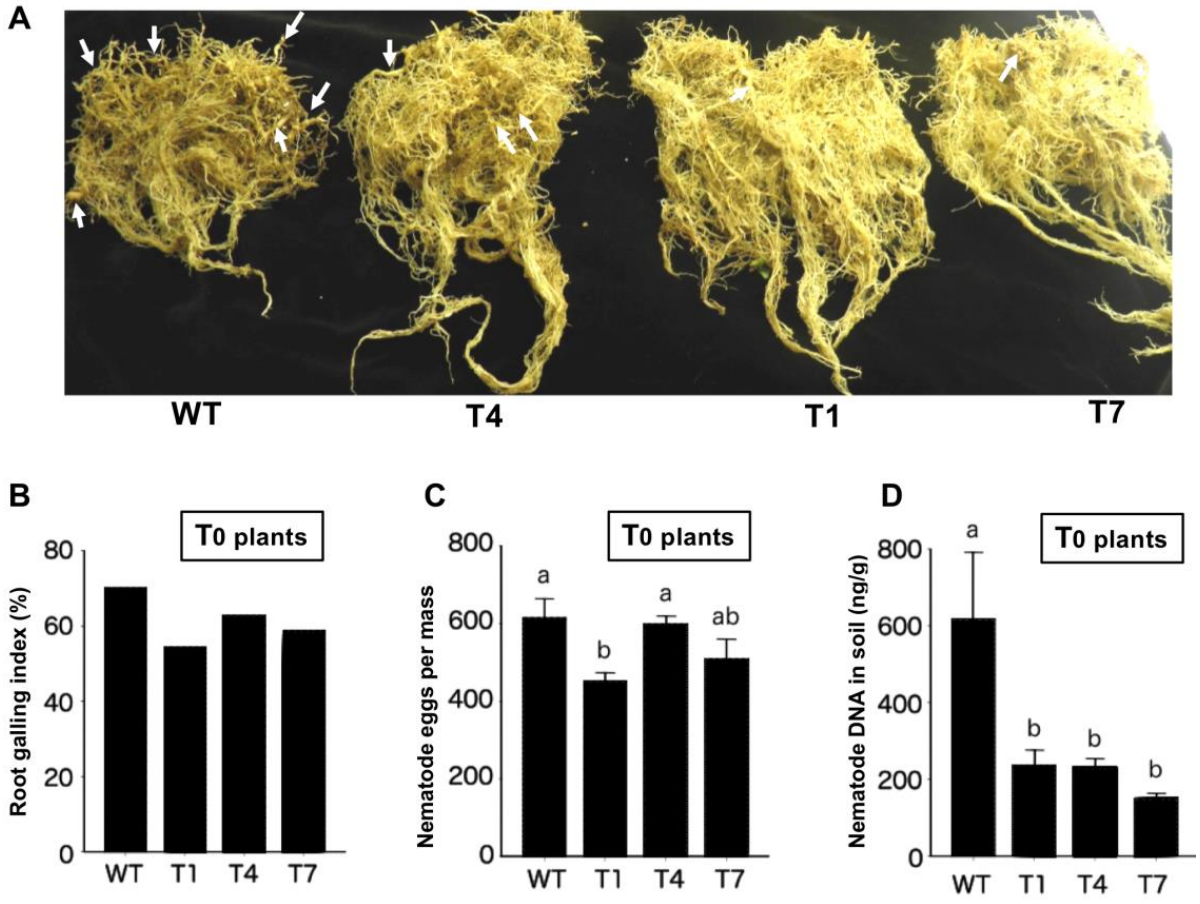


Figure 2-5. Nematode infection analyses in T_0 transgenic tobacco. **A**: WT roots showed more damage (necrosis) and reduced length compared to transgenic lines' roots. Arrows show some gall positions **B**: Root galling indices were generally reduced in transgenic lines compared to WT. **C**: Nematode fecundity, measured by number of eggs per egg mass, was significantly reduced ($p<0.05$) in some 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.

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 2-5c). 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 2-5d). 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 2-6a). 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 2-6b). After 7 weeks of nematode infection, 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 2-6c). 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 2-6d).

Disease condition was more outstanding on WT leaves compared to transgenic plants (Figure 2-7a). 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 2-7b). 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.

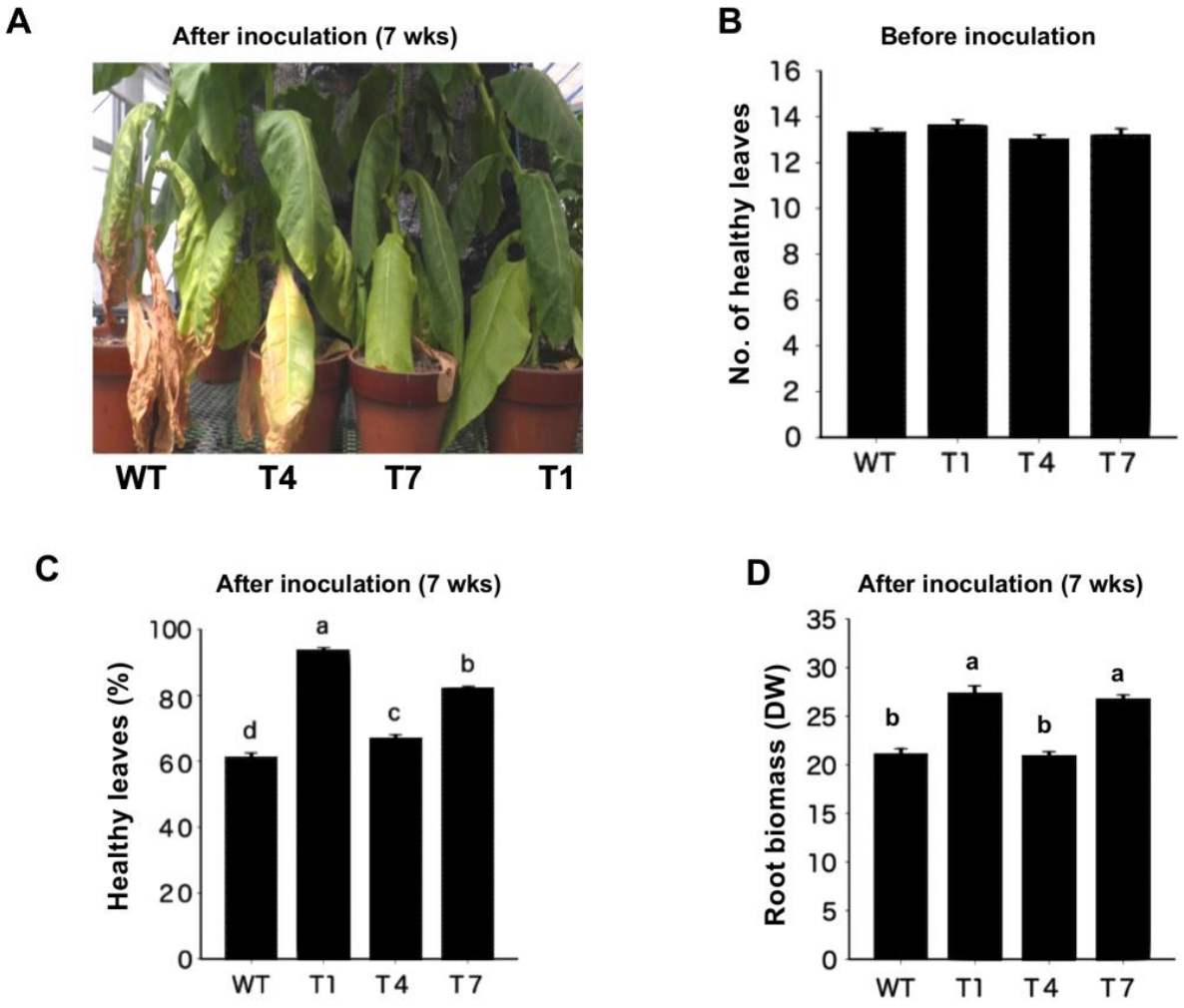


Figure 2-6. Agronomic assessment of T_0 tobacco after 7 weeks of nematode infection. **A** Transgenic lines were comparatively more vigorous than WT. **B** Number of healthy leaves before infection with nematodes was comparable ($p > 0.05$) in all plants. **C** Transgenic lines had significantly higher ($p < 0.05$) percentage of green standing leaves after infection **D** Root biomass was significantly higher ($p<0.05$) in transgenic lines than WT.

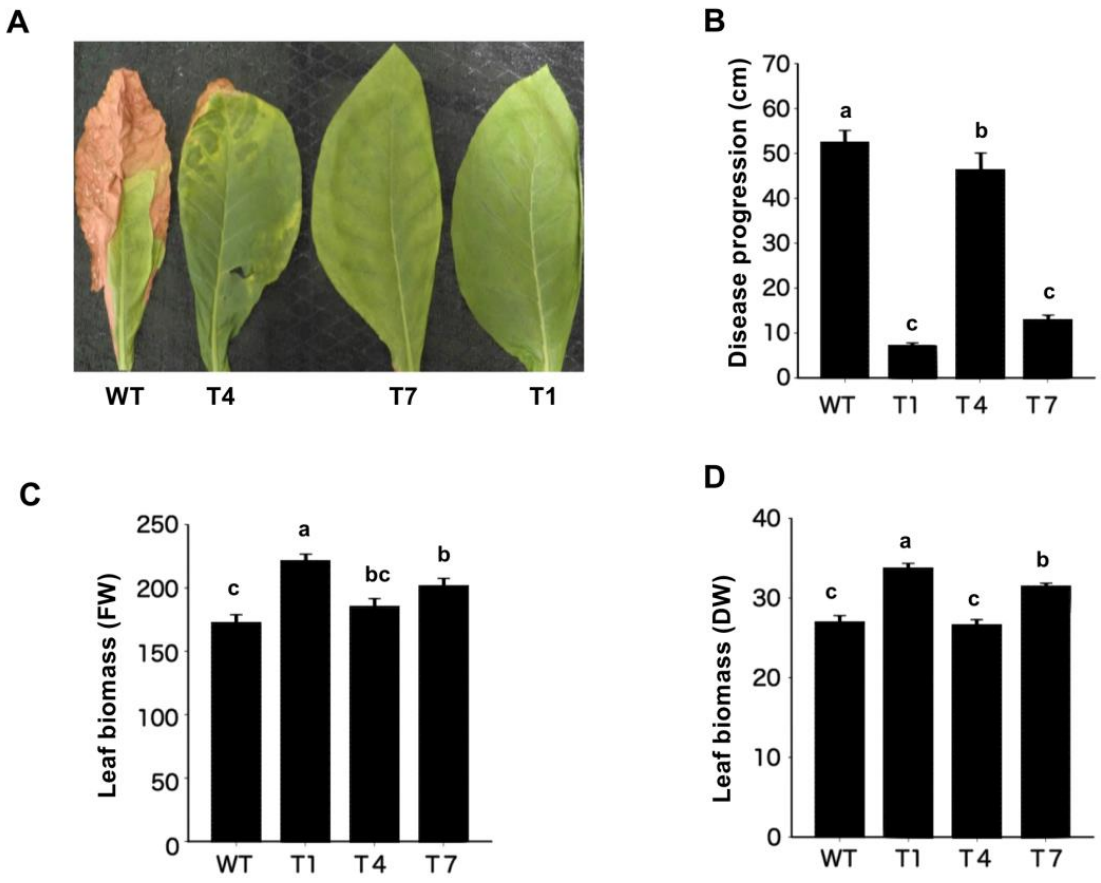


Figure 2-7. Disease progression in T_0 tobacco lines after 7 weeks of nematode infection. **A** WT leaves showed severe leaf disease conditions compared to those of transgenic lines. **B** Disease progression measured as distance from base of each plant to the last diseased leaf was significantly higher ($p<0.05$) 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.

Compared to T4, WT plants showed 6.9% reduction in fresh leaf biomass (Figure 2-7c). 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 2-7d).

Molecular characterization of T_1 tobacco lines

Genomic DNA was extracted from 100 mg young leaves of T_1 tobacco progeny plants generated from transgenic lines T1 and T7. Transgenic line T4 was excluded from further analyses owing to its comparatively weaker T_0 phenotype. PCR amplification was carried out with same primer sets used with T_0 plants. Correct amplicons for hygromycin gene (687 bp) as well as *MiPA* target in both sense (1083 bp) and anti-sense orientations (796 bp) were obtained with the transgenic lines. Wild type plants showed no amplification signal (Figure 2-8a).

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 2-8b). 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 2-8c). The transgenic plants had significantly reduced ($p < 0.05$) number of nematode eggs per mass. Compared to WT plants, nematode eggs per mass on T1 and T7 were reduced by 20.9 and 17.7% respectively (Figure 2-8d). 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 2-8e).

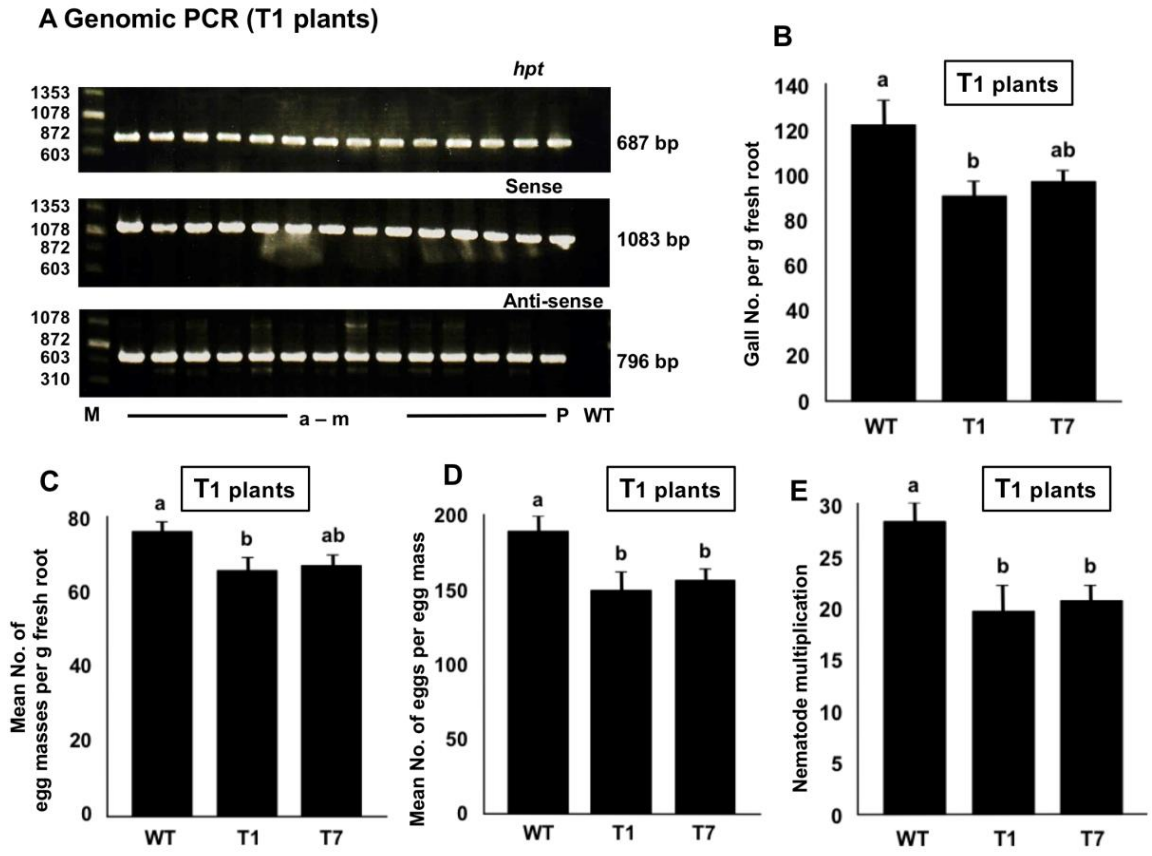


Figure 2-8. Molecular and nematode infection analyses in T_1 transgenic tobacco. **A** PCR amplification of *MiPA* sequence in sense and anti-sense orientations as well as *hpt* gene in genome of T_1 tobacco plants showed correct 1083 bp, 796 bp and 687 bp amplicons respectively. **B** Gall number per 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 replicated and similar results were obtained.

