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Efficacy of PM-RNAi for the Control of *Begomovirus* Vector Whitefly in Transgenic Tobacco Plants

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Abstract

RNA interference (RNAi) holds tremendous potential in controlling various pests and pathogens. It has demonstrated promising results in silencing genes of diverse groups of insects and pathogens. In a previous study, we have demonstrated the potential of *Gossypium hirsutum*- encoded miRNA, ghr-miR166b, in controlling *Bemisia tabaci* (whitefly), the insect vector responsible for transmission of plant infecting, single-stranded DNA containing Begomoviruses. In this communication, efficacy of the overexpressed ghr-miR166b in transformed *Nicotiana tabacum* plants against whitefly infestation, and the inheritance of the transgene in the *N. tabacum* progeny were studied. Integration and expression of the transgene were confirmed by RT-PCR and northern hybridization assays. In transgenic *N. tabacum* lines, inheritance of the transgene was observed in accordance with the *Chi square* test. Further, the role of overexpressing ghr-miR166b in the Transgenic (T_1) plants against *B. tabaci* was investigated. The toxicity of the transgenic lines against *B. tabaci* were tested following feeding of the insects on the individual leaves as well whole plant of *N. tabacum*. Corresponding to highest expression of ghr-miR166b, highest level of whitefly mortality (77%) was achieved on the transgenic line NT-5. Following feeding of viruliferous whiteflies on the transgenic lines, begomoviral DNA was detected in non-transformed control and a transgenic line NT-1. Remarkably, no *Begomovirus* symptoms were seen in the transgenic line NT-1 even four months post infestation with viruliferous whiteflies. This study highlights the importance of Cross-Kingdom RNAi for the development of next generation pest management.

Keywords: *Nicotiana tabacum*; ghr-miR166b; Cross kingdom RNA; *Bemisia tabaci*

Introduction

Insect pests are major threats to the cultivation of agricultural crops, ultimately causing enormous losses to the world's economy. Various approaches, both conventional as well as non-conventional, have been applied to control insects at field levels. Most of them were focused on chewing pests with less or no effect towards sucking pests [1]. The sucking pests directly damages to host plants following feeding on phloem sap and cause indirect losses by acting as a vector of plant viruses. Whitefly (*Bemisia tabaci*) is among the most invasive pests affecting agricultural crops worldwide. They are hemipterous insects of the family *Aleyrodidae*, and are capable of feeding on members of plant families like Solanaceae, Cucurbitaceae, Malvaceae. Apart from physiological disorders such as reduction in sugar content, loss of vigor and drooping in the host plant [2], *B. tabaci* is capable of transmitting more than hundred species of plant viruses including Begomoviruses, Criniviruses, Ipomoviruses, Torradoviruses, and Carlaviruses [3,4]. The *B. tabaci* is the exclusive transmitter of the economically most destructive viruses, belonging to the genus *Begomovirus* [5]. Major control of the pest has been largely dependent on the use of chemical pesticides. The extended use of chemicals has posed serious threats to the environment and has raised an alarming concern towards the development of resistance against widely used chemical insecticides [6].

In recent years whitefly has cost huge losses to cotton (*Gossypium hirsutum*) production in India. *G. hirsutum* cultivation was completely shattered in different regions of the world due to extremely high infestation levels of *B. tabaci*. Therefore, it has become essential to devise and develop effective strategies for the control of the insects.

With the development of advanced high throughput genomic methodologies, new frontiers have been opened to develop plants tolerant to various biotic and abiotic stresses. Biotechnological approaches have long been in use to integrate new traits in crops to render them fit against various stress. The *in vitro* regeneration of plants, however, still remains the major bottleneck for realizing the goal of developing designer crops. Plants like cotton are recalcitrant and not easily

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amenable to propagate in tissue culture and genetic transformation procedures [7]. Their long-life cycle further lengthens the procedure of developing and testing a particular approach. Hence, an efficient strategy to regulate genes of insect vector needs to be established and tested in model plants, such as, tobacco. Tobacco is a natural allotetraploid and is capable of producing seeds in just a short span of three months. The plant is versatile and is easily amenable for genetic engineering [8]. Further, it is susceptible to the viruses transmitted by *B. tabaci*. Therefore, it could greatly contribute in generating plants resistant and competent enough to combat the devastating effects of the viruses and their vectors.

Advances in agricultural biotechnology has contributed to develop novel approaches to address potential challenges of insect pests. RNA interference (RNAi) has been proving its potency in controlling the expression of genes in eukaryotes [9,10]. It has been very efficiently applied in producing plants resistant to various viruses including *African cassava mosaic virus* [11], *Cucumber mosaic virus* [12], *Cotton leaf curl Multan virus* [13-15], *Mungbean yellow mosaic India virus* [16,17], *Tomato yellow leaf curl virus* [18] and many more. However, Begomoviruses have a high rate of recombination and are constantly evolving [19]. Recently, small RNA molecules such as micro (mi) RNA or small interfering (si) RNA) were manipulated for generating protection against pests likes aphids and whiteflies [1,2,20-23]. With significant results, efficient protection against vector of the viruses have been achieved.