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 (Figure 2-9).

Relative expression of *PolA1* in adult feeding female nematodes

We used qRT-PCR analyses to compare *PolA1* 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, *PolA1* expression was significantly reduced ($p < 0.05$) by 34.3 and 31.5% respectively in nematodes feeding on transgenic lines T1 and T7 (Figure 2-10b). Analysis of melt curves for reference and *PolA1* after qRT-PCR showed single peaks that indicate specific target amplification.

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 (Banerjee et al. 2017). In this study, we targeted *M. incognita PolA1* 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::*MiPA* plant expression vector to integrate *MiPA* target sequence in sense and anti-sense orientations (Figure 2-1) such that upon expression, would produce *MiPA* dsRNA.

We confirmed successful transformation of tobacco with this silencing construct via PCR and Southern blot analyses. We also showed evidence of expression of *MiPA* dsRNA via RT-PCR amplification of the GUS linker between the target sequences in the transgenic lines (Figure 2-3). Production of dsRNAs triggers eukaryotic cellular RNA silencing machinery (Mlotshwa et al. 2008) thus, *MiPA* dsRNAs can activate RNAi events in the transgenic tobacco. Detection of *MiPA* siRNAs in the transformed plants (Figure 2-4) is a confirmation that *MiPA* dsRNAs produced by the transgenic plants were processed by dicer into siRNAs. *In planta*-produced siRNAs act as mediators of gene silencing via facilitation of the degradation of complementary genes' transcripts in invading parasites (Dutta et al. 2015; Niu et al. 2012)

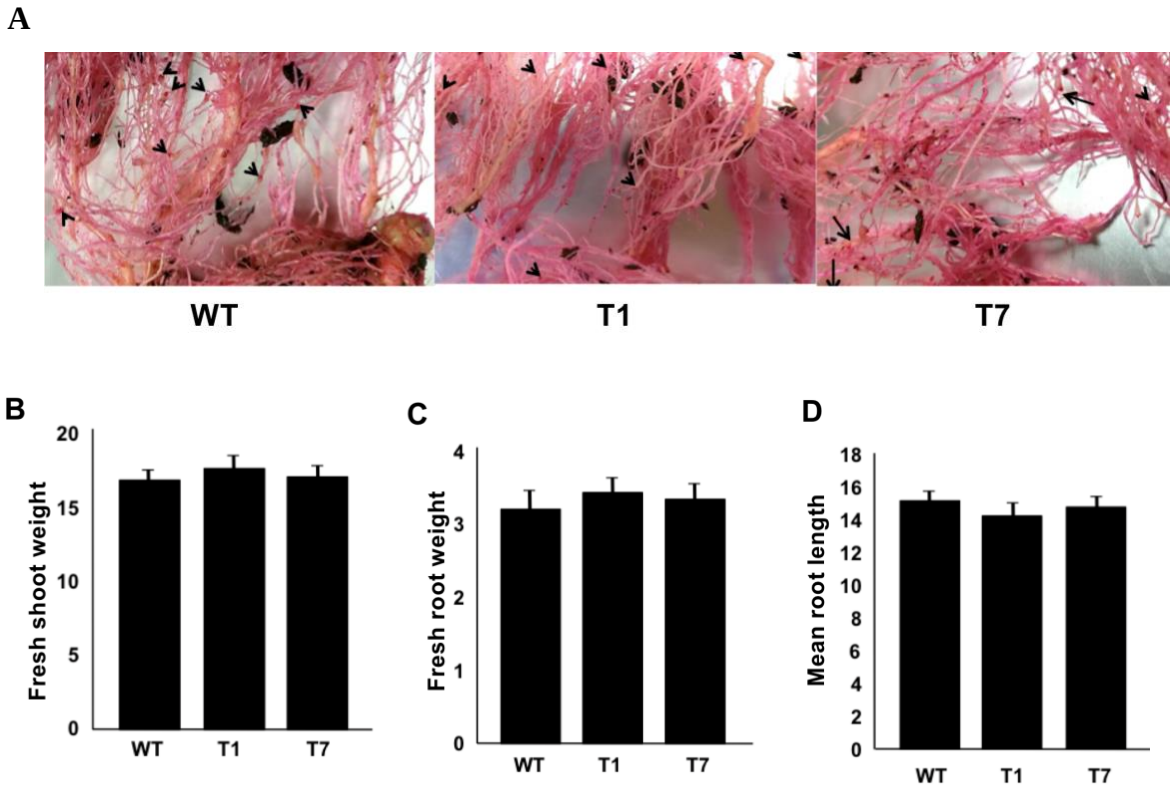


Figure 2-9: Egg mass staining and agronomic characters of T_1 tobacco after nematode infection. A Phloxine B-stained roots of transgenic and WT tobacco plants showing pink egg masses. WT roots had increased number of nematode egg masses (pink arrows) compared to transgenic tobacco roots. Transgenic and 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

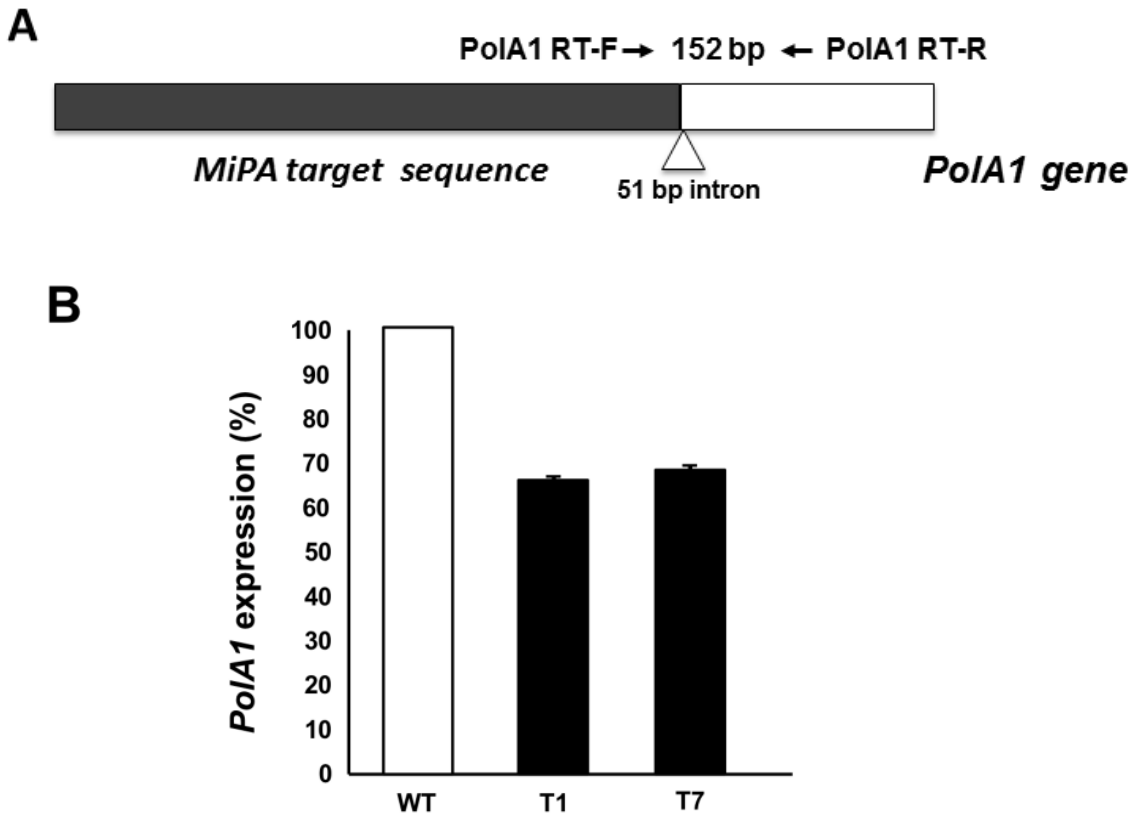


Figure 2-10. ***PolA1*** **transcript expression in adult nematodes feeding on WT and transgenic plants' roots. A** Short fragment (152 bp) of *PolA1* transcript was amplified by RT-PCR using primers PolA1 RT-F and PolA1 RT-R, shown in Figure 2-1. **B** Reduced *Pol*A1 transcript expression in adult 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%).

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 (Figures 2-4, 2-8) is an evidence of superior root reaction to nematode infection among the transgenic lines, which according to Coyne and Ross (2014) depicts a relative indication of resistance. Our T_0 and T_1 transgenic tobacco showed reduced nematode egg masses, and eggs per egg mass compared to wild type plants (Figures 2-4, 2-8). 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 2-4) as well as reduced parasite multiplication on T_1 plants (Figure 2-8) are complementary data that provide evidence of restrained parasite propagation. This is proof of some resistance in plants expressing nematode *MiPA* dsRNA. Suppressed nematode reproduction and development is a phenotype that is consistent with previous successful HIGS studies using different target genes like *MSP* (Steeves et al. 2008; Li et al. 2010; Antonio de Souza Junior et al. 2013; Lourenco-Tessutti et al. 2015).

All T_0 transgenic lines except T4 showed superiority in agronomic vigour and susceptibility to disease conditions compared to WT plants (Figures 2-5 and 2-6). 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 (Mitkowski and Abawi 2003; Rich and Kinloch 2005). The weaker phenotype of T4 lines may have to do with reduced amount of processed *MiPA* siRNAs (Figure 2-4), 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 (Papolu et al. 2013). Reduced silencing efficiency due to relatively low siRNA production may be attributable in part to inefficient processing of *PolA1* dsRNA to siRNAs by dicer enzymes. Moderate silencing of *NtFAD3* gene in tobacco was attributed to inefficient dsRNA processing by dicer (Hirai et al. 2007).

T_1 tobacco plants, however, were comparable to WT in agronomic traits evaluated after 35 days of nematode infection (Figure 4). 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 (Coyne and Ross 2014). 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 might have been 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 *MiPA* transcripts relative to those on WT (Figure 2-10). Down-regulation of target gene transcripts in parasites has been characterized as an indicator of effective host induced RNAi in transgenic plants (Papolu et al. 2013). Effective silencing of *PolA1* gene in the feeding nematodes provides indirect 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 *PolA1* plays a crucial reproductive and developmental function in these pests.

According to Ghang (2017), a good candidate gene for HIGS to achieve durable resistance must be one for which the host pest or pathogen cannot risk its mutation. *PolA1* satisfies this requirement due to its single copy existence and essentiality in eukaryotic survival. Hence, although some previously evaluated candidate genes (Xue et al. 2013; Dutta et al. 2015; Shivakumara et al. 2017) showed greater potency for nematode control than was obtained in this study with *PolA1*, the potential durability of *PolA1*-conferred resistance will be a crucial benefit for crop production. The high homology of our target sequence among *Meloidogyne* species (Appendix 4) is an additional benefit that can extend durable resistance across all root-knot nematode species in the *Meloidogyne* genus.

Chapter III: Expression of *Meloidogyne incognita PolA1* hairpin RNA reduced nematode multiplication in transgenic tomato

Introduction

Engineering resistance in plants through biotechnology is currently gaining acceptance as an effective strategy for the control of root knot nematodes, *Meloidogyne spp.* (Dutta et al. 2015). Host induced gene silencing (HIGS) is one of such strategies in which susceptible host plants are transformed with an RNAi construct that produces hairpin or double stranded RNA (dsRNA) of a target nematode gene after transcription (Hirai et al. 2007). The expressed dsRNA are then used as trigger to activate a network of highly related pathways that repress gene expression in eukaryotes using small regulatory RNAs (Vaucheret 2006). Typically, the dsRNA is rapidly processed into 21- 24 bp RNA duplexes called siRNAs which then combine with RNA-induced silencing complex (RISC) and guide the sequence-specific recognition and degradation of complementary mRNAs (Sen and Blau 2006).

Nematodes attacking such engineered plants can ingest either processed siRNAs or dsRNA during feeding and activate the parasites' cellular RNAi machinery which represses expression of targeted genes. When the repressed gene is essential for survival, lethal effects may be induced in the nematodes which in turn confer protection on the expressing host plant against invading nematodes (Yadav et al. 2006; Huang et al. 2006). Successful utilization of this strategy to control root knot nematodes has the selection of appropriate target nematode gene as a key consideration (Ajjappala et al. 2012). Dutta et al. 2015 reviewed the RNAi efficacy of a number of nematode genes including housekeeping, development and parasitism.

Root-knot nematodes are known to parasitize a wide range of food and commercial crops causing severe economic implications due to significant yield losses (Bird and Kaloshain 2003). Control of these parasites through HIGS promises a multi-beneficial, less

risky alternative for achieving durable, broad-spectrum resistance than conventional chemicals use (Thomas and Cottage 2006). Different authors, including Shivakumara et al. (2017) and Chaudhary et al. (2019), have reported nematode resistance in plants expressing dsRNA of the various parasitism-related and house-keeping nematode genes targeted. However, the need to identify more effective nematode genes whose repression through silencing can mitigate parasite propagation on susceptible plants remains crucial.

Meloidogyne incognita PolA1 is a single-copy nuclear gene that encodes the largest subunit of the multi-subunit RNA polymerase I holoenzyme complex responsible for the synthesis of ribosomal RNA precursor. The *PolA1* gene contains a sequence specific to each eukaryotic organism (Nakamura 2010). Given the importance of this gene to eukaryotic survival and propagation, it will be a potentially effective target for host induced silencing to confer protection on plants against nematodes without the laborious and time-consuming survey of specific target genes for siRNA silencing. We considered that species-specific sequence (*MiPA*) of *Meloidogyne incognita PolA1* gene would be an effective target of siRNA without off-target effect on the host plant. This study was designed therefore to evaluate the suitability of this gene for HIGS by assessing root knot nematode resistance in transgenic tomato expressing double stranded *MiPA* RNA of nematode *PolA1* gene.

Materials and methods

Identification of target sequence for RNAi

A species-specific amino acid (MiPTAG) sequence in the C-terminal region of RNA polymerase I largest subunit (POLA1) of *Meloidogyne incognita* was earlier identified (Nakamura 2010). As shown in Appendix 5, MiPTAG sequence was used as query to identify a 472 bp *MiPA*-specific coding sequence of nematode *PolA1* gene as target from the genomic sequence CABB01001461 (NCBI). This target sequence did not show perfect match of more than 20 bp stretch to whole genomic sequence of tomato through blast server of NCBI web page (data not shown).

Cloning of *MiPA* target sequence

MiPA sequence was synthesized and cloned into the multiple cloning site of pUC57 vector. *MiPA* target sequence was then PCR-amplified using MiPA-5P and MiPA-3P primers (Table 1), and inserted into the entry vector, pCR8, by TA cloning. A unique *Bgl*II restriction site was added to the 5'-end of MiPA-5P (Table 1) to aid in downstream vector analyses. *MiPA* target on pCR8 was inserted into pANDA35HK RNAi binary vector (Figure 3-1) using Gateway LR Clonase II enzyme mix (Thermo Fisher Scientific). pANDA35HK::*MiPA* plasmids from transformed *E. coli* was checked by PCR and restriction analyses to confirm *MiPA* integration in both sense and anti-sense orientations. pANDA35HK::*MiPA* was then transformed into *Agrobacterium tumefaciens* by freeze-thaw method.

Table 1

List of oligonucleotide primers used for cloning, PCR amplification, probe labelling and qRT-PCR

Gene	Primer name	Primer sequence (5′ – 3′)	Product length (bp)
MiPA	MiPA5P MiPA3P	*tagatct*ATTTGTTTCGTCCTGAAGTA AGTTCGATTTGCAGCCTCTACACC	472
HPT	HPT5P HPT3P	GTGTCACGTTGCAAGACCTG CGAGTACTTCTACACAGCCA	687
MiPA-Sense	GUSL5P MiPA3P	TGATAGCGCGTGACAAAAACCACCCAAG AGTTCGATTTGCAGCCTCTACACC	1083
MiPA-Antisense	MiPA3P GUSL3P	AGTTCGATTTGCAGCCTCTACACC AAGGCCGACAGCAGCAGTTTCATCAATCA	796
GUS	GUS5P GUS3P	CATGAAGATGCGGACTTACG ATCCACGCCGTATTCGG	636
EF1A	EF1A5P EF1A3P	ACTGTGCTGTCCTGATTATTGACT GGACCAAAAGTAACAACCATACCA	471
35SP-GUSL	35SP GUSL3P	GATGTGATATCTCCACTGAC AAGGCCGACAGCAGCAGTTTCATCAATCA	956
MiPA1-RT-PCR	MiPA1F MiPA1R	AATTGATGTGCCTGCTTTAGTTGA TTTCCCATTACGTTCTTCACTTCT	105
MiPA2-RT-PCR	MiPA2F MiPA2R	AGGCTTTCTACAATCGAGTACAAT TCAATTCCATAAACGCCGAATACA	152
EF1A-RT	EF1RT-F EF1RT-R	GAAAGACTTTGTTGGAAGCCCTTG GGGAACAGTTCCAATACCTCCAAT	122

*_tagatct_ – Unique *Bgl*II restriction enzyme site incorporated into the forward primer sequence

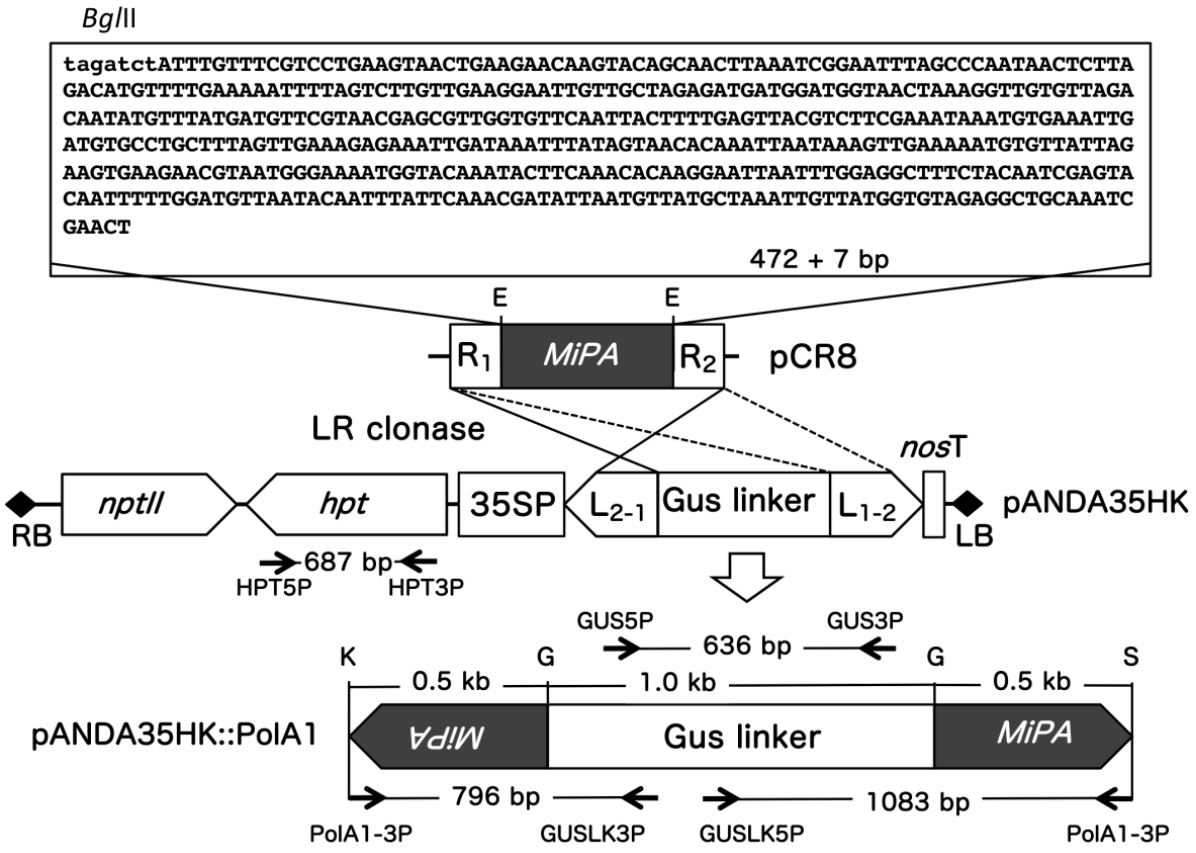


Figure 3-1. Schematic representation of RNAi binary vector containing *MiPA* target sequence. A target *MiPA* sequence (472 bp) of nematode *PolA1* gene cloned into pCR8 plasmid was transferred into pANDA35HK binary vector using LR clonase to integrate them in both sense and anti-sense orientations. *MiPA*: *Meloidogyne incognita PolA1* target sequence, 35SP: cauliflower mosaic virus 35S promoter, nosT: nopaline synthase gene terminator, *nptII*: kanamycin resistance cassette, *hpt*: hygromycin resistance cassette, RB: right border, LB: left border, arrows: primer positions

Agrobacterium transformation of tomato

Tomato (*Solanum lycopersicum* Mill. CL5915) seeds were surface sterilized by immersing in absolute ethanol for 5 seconds. This was followed by agitation in 1% sodium hypochlorite (5% available chlorine) with a drop of Tween 20 for 10 min. Seeds were rinsed 10 times in sterile distilled water and germinated on half strength MS medium containing 1% sucrose. *Agrobacterium*-mediated transformation was carried out with cotyledonary leaf explants as described by Kaur and Bansal (2010) and Kong et al. (2014). Selection was done by transferring agro-infected explants to MS medium containing 1.5 mg/L zeatin, 0.1 mg/L IAA, 50 mg/L kanamycin and 20 mg/L meropenem. Subsequent sub-cultures were carried out every two weeks on same medium until shoot formation. Shoots produced were excised and transferred to rooting medium – half strength MS containing 50 mg/L kanamycin and 20 mg/L meropenem. Escape plantlets were eliminated by repeated excision and transfer of shoots to fresh rooting media containing antibiotics.

PCR analysis of putative transgenic tomato plants

Genomic DNA was extracted from 100 mg putative transgenic young leaves using a modified Sodium Dodecyl Sulfate (SDS) method according to Ahmed et al. (2009). We amplified *MiPA* sequence in both sense and antisense orientations using the primer pairs, GUSL5P and MiPA3P, MiPA3P and GUSL3P respectively (Figure 3-1). We also amplified hygromycin selectable marker gene (HPT) using primers HPT5P and HPT3P. A list of all primer sequences used in this study is presented in Table 1.

Southern hybridization analysis

We extracted genomic DNA from PCR positive lines using the cetyl trimethyl ammonium bromide (CTAB) method according to Rogers and Bendichl (1985). 15 µg genomic DNA from both transgenic and wild type plants was digested overnight with *XhoI* (TAKARA) at 37ºC. Plasmid DNA was also digested with *XhoI* and used as positive control. Restricted DNA fragments were separated on 0.7% agarose gel at 50V for 4 hours and transferred to a nylon membrane overnight by capillary method. Fixing of DNA to membrane was done by exposure under a UV transilluminator for 3 min. *MiPA* probe (472 bp) was labelled by PCR reaction incorporating a digoxigenin-labelled nucleotide as in PCR DIG Probe Synthesis Kit (Roche) protocol. Hybridization and stringency washes were carried out following the DIG manual while detection was done by chemiluminescence using CDP-Star, according to manufacturer's instructions.

Expression analysis of *MiPA* RNAi cassette

Semi-quantitative reverse transcription PCR (RT-PCR) was used to analyze the expression of the *MiPA* RNAi cassette in transgenic plants. We extracted total RNA from 100 mg young leaves of transgenic and WT plants using the RNeasy Plant Mini Kit (Qiagen, Germany). Contaminating genomic DNA was eliminated by DNase I enzyme (TAKARA) treatment. One microgram of the purified RNA was used as template to synthesize first strand cDNA using Superscript III First-Strand cDNA Synthesis Kit (Invitrogen). Two microliters of cDNA was used as template in 50 µl total volume for PCR amplification of a fragment of the GUS linker (Figure 3-1) using primers GUS-5P and GUS-3P. Elongation factor 1α (*EF1a*) was used as an internal control gene.

Northern analysis for detection of *MiPA*-specific siRNA

We used Northern analysis to check for the production of siRNAs specific to *MiPA* target in our T_0 transgenic plants. Small RNAs (< 200 bp) were extracted from the selected 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 10V/400mA. Northern hybridization was done with a 472bp DIG-labelled *MiPA* RNA probe obtained via *in vitro* transcription of the *MiPA* target gene using T7 RNA polymerase according to DIG Northern Starter Kit Version 10 (Roche) protocol. Pre-hybridization (30 min) and hybridization (overnight) were performed at 50ºC. 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.

Generation of T_1 transgenic plants and nematode bioassay

Selected T_0 plants (T1, T2 and T3) were acclimatized according to Cruz-mendivil et al. (2011). Tomato plantlets having up to 15 leaves, 10 cm root length and at least 5 cm in height were gently removed from culture vessels and their roots washed free of agar. The plantlets were then transferred to pots containing sterile vermiculite and irrigated with 20 ml of half strength MS medium every 3 days for 14 days. Pots were covered with transparent polythene and kept in a growth room (25ºC, 65% relative humidity and 16:8 hour photoperiod) throughout the acclimatization period. The polythene covers were perforated every two days and completely removed at the end of the acclimatization period. The plantlets were then

transferred to pots containing 2000 ml of commercial garden soil in the green house and grown till they produced T_0 seeds.

We generated T_1 progeny plants by germinating the seeds of T1 and T2 lines in half strength MS medium containing 1% sucrose, 0.8% agar and 100 mg/L kanamycin. T3 plants produced no seeds and were thus excluded from further analyses. Segregation analysis was conducted by scoring the number of resistant and non-resistant seedlings at 14 days after sowing. Transgenic plants showing 3:1 segregation ratio were selected for further analysis. We genotyped the T_1 plants by PCR to confirm presence of *MiPA* gene in sense and anti-sense orientations. Hygromycin marker gene (HPT) was also amplified.

We transferred T_1 plants showing presence of all three amplicons to 300 ml autoclaved garden soil and acclimatized them under high humid conditions in growth room for 10 days. Further acclimatization was done in the green house for 11 days after which the plants were infected with approximately 1500 freshly extracted eggs of *M. incognita* (Appendix 6). Infection was done by adding the egg inoculum suspension to three holes made in the soil around the root system of each plant. Nematode-infected plants were grown in confinement for 49 days. After 30 days of infection, the test plants were analyzed for the number of nematode galls formed on each plant root system. Other nematode parasitic parameters (number of nematode egg masses/plant, number of nematode eggs/egg mass/plant and nematode multiplication factor) were evaluated at the end of 49 days infection period when the egg masses became visible after staining.

Nematode gall count was done by root inspection and with the aid of magnifying lens. Egg mass count was done after staining the roots with 15 mg/L Phloxine B for 20 mins to reveal pink-coloured egg masses. Egg number per mass was evaluated by counting from ten randomly selected stained egg masses after egg mass treatment with 1% sodium hypochlorite (Coyne and Ross 2014). Nematode multiplication was evaluated by multiplying the number of egg masses/plant by number of eggs/egg mass/plant and dividing by initial amount of egg inoculum used for infection. Agronomic characters evaluated include shoot and root weights, plant height and root length. Nematode infection experiment was conducted twice and 10 replicate plants per treatment were analyzed.

Relative expression of *MiPA* target sequence in adult feeding female nematodes

Adult female nematodes feeding on roots of transgenic and WT tomato plants were isolated under a stereo microscope (Olympus SZX9), frozen in liquid Nitrogen and stored at -80ºC. Total RNA was purified from the nematodes using ISOGEN (Nippon Gene), and 300 ng total RNA from each sample was converted to cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). We performed qRT-PCR for 2 target regions on the *MiPA* sequence using SYBR Green technology in StepOnePlus™ thermal cycler (Applied Biosystems). *MiPA1* is a 105 bp exon on the target sequence while *MiPA2* is a 203bp region partly on and outside the target sequence and contains an interrupting 51bp intron (Figure 2-6a, Appendix 7). The interrupting intron sequence is spliced out after transcription so that a 152 bp exon region is amplified on the cDNA by PCR.

PCR cocktail for each reaction was prepared by mixing 10 µl KOD SYBR qPCR Mix (TOYOBO), 0.2 µM each of forward and reverse primers (MiPA1-F and MiPA1-R for

MiPA1 or MiPA2-F and MiPA2-R for *MiPA2*), 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 98ºC for 2 min, followed by 40 cycles of 98ºC for 10s, 55ºC for 10s and 68ºC for 30s in a 96-well µltra Amp PCR plate (Sorenson Bioscience). Specificity of the amplifications was assessed by melt curve analysis at 60-95ºC after 40 cycles.

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

Statistical analyses

All experimental units were laid in a completely randomized design (CRD) in the glass house. Data generated after nematode infection were analyzed by 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).

Results

Genomic PCR analyses of T_0 transgenic tomato

We used PCR to genotype the primary transgenic tomato selected after *Agrobacterium* transformation. With pairs of primers GUSL5P and MiPA3P or MiPA3P and GUSL3P (Figure 3-1), we amplified a 1083bp or 796bp sequence corresponding to the sense or anti-sense orientation of our target sequence. A 687bp sequence of the hygromycin gene (HPT) was also amplified using primers HPT5P and HPT3P. Sense, anti-sense and *hpt* amplicons for 10 selected events are shown in Figure 3-2a.

Southern analysis of T_0 transgenic tomato

We analyzed *MiPA* T-DNA integration pattern in the PCR-confirmed T_0 transgenic tomato by Southern hybridization. Using *MiPA* specific probe, lines T1, T2 and T9 showed single copy integration of the *MiPA* RNAi transgene while lines T3 and T7 showed triple copy transgene integration. Plasmid control (P) also showed hybridization signal with *MiPA* probe while wild type plants showed no hybridization signal (Figure 3-2b).

MiPA expression analysis in T_0 transgenic tomato

Complementary DNA (cDNA) obtained from selected T_0 events (T1, T2 and T3) were used in a reverse-transcription PCR (RT-PCR) analysis to confirm expression of the *MiPA* dsRNA in the transgenic lines.

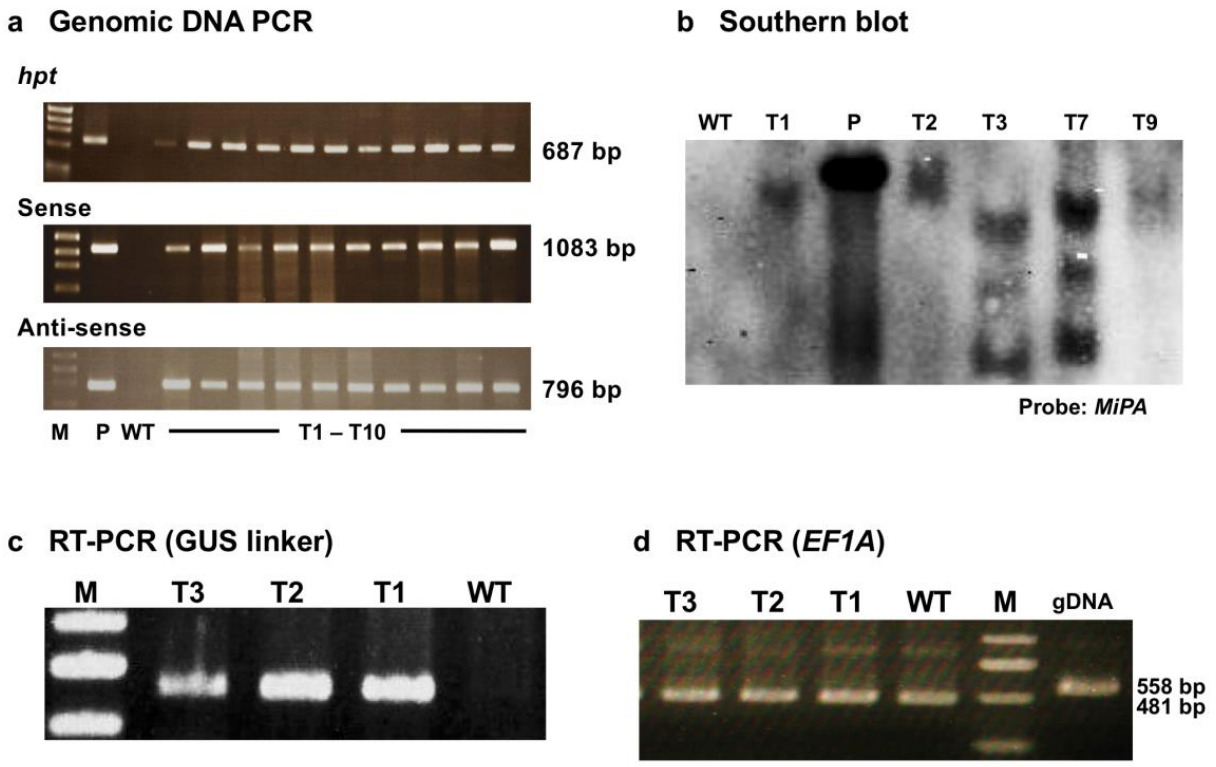


Figure 3-2. Molecular analyses of T_0 transgenic tomato. **a**: PCR amplification of *hpt* marker gene, sense and anti-sense orientation of *MiPA* target fragment. Plasmid control (P) showed similar band size while the wild type plants (WT) showed absence of target amplicons. M: ΦX174 *Hae*III digest marker. **b**: Southern blot analysis. Probe used for hybridization was specific to *MiPA* target gene. 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 *MiPA* hairpin RNA. **d**: RT-PCR of elongation factor 1α (*EF1a*), used as control, was amplified from cDNA (481 bp) of selected transgenic and wild type tomato lines and from genomic DNA control (558 bp). M: DL 2,000 DNA Marker (TaKaRa) **b**: Southern blot analysis. Probe used for hybridization was specific to *MiPA* target sequence. Wild type plants (WT) showed no hybridization signal.

Using primers GUS5P and GUS3P (Figure 3-1), we amplified a 636 bp fragment corresponding to the GUS linker region between sense and anti-sense orientations of the *MiPA* target sequence in all transgenic lines (Figure 3-2c). Wild type plants showed no amplification for the GUS linker region. Elongation factor (*EF1a*) used as internal control reference gene was amplified using primers EF1A5P and EF1A3P (Table 1). Both transgenic and wild type plants showed a 481bp amplicon of *EF1a*. A genomic DNA control, with an interrupting 77 bp intron, was amplified with the same primers and showed a 558 bp amplicon (Figure 3-2d).

Detection of *MiPA*-specific siRNA in T_0 tomato

Using DIG-labelled *MiPA* RNA probe, we detected siRNA hybridization signals with the transgenic lines that expressed dsRNA of *MiPA* target sequence (Figure 3-3). There was no such hybridization signal detected with WT plants. Ethidium-bromide staining of the polyacrylamide gel after resolution confirmed equal loading of small RNAs across all samples.

Molecular characterization of T_1 tomato lines

We extracted genomic DNA from 100 mg young leaves of T_1 tomato progeny plants generated from transgenic lines T1 and T2. PCR amplification was carried out with same primer sets used with T_0 plants. Correct amplicons for hygromycin gene (687 bp) as well as *MiPA* transgene in both sense (1083 bp) and anti-sense orientations (796 bp) were shown by the transgenic lines. Wild type plants showed no amplification signal (Figure 3-4a).

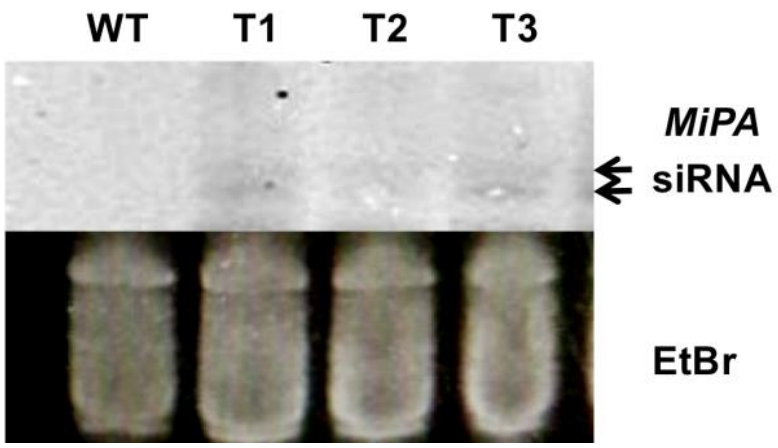


Figure 3-3. Detection of *MiPA*-specific siRNAs in transgenic tomato. *MiPA*-specific siRNAs (arrows) were detected in transgenic tomato (T1, T2, T3). WT plant showed no hybridization signal. DIG-labelled transcribed *MiPA* RNA was used as probe. EtBr shows equivalent loading of samples.

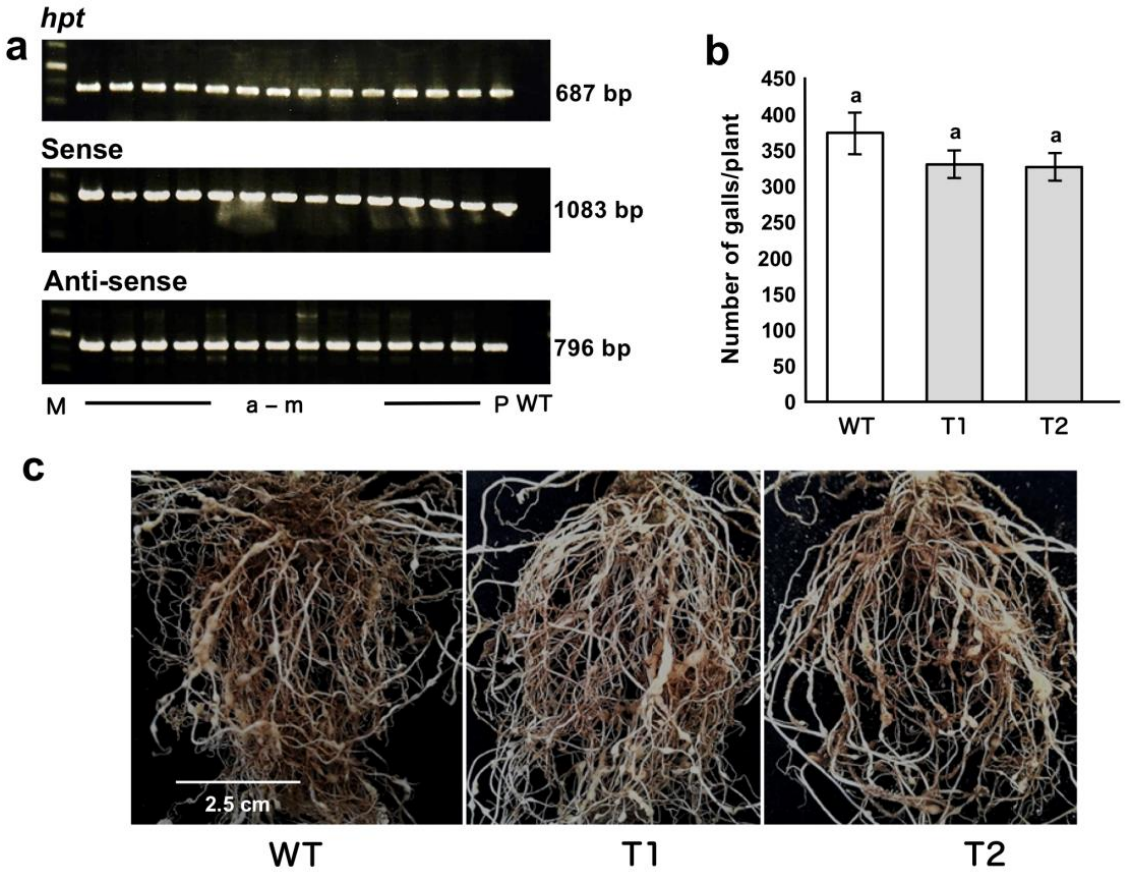


Figure 3-4. Molecular and nematode gall analyses in T_1 transgenic tomato. **a:** PCR amplification of *hpt* marker gene, sense and anti-sense orientation of *MiPA* target fragment. Plasmid control (P) showed similar band size while the wild type plants (WT) showed absence of target amplicons. M: ΦX174 *Hae*III digest marker. **b:** Gall number per plant was not significantly different ($p > 0.05$) in both transgenic lines and WT. **c:** Nematode galls on WT and transgenic tomato plants' roots reveal similarity in galling index and pattern

Bio-efficacy of T_1 tomato against *M. incognita*

After 30 days of infection by *M. incognita*, mean number of nematode galls formed on the root system did not differ significantly ($p > 0.05$) among the tomato lines. WT tomato had a mean of 374 galls per root, while transgenic lines T1 and T2 had 330 and 327 galls respectively (Figure 3-4b). Figure 2-4c is a visual assessment of WT and transgenic plants' roots revealing similarity in galling pattern. All root systems were characterized by presence of both single and multiple fused galls.

Figure 3-5a is the result of root observation after Phloxine B staining showing pink stained egg masses on WT plants as well as on both T1 and T2 lines. Mean number of nematode egg masses per plant was significantly increased ($p<0.05$) in WT tomato compared to transgenic lines T1 and T2 (Figure 3-5b), while mean number of nematode eggs per egg mass showed marginal differences ($p>0.05$) among the lines (Figure 3-5c). Nematode multiplication was significantly increased ($p < 0.01$) in WT tomato (32.3) compared to transgenic lines T1 (23.2) and T2 (20.3) (Figure 3-5d).

Mean shoot and fresh root weights among the lines did not differ significantly ($p > 0.05$). WT tomato plants had a mean fresh shoot weight of 15.1 g, while RNAi lines T1 and T2 had mean fresh shoot weights of 13.8 and 13.9 g, respectively (Appendix 8). Mean fresh root weight in WT tomato was 3.0 g, but averaged 2.9 g in T1 and T2 RNAi lines. Mean shoot and root lengths were also not significantly different ($p > 0.05$). WT tomato plants had a mean shoot length of 26.8 cm, while RNAi lines T1 and T2 had mean lengths of 25.3 and 25.7 cm, respectively. Mean root length among WT tomato was 25.6 cm, while mean root lengths among RNAi lines T1 and T2 were 25.7 and 25.8 cm, respectively.

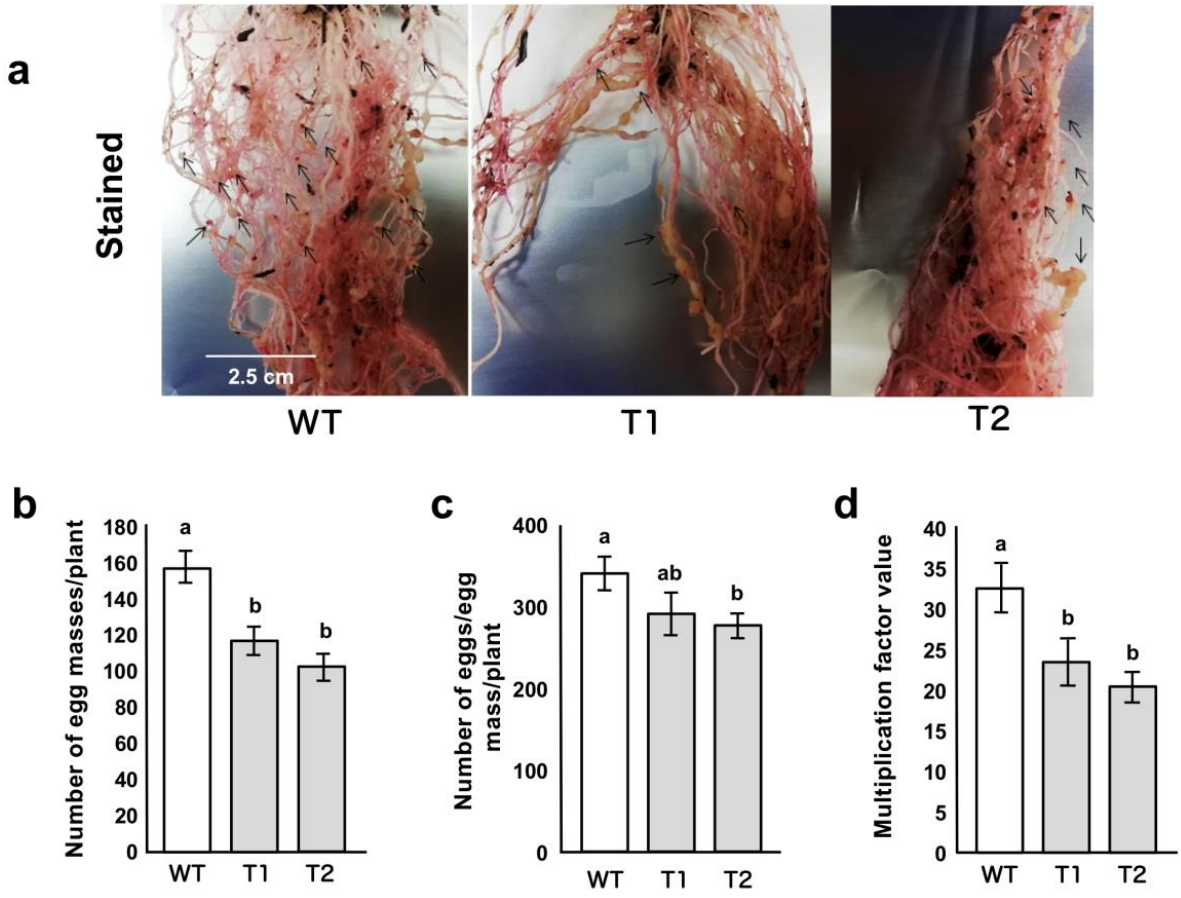


Figure 3-5. Nematode egg masses on root samples of WT and T_1 transgenic tomato

a: Acid fuschin-stained roots of WT and transgenic tomato showing pink egg masses. Arrows indicate the pink stained egg masses **b:** Mean number of egg masses per plant was significantly reduced ($p<0.05$) in transgenic lines compared to WT plants. **c:** Mean number of eggs per egg mass was not significantly different ($p > 0.05$) across the tested lines. **d:** Overall nematode multiplication was significantly increased ($p < 0.05$) on WT roots compared to those of transgenic lines. Bar means with different alphabets are significantly different ($p < 0.05$). Infection experiment was replicated and similar results were obtained.

Relative expression of *MiPA* target sequence in adult feeding female nematodes

Quantitative reverse transcription PCR **(**qRT-PCR) was used to analyze the expression of two target regions – *MiPA1* and *MiPA2* (Figure 3-6a) on *M. incognita PolA1* gene. Results showed that relative to expression in adult female nematodes feeding on roots of WT plants, nematodes feeding on roots of T1 and T2 tomato plants showed significant reduction ($p < 0.05$) in *MiPA1* expression. Nematodes from T1 line showed 64.8% expression while those on T2 line showed 63.8% expression, relative to 100% expression in nematodes from WT plants (Figure 3-6b). With respect to *MiPA2* expression, a 62.5 and 61.5% expression were obtained with nematodes on T1 and T2 plants respectively relative to WT (Figure 3-6c). Melt curve analysis for both *MiPA* and reference gene sequences after qRT-PCR showed single peaks (Appendices 9-11) indicating that the amplification was specific to the targets. Standard curves obtained with the different primer sets (Appendices 12-14) showed strong linear relationships between the log DNA concentration and mean Ct values.

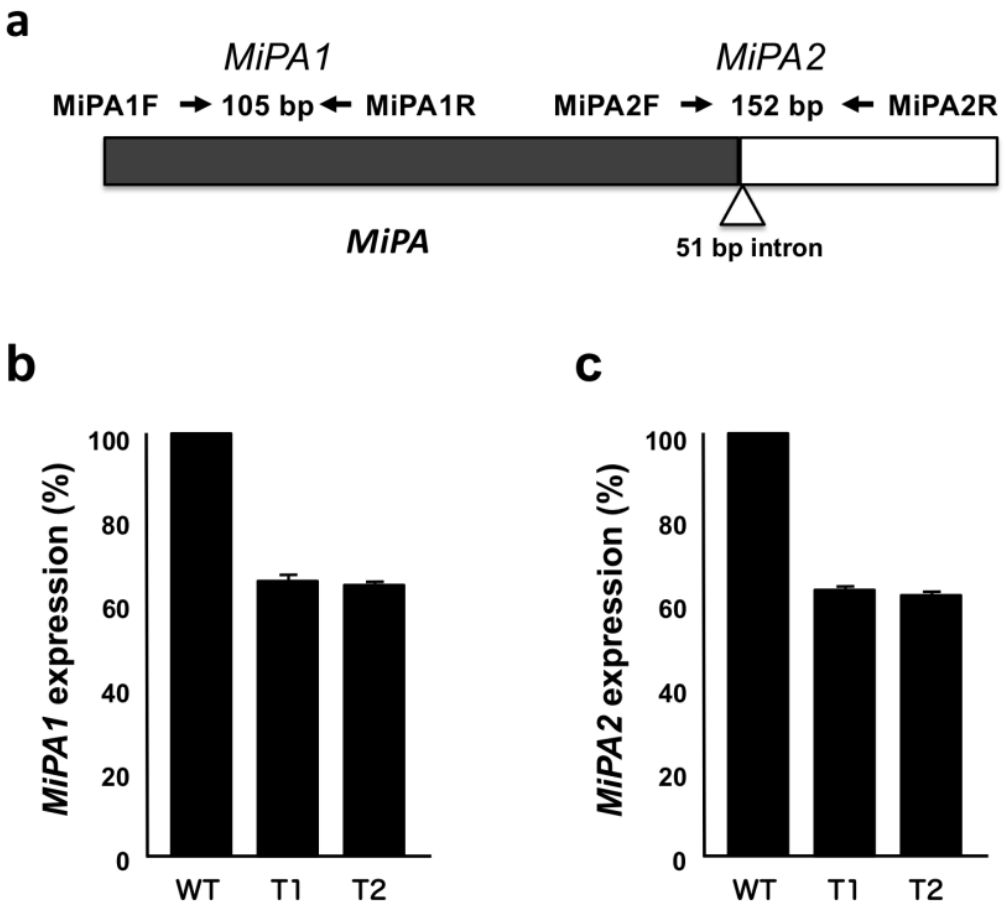


Figure 3-6. *MiPA* transcript levels in adult nematodes feeding on WT and transgenic plants' roots a: *MiPA* target sequence showing primer positions for two targets of qRT-PCR. Target 1 (*MiPA1*) is a 105 bp exon region while target 2 (*MiPA2*) is a 152 bp region excluding a 51 bp spliced out intron. **b:** Reduced *MiPA*1 transcript expression in adult female nematodes isolated from transgenic roots relative to those from WT roots. **c:** Reduced *MiPA*2 transcript expression in adult female nematodes extracted from transgenic 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%).

Discussion

Expressing dsRNA with sequence identity to a target nematode gene in plant roots can reduce the expression of such genes in feeding nematodes through RNA silencing. Depending on the targeted gene, transcript downregulation can produce lethal effects in these parasites and reduce infection or multiplication on plants (Fairbain et al. 2007). Identifying genes with potential value to control nematodes through RNA silencing is important for sustainable agriculture. In this study, therefore, we targeted species-specific sequence (*MiPA*) of nematode *PolA1*, an essential gene encoding the largest sub-unit of RNA polymerase I enzyme which synthesizes ribosomal RNA for RNAi-mediated control of nematodes.

We constructed pANDA35HK::*MiPA* plant expression vector to form an inverted repeat which will express dsRNA of *MiPA* target sequence (Figure 3-1). Successful tomato transformation with this silencing construct was confirmed via PCR amplification and Southern blot analysis (Figure 3-2a, 3-2b). Expression of the silencing construct was shown via RT-PCR amplification of the GUS linker between sense and anti-sense orientations of *MiPA* target sequence in the transgenic lines (Figure 3-2c). This expression is a confirmation of *MiPA* dsRNA production in the transgenic plants. Double-stranded RNAs are known to trigger cellular RNA silencing mechanism (Miki and Shimamoto 2004; Mlotshwa et al. 2008).

Expressed dsRNA in eukaryotes are typically processed into siRNAs that mediate gene silencing. Detection of *MiPA*-specific siRNAs in our transgenic plants (Figure 3-3) provided confirmation that the expressed *MiPA* dsRNA were processed by dicer in the plants. The role of gene-specific siRNA production by transgenic plants in facilitating degradation of complementary genes in attacking parasites has been established (Dutta et al. 2015; Ntui et al.

2015). Nematodes in particular are known to ingest siRNAs while feeding on transgenic plants, resulting in down-regulated expression of complementary endogenous genes (Nowara et al. 2010; Nui et al. 2012). Reduced host parasitism and attenuated developmental effects have been reported in such nematodes when essential genes are targeted (Huang et al. 2006; Papolu et al. 2013).

We evaluated transgenic and WT plants for nematode resistance and observed that all lines showed root gall formation (Figure 3-4c), indicating a lack of complete resistance to nematode infection. However, although total nematode galls per plant did not differ significantly among the lines (Figure 3-4b), transgenic lines T1 and T2 had 12.0 and 12.8% reduction respectively in overall gall formation per plant compared to WT plants. Nematode *PolA1* is not a parasitism-related gene and thus not involved during the infection process of nematode on host plants. This may explain, in part, the formation of galls on all plants including transgenic lines.

Galling assessment, according to Dong et al. (2007) and Coyne and Ross (2014), only reflects the ability of a plant to lessen or overcome the attack by root-knot nematodes but does not directly indicate other nematode parasitic success such as reproduction. Egg mass number provides a higher level of resistance indication (Coyne and Ross 2014). It is a reproduction-related parameter useful for evaluating the number of nematodes able to reach maturity on a given plant. Compared to WT plants, transgenic lines T1 and T2 showed 25.3 and 34.4% reduction in mean number of egg masses per plant root (Figure 3-5b). This finding suggests that younger female nematodes might have been developmentally retarded and could not reach maturity. Retarded nematode development can be expected with transcript degradation of an important house-keeping gene as *PolA1* gene. Reduced egg mass production is a

consistent observation in reports where important parasitism-related (Dutta et al. 2015) and house-keeping genes (Papolu et al. 2013) in nematodes were silenced.

Egg count per mass is also useful in establishing female nematode fecundity (reproductive ability). T1 and T2 transgenic lines showed 14.1 and 18.7% reduction in mean number of eggs per egg mass, respectively (Figure 3-5c) compared to WT. Such attenuated reproductive capacity was also reported by Antonio de Souza Junior et al. 2013 in nematodes feeding on tomato plants expressing dsRNA of different *M. incognita* proteases, though to a greater degree. Suppression in nematode reproduction is regarded as an attribute of plant resistance to nematodes (Boerma and Hussey 1992). Compared to WT plants, nematode multiplication on transgenic lines T1 and T2 was significantly reduced by 28.3 and 37.2%, respectively (Figure 3-5d). This finding provides evidence that our transgenic plants expressing *MiPA* dsRNA had reduced nematode propagation and hence some degree of resistance to the parasites.

In order to validate our findings and provide evidence of HIGS, we quantified *MiPA* transcript levels in feeding female nematodes isolated from roots of transgenic and WT plants. Our qRT-PCR analysis showed a 35.2 – 36.2% and 37.5 – 38.5% reduction respectively in *MiPA*1 (Figure 3-6b) and *MiPA2* (Figure 3-6c) transcript expression in adult female nematodes feeding on roots of transgenic plants relative to nematodes feeding on WT roots. Down-regulation of target gene transcripts in parasites typically indicates effective host induced RNAi in transgenic plants (Papolu et al. 2013). Our qRT-PCR results thus provide the evidence of *MiPA* silencing in these plant parasitic parasites and corroborates reduced nematode multiplication on the transgenic tomato lines in our bio-efficacy studies.

In planta expression of dsRNA of nematode splicing factor and integrase (Yadav et al. 2006), parasitism gene *16D10* (Huang et al. 2006) and protease gene (Dutta et al. 2015) all produced higher nematode resistance than we obtained with *PolA1* gene. However, if these genes are not present in the nematodes in single copies, their nucleotide sequences may have a higher probability of mutation compared to *PolA1* which occurs as a single copy per haploid genome and critical for survival due its role in synthesis of *45S rRNA*. *PolA1* thus have a possible advantage of being used as a universal target of HIGS unlike highly mutable genes where RNAi silencing induced can be easily lost with alteration in target sequence.

MiPA-mediated silencing may, however, be enhanced with the use of more effective intron-containing RNAi vectors. Exon linkers such as *gus* used in the pANDA35HK: *MiPA* vector is useful for confirming and quantifying expression of the RNAi construct via RT-PCR (Miki and Shimamoto 2004). However, intron spacers induce enhanced RNAi effects than exon counterparts (Smith et al. 2000; Wesley et al. 2001), possibly due to promotion of self-annealing of sense and anti-sense regions of the transcript after splicing (Hirai et al. 2007).

Chapter IV: General Discussion

Engineering plants through biotechnology for the delivery of RNA interference to silence essential nematode genes is currently applied as a valuable resistance strategy against root-knot nematodes. This strategy, also referred to as host-induced gene silencing, can potentially deliver protection to plants against a wide range of pathogens without posing environmental risk associated with the use of chemical nematicides (Roderick et al. 2018). Considering that HIGS operates in a sequence-specific manner and does not involve the transgenic production of any novel proteins or peptides, arguments about its biosafety and potential reduction of regulatory hurdles has been made (Roderick et al. 2018). HIGS approach, if commercialized, may therefore be useful for improving global agricultural productivity and addressing food security concerns in developing countries through reduction of pathogen-induced crop damages.

This study evaluated the potential effectiveness of the *M. incognita*-specific sequence of the *PolA1* gene in conferring root-knot nematode resistance on two economically important crops – tobacco and tomato using the HIGS approach. The choice of an appropriate essential nematode target gene is critical to the successful application of HIGS. In the past, different categories of nematode genes including those involved in parasitism, house-keeping and development have all been used as targets of HIGS with different ranges of resistance achieved. However, the need to evaluate more candidate nematode genes that can potentially produce more robust and broader range resistance against *M. incognita* underpinned the choice of *PolA1* gene as target. Apart from its essentiality in the synthesis of 45S rRNA in eukaryotes, the *PolA1* presents as a single copy gene, making its silencing potentially detrimental to nematodes.

Although the *PolA1* is an important housekeeping gene in eukaryotes, Nakamura (2000) found that POLA1 protein contains species-specific amino acid sequence at the C-terminal region. It was, therefore, considered possible that species-specific sequence within *PolA1* gene of plant pathogens can be universal target sequence for RNAi, thus eliminating the usually time-consuming and laborious process of identifying RNAi target sequences. Consequently, a 472 bp *M. incognita*-specific sequence of the *PolA1* gene was selected from the conserved amino acid sequence in the C-terminal of POLA1 protein as target for HIGS. This target sequence was cloned into Gateway pCR8 entry vector which carry 2 recombination sites (attL1 and attL2) for LR clonase reaction. The target sequence was then transferred into pANDA35HK RNAi destination vector by recombinase reaction. This reaction produced an *M. incognita PolA1* silencing construct with the target sequence in opposite orientations, and the *gus* linker sequence flanked by the 2 inverted repeats (Figure 2-1). The constructed pANDA35HK::*PolA1* binary vector was then mobilized into *Agrobacterium tumefaciens* strain EHA 105 by freeze thaw method.

In Chapter II of this study, transgenic tobacco plants harboring the pANDA35HK::*PolA1* silencing vector were generated by the leaf disc transformation method using *A. tumefaciens* strain EHA 105. Single and double copy primary transgenic lines that showed transgene presence as well as expression into dsRNA and siRNA were selected. Stable integration and inheritance of the RNAi transgenes was ascertained in the T_1 transformants. Resistance screening in selected T_0 lines indicated a reduction in root damage and galling intensity, nematode egg production, and overall disease condition in some of the lines compared to the wild type. In selected T_1 plants, resistance screening showed a

significant reduction in all nematode parasitic parameters measured relative to WT as well as in *PolA1* expression of feeding nematodes although agronomic characters were comparable.

In Chapter III of this study, further experiments were conducted to understand the efficacy of the pANDA35HK::*PolA1* silencing construct for root-knot nematode resistance in another susceptible host plant. Tomato was therefore transformed using the cotyledonary leaf disc method by *A. tumefaciens* strain EHA 105. T_1 transgenic plants of selected single copy lines showing presence of the transgene and its expressed dsRNA were generated. The selected lines were then subjected to root knot nematode infection and no significant difference in gall formation was observed relative to WT after 4 weeks. However, after 7 weeks, number of egg masses, number of eggs per egg mass and parasite multiplication were significantly reduced in the transgenic lines compared to WT. Female nematodes extracted from roots of transgenic plants showed significant reduction in *PolA1* expression at 2 target regions on the selected *PolA1* sequence.

In the two studies presented, it is evident that the transgenic tobacco and tomato plants showed partial resistance to root-knot nematode infection. This finding is consistent with previous reports of HIGS studies involving other target nematode genes including house-keeping (Yadav et al. 2006, Niu et al. 2012; Walawage et al. 2013), development-related (Ibrahim et al. 2011; Papolu et al. 2013) and parasitism-related (Huang et al. 2006; Dutta et al. 2015; Shivakumara et al. 2017; Chaudhary et al. 2019). According to Rosso et al. (2009), complete resistance to nematodes may not be practicable to achieve due to the transient nature of RNAi effect. However, Dutta et al. (2015) recommended strategies like the use of constructs of chimeric RNAi that target multiple genes for improving resistance obtained in HIGS studies. Improving the silencing efficiency of RNAi constructs through the use of

intron linkers may also be a useful strategy to obtain superior *PolA1*-mediated resistance. Gene constructs containing intron linkers have been reported to significantly increase silencing efficiencies (Fire et al. 1998; Smith et al. 2000).

According to Smith et al. (2000), the enhanced efficiency of intron-containing constructs may have to do with a possible alignment of the complementary arms of the hairpin by the spliceosome during intron excision. Such alignment could promote duplex formation in an environment that favours RNA hybridization. It is also possible that intron splicing may transiently increase the amount of hairpin RNA through the facilitation of the hairpin's passage from the nucleus. In this study, the exon linker-containing pANDA35HK RNAi vector was used in order to facilitate quantification of the trigger dsRNA (Miki and Shimamoto, 2004). Although variants of this vector have been successfully used for knocking down expression of endogenous host plants genes (Miki et al. 2005), there is no known literature on the use of pANDA35HK vector to achieve HIGS of pathogen genes. This study may therefore be the first attempt to achieve HIGS using this vector although a substitution of its exon linker with an intron may improve its use as a HIGS vector.

Regarding resistance to root-knot nematodes by the host plants, we observed that the transgenic plants displayed resistance more by suppressing nematode reproduction through reduction in egg masses, number of eggs per egg mass and multiplication factor. According to Dong et al. (2007), while gall number and degree of galling may reflect the ability of a plant to lessen or overcome attack by the root-knot nematodes, they do not indicate nematode reproduction directly. Cook and Evans (1987) presented the most widely used definition of resistance in plant nematology as the ability of a plant to inhibit the reproduction of a nematode species relative to reproduction on a plant lacking such resistance. Williamson and

Kumar (2006) clarified that resistance may not generally imply plants protection against nematode invasion but is characterized by a failure to support development of a reproducing female. Therefore, although T_1 transgenic tomato lines did not differ significantly with WT plants in average number of nematode galls formed, the significant reduction in nematode egg parameters and multiplication can be taken as evidence of resistance.

According to Wingard (1953), resistance of plants to pathogens generally can often refer to the capacity of the host to lessen, inhibit or overcome attack by the pathogen. By this understanding, we can further infer that the overall superior agronomic phenotype of T_0 tobacco plants infected with *M. incognita* connotes resistance. However, the disease conditions observed on T_0 tobacco plants presented symptoms that are partly consistent with both nematode and fungal infection. It is therefore suspected that a disease complex caused by interaction between the root-knot nematodes and a secondary fungal infection may have been responsible. According to Rich and Kinloch (2005), nematode attacks on highly sensitive and susceptible plants like tobacco produce both direct and indirect effects. Indirect effects occur when the nematodes make the crop more susceptible to other diseases like brown spot, blank shank and Fusarium wilt, thereby initiating a disease complex. Such interactions have been reported to cause even greater damage to the host plants (Bertrand et al. 2002; De et al. 2001), as was also evident in this study where leaf biomass, for example, an important economic trait for tobacco growers, was reduced substantially in WT and T4 plants. Transgenic plants also showed substantially improved reproductive fitness as they flowered earlier than WT plants (data not shown).

Reduced *PolA1* expression in adult feeding female nematodes extracted from transgenic roots provides an ultimate indication of HIGS in the pathogens. It is difficult to

understand the exact means through which silencing occurred – whether the nematodes directly ingested host plants-processed siRNAs or dsRNAs and processed them into siRNAs. However, following reports by Li et al. (2007) and Zhang et al. (2012) that root-knot nematodes are capable of ingesting large biomolecules efficiently via their stylet, either or both of the means appears possible. Improving the silencing efficiency of *PolA1*-containing vector may thus provide more robust protection against *M. incognita* and other species of root-knot nematodes given the homology of our target.

Summary

Root-knot nematodes are top priority nematode pests that significantly constrain agricultural productivity globally and threaten food security 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, called host induced gene silencing (HIGS), is currently gaining attention as potential alternative to addressing the limitations of conventional nematode control methods. However, identifying target genes whose knock-down in the parasites through HIGS can effectively protect host plants is critical to success of this strategy.

In this study, the effectiveness of root knot nematode *PolA1*, an essential single copy nuclear gene encoding the largest subunit of RNA polymerase I enzyme, as a target in conferring nematode resistance on *Agrobacterium*-mediated transformed tobacco and tomato plants was evaluated. Transgenic plants were characterized by PCR and southern blot analyses to confirm presence of *PolA1* RNA silencing construct as well as T-DNA integration patterns. *PolA1* dsRNA expression was confirmed by RT-PCR analyses while detection of *PolA1*-specific siRNAs was done via Northern blot.

Nematode infection bioassay revealed significant reduction in nematode fecundity and multiplication in the transgenic plants expressing *M. incognita*-specific dsRNA of *PolA1* gene compared to wild type plants. T_0 tobacco expressing *PolA1* dsRNA showed significantly improved agronomic characters over WT plants. QRT-PCR analyses showed a significant reduction in *PolA1* transcript expression in nematodes feeding on roots of transgenic plants thus providing evidence of HIGS. Taken together, these results show that *PolA1* is a

potentially effective target for HIGS-mediated reduction of root knot nematode propagation on transgenic tobacco and tomato plants although the silencing efficiency may be improved by the use of intron-containing RNAi vectors. Given the homology of the target sequence among *Meloidogyne* species, *PolA1* silencing could be broad range against other species of root-knot nematodes aside *M. incognita* and thus useful to improve agricultural productivity.

Appendix 1

One internode stem cuttings (rooted)

↓

Sterile vermiculite + ½ MS (2 weeks)

↓

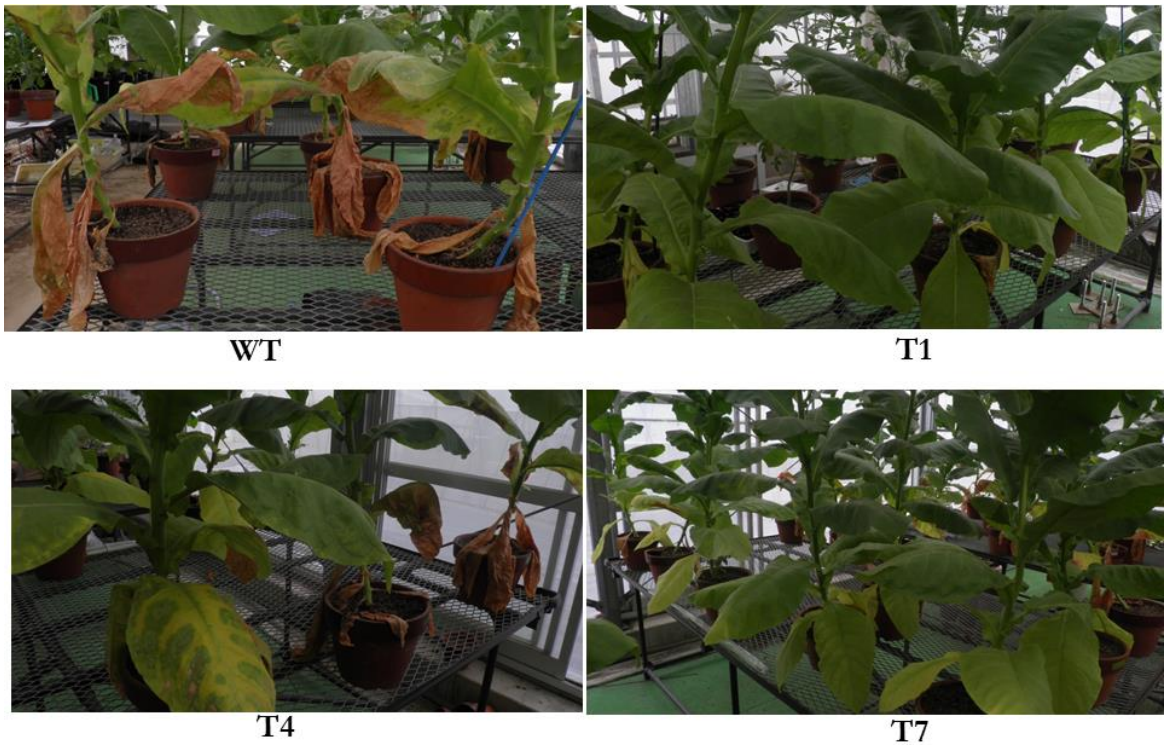
Green house acclimatization (10 days)

↓

Inoculation with *M. incognita* (10,000 eggs)

Establishment of T_0 tobacco prior to *M. incognita* infection

Appendix 2



Transgenic (T1, T4, T7) and WT tobacco plants after 7 weeks of *M. incognita* infection

Appendix 3

300 ml garden soil (approximately 500 *M. incognita* eggs)

Seedling selection in 100 mg/L Kanamycin

↓

Molecular confirmation by PCR

↓

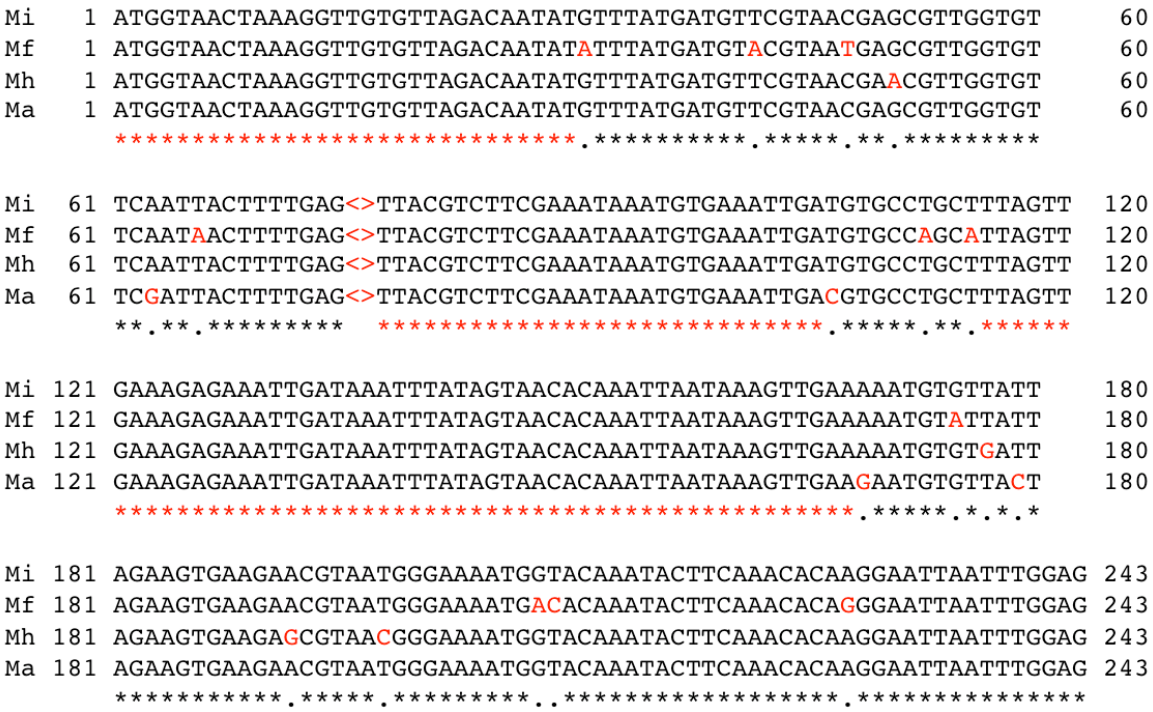
Seedling establishment and acclimatization

↓

Final acclimatization in green house and infection

Establishment of T_1 tobacco prior to *M. incognita* infection

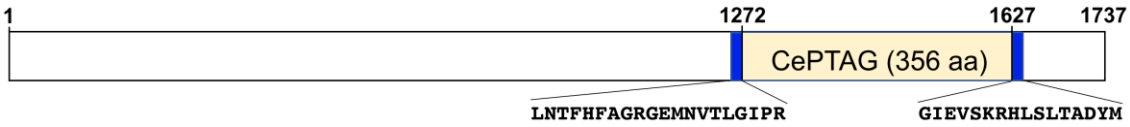
Appendix 4



Alignment of partial ntag sequence of *PolA1* gene in four species (*M. incognita*, *M. floridensis*, *M. hapla* and *M. arenaria*) of root-knot nematode. Red portions signify >23 bp sequences capable of producing siRNAs against the four species

Appendix 5

A



B

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CePTAG      1 LREILMTASKSIATPSASIAVIAGTSRDRIDSIKRELDRVYLKQLLKNFSLEEKITLTQNQSC-RRYHLRIDILAAEKRE     79
MiPTAG      1 LREILMTQGKSIATPMAEICIRPEVTEEQVQQLKSEFSPITLRHVLKNFSLVEGIVARDDGLCYRQYIVRLELLKNKKRD     80
              *******  ****** * *             * *     *   ****** * *       * * *  *   *   **

CePTAG     61 LGARHLKRSQIMEEIEKRFILRVAQAIKKKYHEITDYQQMSHRTMRQGNMAAGIETGGTKNRGLQGPDNGDSSDEEADGG    159
MiPTAG     61 QIARHLRRKKIMLEIESRFVPLLSTHLIKRDKDVFEQQQIQQRKILGNDNDAGGEQVETKKKGQDEFLASDEEDAMADLQ    160
               **** *  ** *** **         *        **   *        ** *   **  *       * *  **

CePTAG    120 READAAEARLHRRHRDEGADYEGEDEER----------VEVRE-EEEPMDSDSEDVKKEGLDGEDQTTEPLLVNSSR---    225
MiPTAG    121 GELDAHEERLNRRHLDGMAEYEGEEQDRGDSGIVDEFDDELIEAEEEKEDSEAELVNDDDKDDNEEEDPNNIKNEHKFIV    240
               * ** * ** *** *  * ****   *           *  * ***  **  * *     *           *

CePTAG    226 -IQSVQRLSEN-ISSYTYDVKSNKWCEVVFELPLRNKTKMDVSSIVEKEVELFIVHQTPGIERCVETTEQKNGKEMTILQ    303
MiPTAG    241 DEERVNMVTKGCVRQYVYDVRNERWCSITFELRLRNKCEIDVPALVEREIDKFIVTQINKVEKCVIRSEERNGKMVQILQ    320
                  *          * ***    **   *** ****   **   ** *   *** *    * **   *  ***   ***

CePTAG    284 TQGVNLAAFFKHADVLDVNSVYSNDLNLILENYGVEACSKAITTEMNNVFAVY                              356
MiPTAG    301 TQGINLEAFYNRVQFLDVNTIYSNDINVMLNCYGVEAANRTIVKEMNNVFGVY                              373
              *** ** **      ****  **** *  *  *****    *  ****** **
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C

```
        10        20        30        40        50        60        70        80        90       100
ATTTGTTTCGTCCTGAAGTAACTGAAGAACAAGTACAGCAACTTAAATCGGAATTTAGCCCAATAACTCTTAGACATGTTTTGAAAAATTTTAGTCTTGT
         R  P  E  V  T  E  E  Q  V  Q  Q  L  K  S  E  F  S  P  I  T  L  R  H  V  L  K  N  F  S  L  V

       110       120       130       140       150       160       170       180       190       200
TGAAGGAATTGTTGCTAGAGATGATGGATGGTAACTAAAGGTTGTGTTAGACAATATGTTTATGATGTTCGTAACGAGCGTTGGTGTTCAATTACTTTTG
  E  G  I  V  A  R  D  D  G     V  T  K  G  C  V  R  Q  Y  V  Y  D  V  R  N  E  R  W  C  S  I  T  F

       210       220       230       240       250       260       270       280       290       300
AGTTACGTCTTCGAAATAAATGTGAAATTGATGTGCCTGCTTTAGTTGAAAGAGAAATTGATAAATTTATAGTAACACAAATTAATAAAGTTGAAAAATG
E  L  R  L  R  N  K  C  E  I  D  V  P  A  L  V  E  R  E  I  D  K  F  I  V  T  Q  I  N  K  V  E  K  C

       310       320       330       340       350       360       370       380       390       400
TGTTATTAGAAGTGAAGAACGTAATGGGAAAATGGTACAAATACTTCAAACACAAGGAATTAATTTGGAGGCTTTCTACAATCGAGTACAATTTTTGGAT
  V  I  R  S  E  E  R  N  G  K  M  V  Q  I  L  Q  T  Q  G  I  N  L  E  A  F  Y  N  R  V  Q  F  L  D

       410       420       430       440       450       460       470
GTTAATACAATTTATTCAAACGATATTAATGTTATGCTAAATTGTTATGGTGTAGAGGCTGCAAATCGAACT
 V  N  T  I  Y  S  N  D  I  N  V  M  L  N  C  Y  G  V  E  A  A  N  R  T
```

Identification of target sequence (*MiPA*) for RNAi. a: Schematic representation of *Caenorhabditis elegans*-specific amino acid (CePTAG) sequence in the largest subunit of RNA polymerase I (NP_001022450). CePTAG sequence is located between two highly conserved amino acid sequences (LNTFHFAGRGEMNVTLGIPR, GIEVSKRHLSLTADYM). *Meloidogyne incognita*-specific amino acid (MiPTAG) sequence was also identified in similar fashion. b: CePTAG and MiPTAG sequences showed low homology (39 %) indicating species-specificity. Amino acid sequences (blue) of MiPTAG were selected to identify *MiPA* target sequence. c: *MiPA* target sequence and corresponding amino acid sequence. Red characters indicate positions of junction of two linked sequences or introns in the genomic sequence.

Appendix 6

300 ml garden soil (approximately 1500 *M. incognita* eggs)

Seedling selection in 100 mg/L Kanamycin

↓

Molecular confirmation by PCR

↓

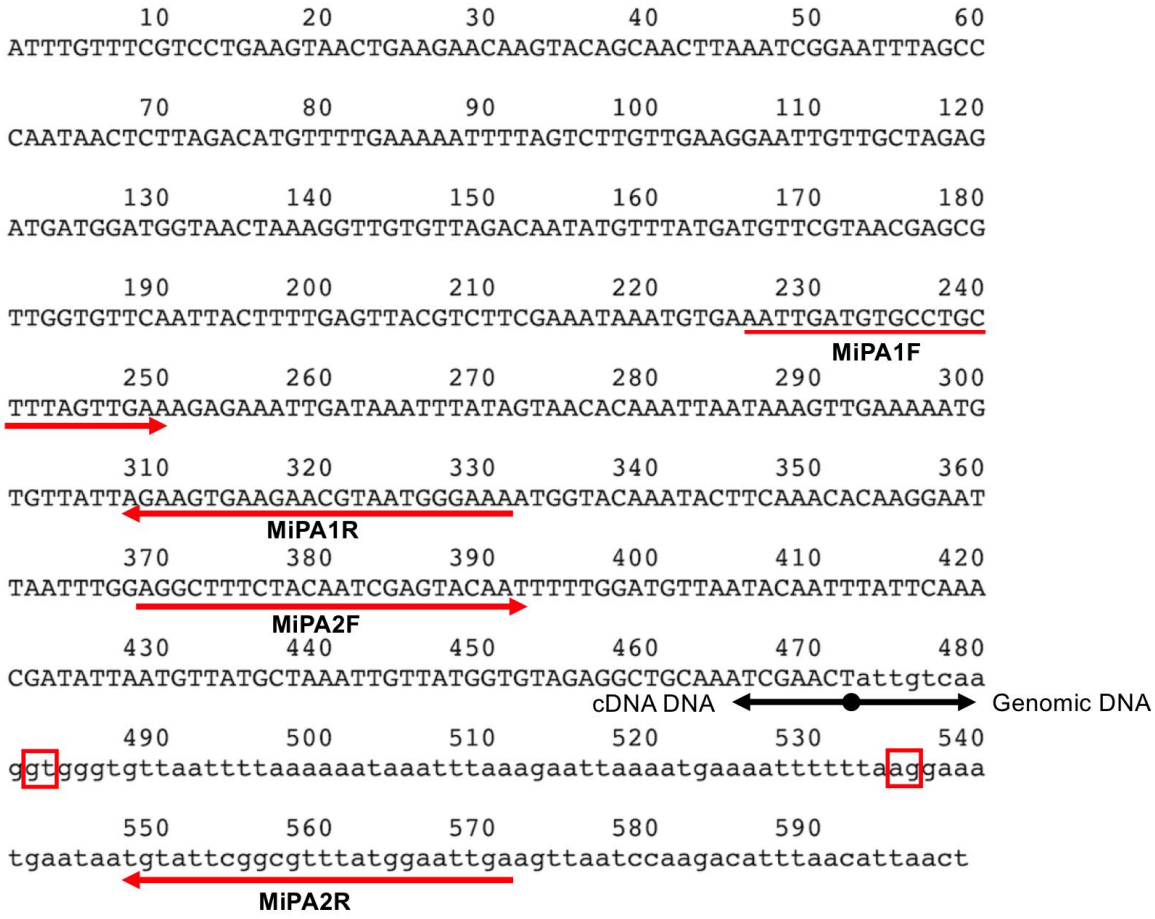
Seedling establishment and acclimatization

↓

Final acclimatization in green house and infection

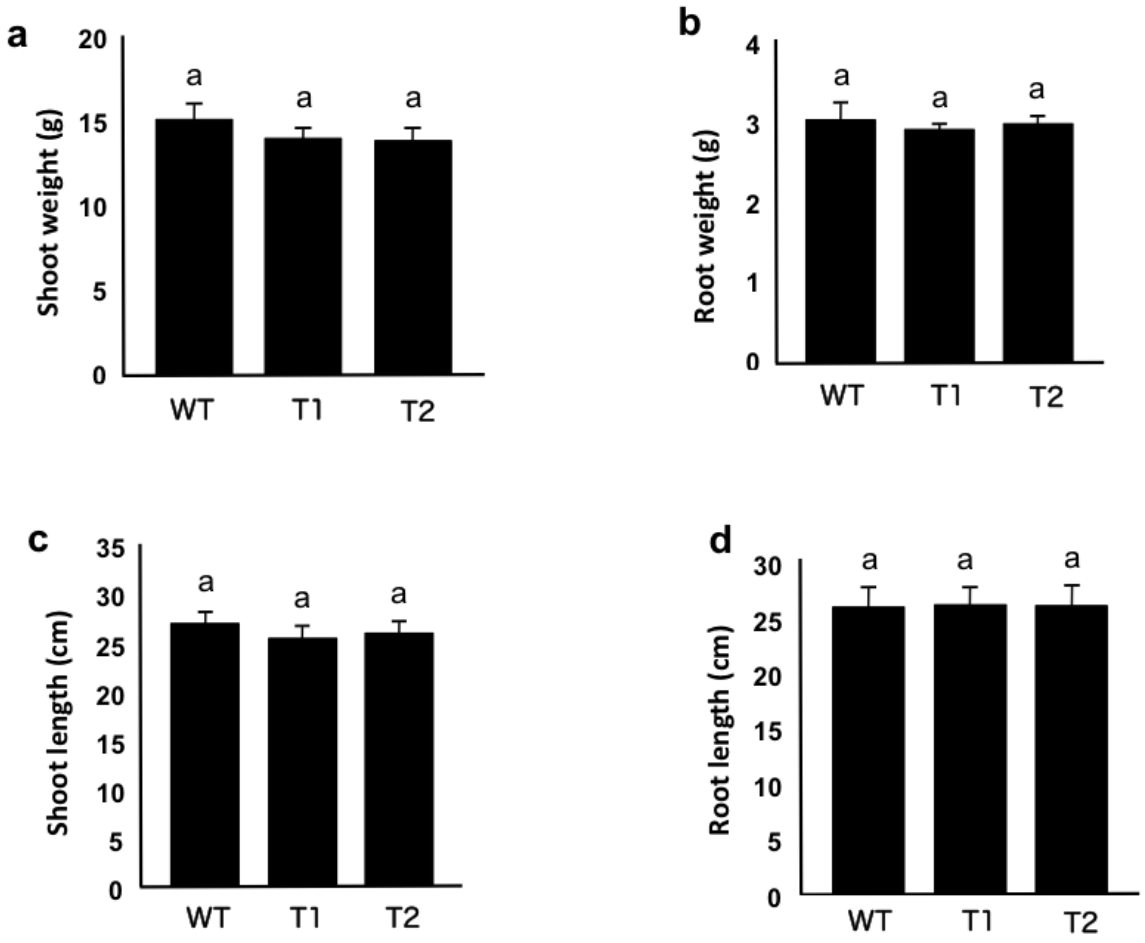
Establishment of T_1 tomato prior to *M. incognita* infection

Appendix 7



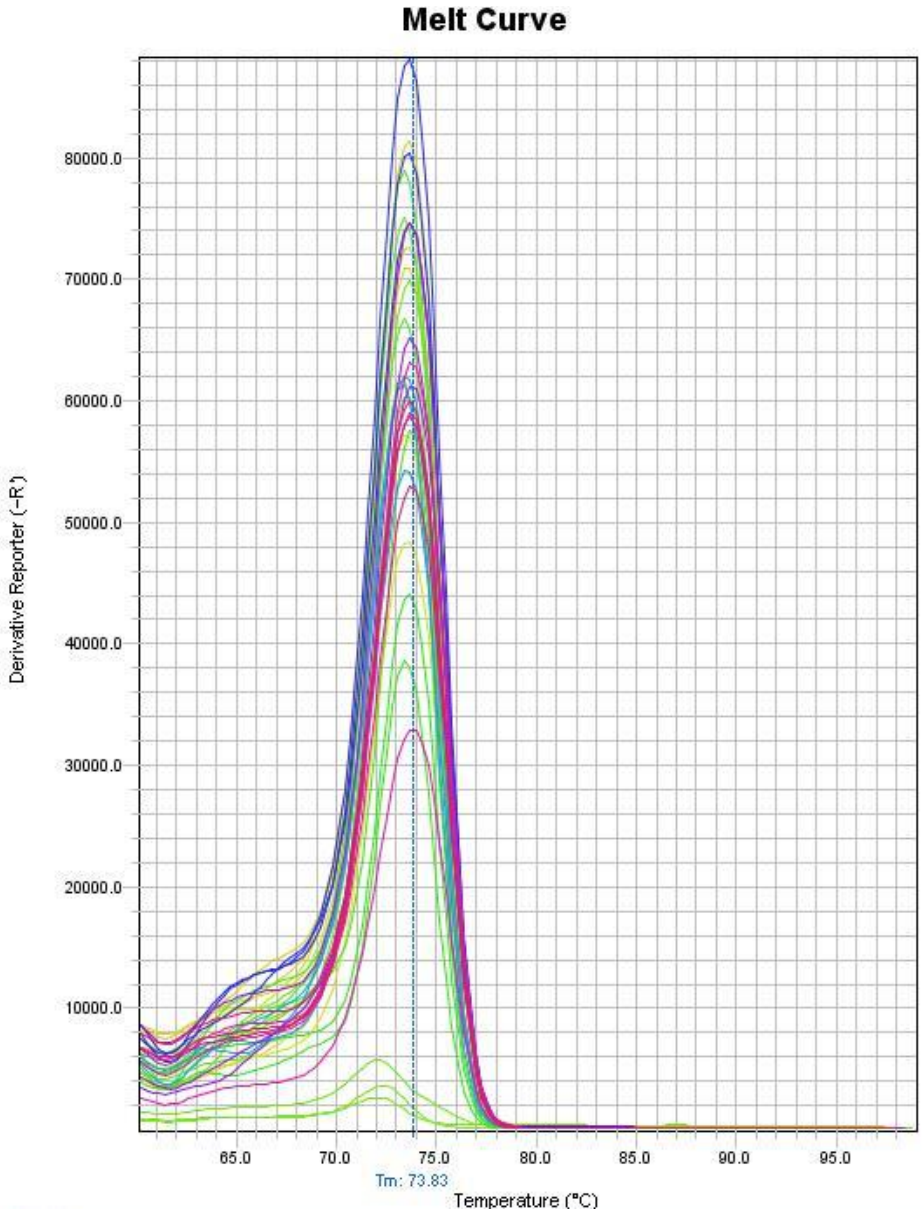
Positions of primers used for qRT-PCR of *MiPA* target sequence. *MiPA1* (105 bp) and *MiPA2* (152 bp) were amplified using primers MiPA1F-MiPA1R and MiPA1F-MiPA2R on *MiPA* cDNA (upper case) and downstream genomic (lower case) sequences, respectively. Boxes indicate donor and acceptor sites of spliced out intron sequence (51 bp).

Appendix 8



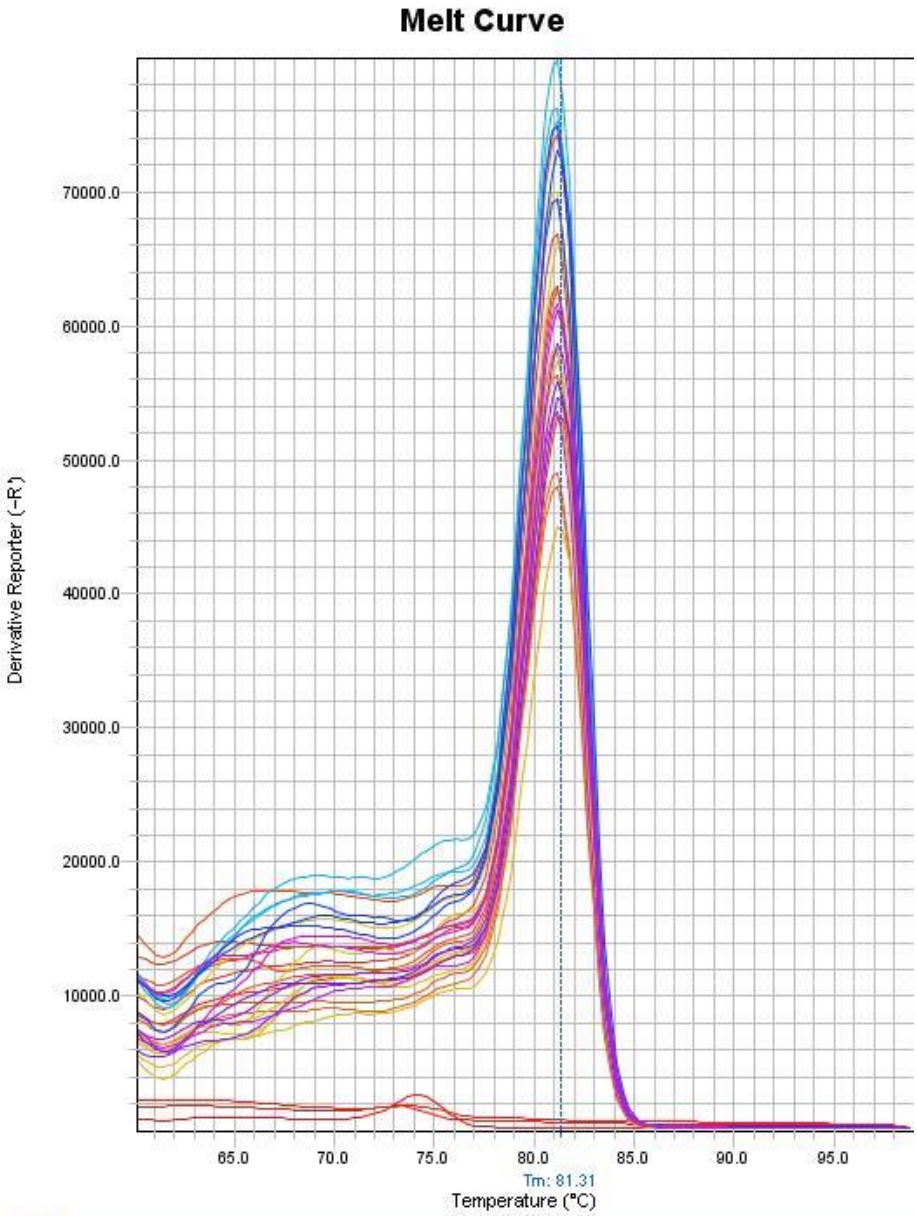
Agronomic traits analyses in T_1 transgenic tomato after nematode infection All agronomic traits evaluated – shoot weight (a), root weight (b), shoot length (c), and root length (d) were comparable ($p > 0.05$) in both transgenic lines and WT plants.

Appendix 9



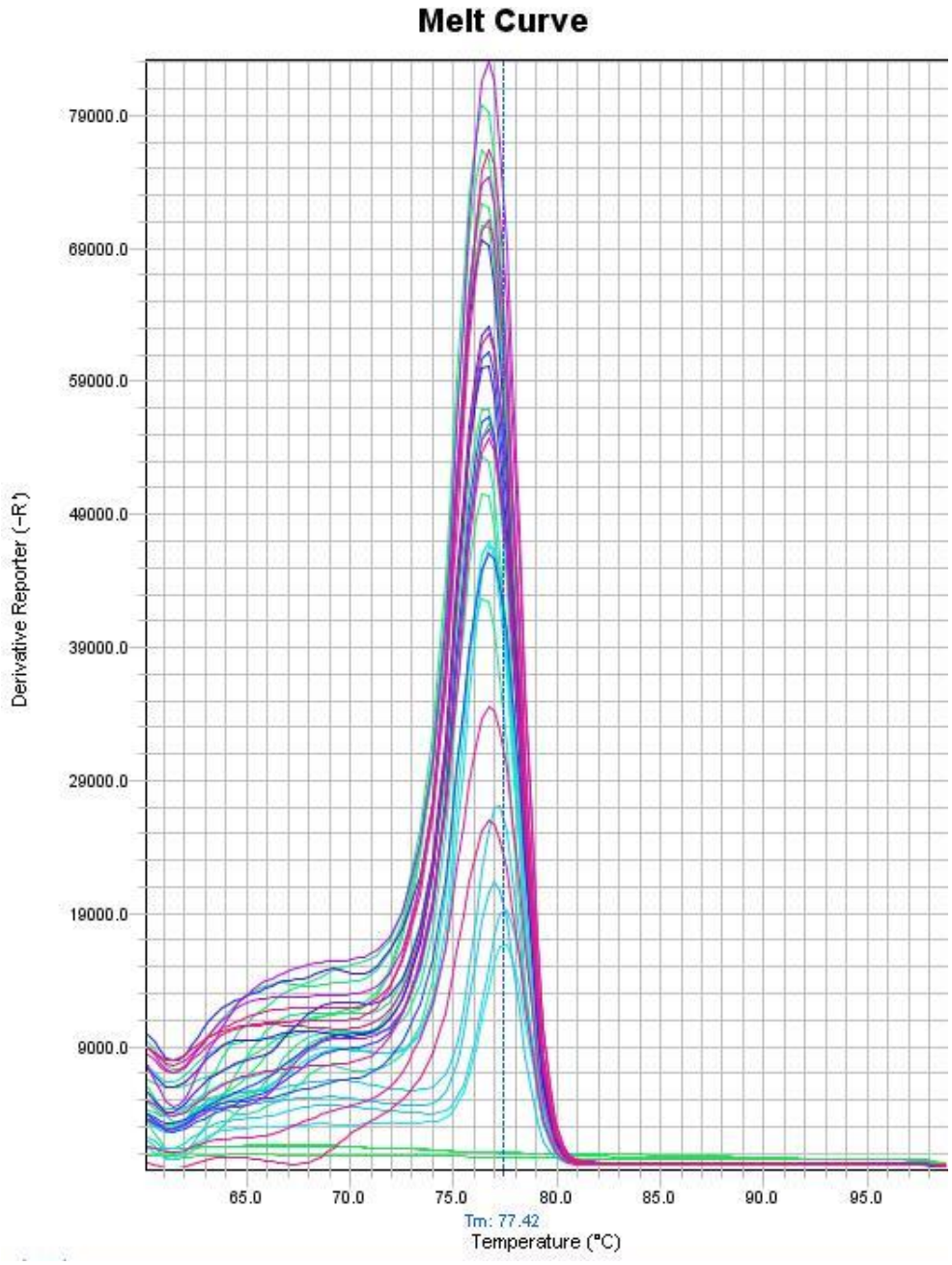
Post amplification melt curve analysis with *MiPA1* primers showing single peak

Appendix 10



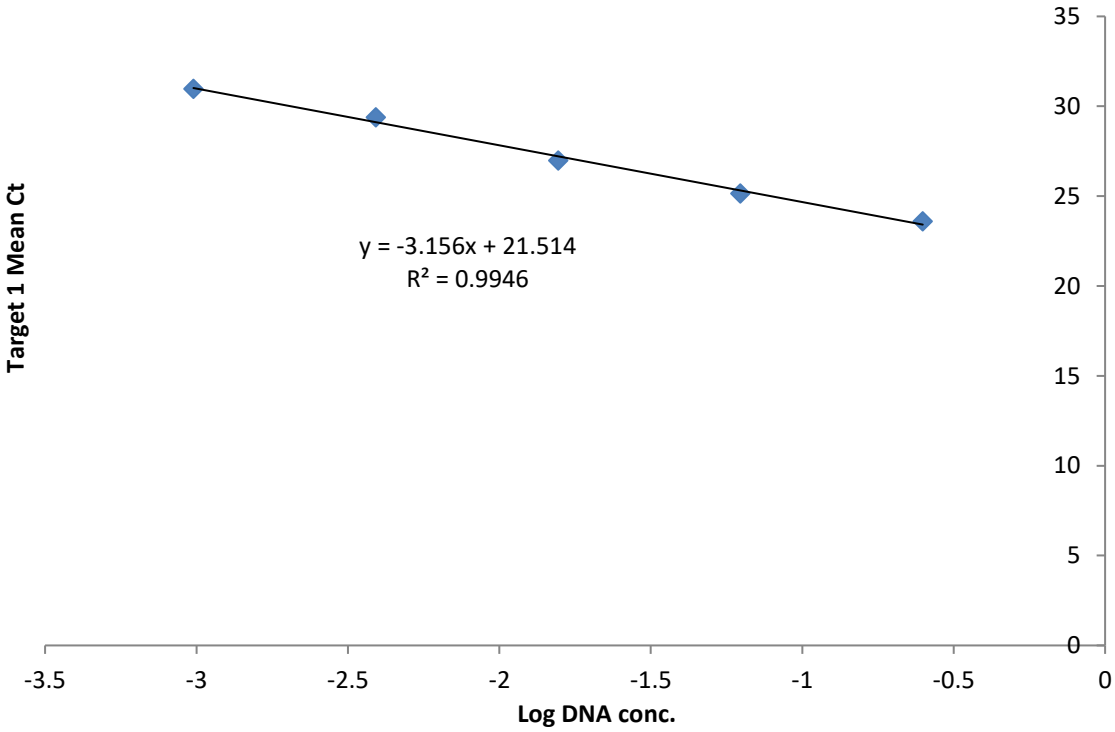
Post amplification melt curve analysis with *MiPA2* primers showing single peak

Appendix 11



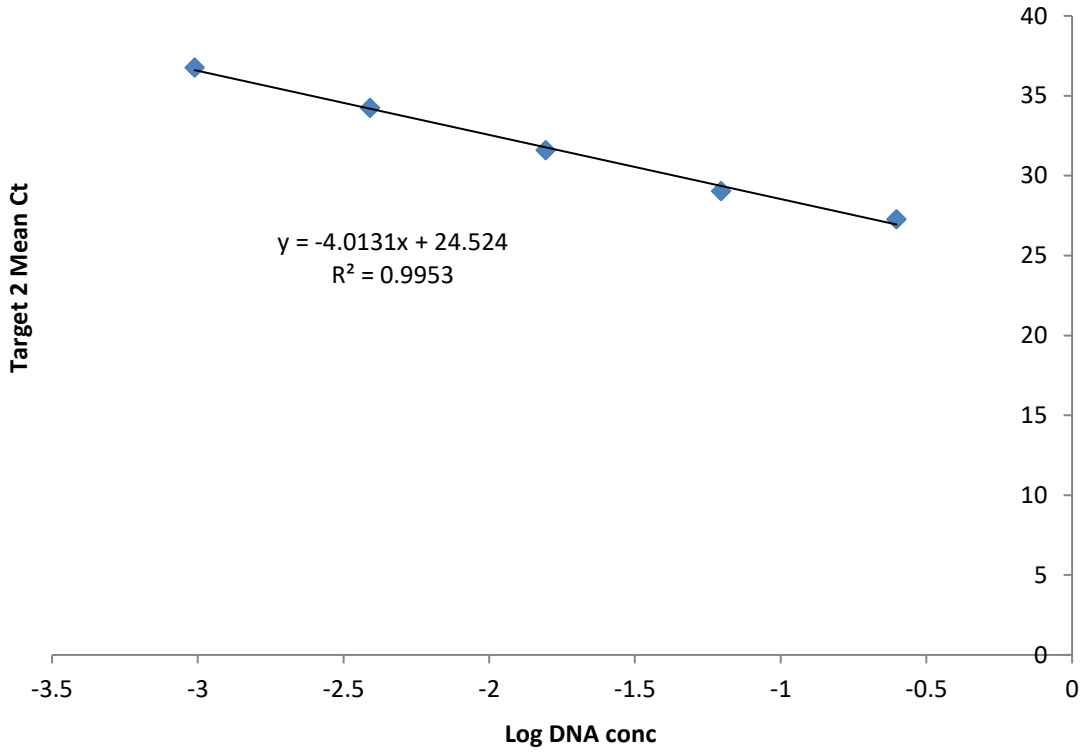
Post amplification melt curve analysis with *EF1* (reference gene) primers showing single peak

Appendix 12



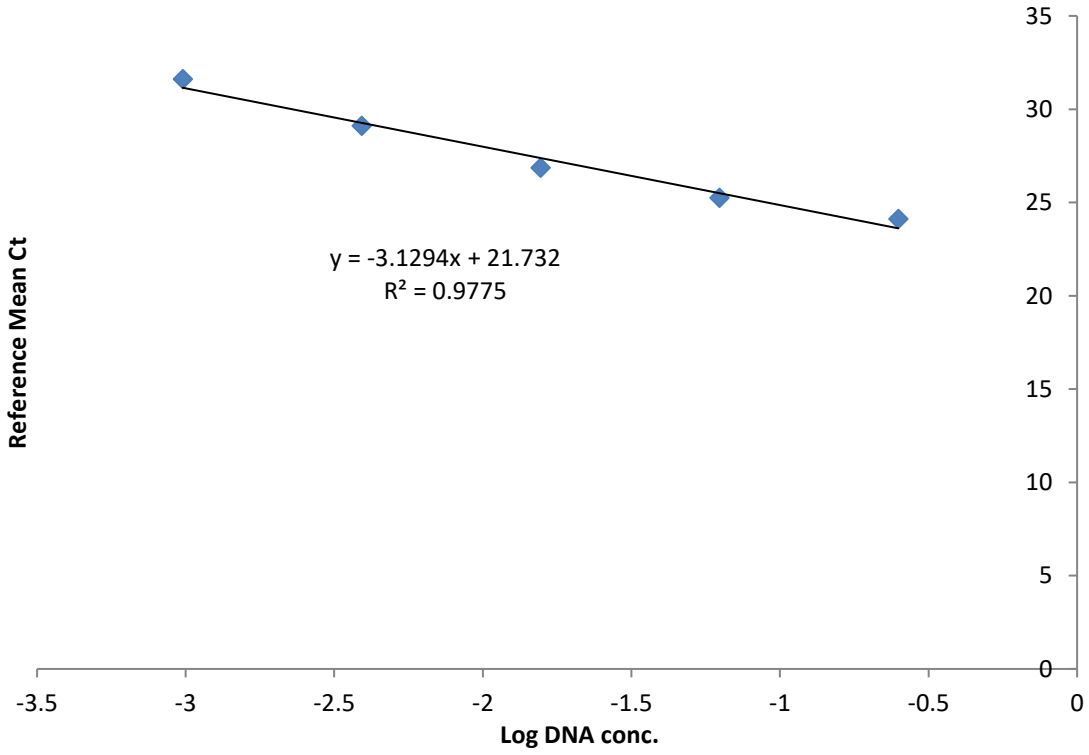
Standard curve of log DNA concentration vs mean Ct values with *MiPA1* primers

Appendix 13



Standard curve of log DNA concentration vs mean Ct values with *MiPA2* primers

Appendix 14



Standard curve of log DNA concentration vs mean Ct values with *EF1* primers

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