In our previous study, based on the assumption that host-encoded miRNAs are capable of downregulating the expression of the genes of infecting pests and pathogens [24-30], *G. hirsutum*- encoded miRNA, *viz* ghr-miR166b, was *in silico* identified and selected for its potential to target mitochondrial ATP synthase of the *B. tabaci*. Further, overexpression of ghr-miR166b in cotton plants did not exhibit any phenotypic abnormality. The transgenic cotton plants showed effective control against the insect. In present study, the seeds obtained from the Transgenic (T_0) *N. tabacum* lines overexpressing ghr-miR166b, were subjected to biological and molecular assays for confirming the presence of transgene in the progeny. Inheritance of the transgene in the transgenic progeny was studied. Further, the efficacy of the Transgenic (T_1) *N. tabacum* lines against the insect vector, *B. tabaci* was evaluated. The transmissibility of the *Begomovirus* in the transgenic lines, post infestation with viruliferous *B. tabaci* was also investigated.

Materials and Methods

Plant and insect culture

The seeds obtained from Transgenic (T_0) tobacco (*Nicotiana tabacum* L. cv. Xanthi) lines, namely, NT-1, -2, -3, -4, -5 carrying transgene (ghr-miR166b cassette) were sown on MS medium under controlled conditions of 26°C ± 2°C and 60% to 70% relative humidity with a photoperiod of 16 h in culture room. Non-transformed tobacco served as controls.

Whitefly culture was maintained for several generations on potted tobacco and cotton plants (26°C ± 2°C and 60% to 70% relative humidity).

Detection of transgene in transformed tobacco

In order to check the presence of transgene in the transgenic progeny, seeds of Transgenic (T_0) *N. tabacum* cv. Xanthi lines (NT-1, -2, -3, -4 and -5) were raised on kanamycin (50 mg/L) supplemented MS medium, after the dormancy period was over. Around 25 seeds of each of the 5 transgenic *N. tabacum* lines were used. Once the plants were established, genomic DNA was isolated from the fresh leaves of the *in vitro* raised plants using DNeasy Plant Mini Kit (Qiagen, Germany) according to manufacturer's instructions, and subjected to detection of *npt* II gene through PCR amplification. The reaction mix consisted of 0.5 µl of Taq DNA polymerase (1.5 U), 0.5 µl dNTPs (50 mM), MgCl2 (1.5 mM) and 0.5 µl of each *npt* II For and Rev primer (50 pmol, Suppl Table 1), and the final volume of 25 µl was made up with double-distilled H_2O. The PCR conditions were: Initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 2 min and extension at 72°C for 1 min, followed by a final extension of 5 min at 72°C to extend any premature DNA synthesis.

Semi-quantitative RT-PCR amplification of precursor miR166b

Total RNA was isolated from the leaves of all the five *N. tabacum* lines and non-transformed control tobacco plant using RNeasy Plant Mini Kit (QIAGEN, Germany) according to manufacturer's instructions. Five µg of total RNA was subjected to cDNA synthesis by reverse transcription and oligo dT primers using M-MuLV RT-PCR Kit (Merck, Germany) following manufacturer's instructions. Semi-quantitative PCR was performed employing ghr-MIR166b gene specific primers (166 RT For and 166 RT Rev) as described by Wamiq and Khan [23]; (Suppl Table 1) and reference gene Actin primers (Actin for and Actin Rev) as described by Khan et al., [31], (Supp Table1). The cycling conditions were: 5 min of initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 30 sec, 30 sec of annealing at 55°C, extension of 1 min at 72°C and final extension of 5 min at 72°C. The PCR product was electrophoresed on 1.5% agarose gel, stained with Ethidium Bromide (EtBr) and visualized in gel documentation system (BIO-RAD, USA).

miRNA isolation and Northern blot analysis of transgenic *N. tabacum* plants

The transgenic (T_1) *N. tabacum* lines (NT-1, -2, -3, -4, -5) were analyzed for the presence of mature ghr-miR166b by northern blot analysis of small RNAs (sRNA). The mirVANA RNA isolation kit (Ambion, UK) was used to isolate sRNAs from non-transformed and Transgenic (T_1) *N. tabacum* plants, according to manufacturer's instructions. 30 µg of RNA was resolved on 20% denaturing gel and blotted onto Hybond-N+ membrane (GE Healthcare, UK) [32]. The digoxigenin labelled PCR amplified ghr-MIR166b DNA probes were prepared according to manufacturer's instructions using DIG DNA labelling and detection kit (Roche Diagnostics). The hybridization was performed at 42°C for 16 h, followed by stringency wash, at 45°C for 10 min, with 2X SSC and 0.1% SDS. The membrane was then incubated with NBT/BCIP (color substrate) at 37°C for color development. Equal amount of RNA loading was assessed by staining the corresponding RNAs with EtBr.

Testing toxicity of transgenic *N. tabacum* plants against whitefly infestation

Leaf feeding bioassay: Leaves of transgenic (T_1) lines (NT-1, -2, -3, -4, -5) and the non-transformed control tobacco plants were tested to check their toxicity against *B. tabaci*. Three plants of each transgenic line, and three fresh leaves of each plant were placed on solidified agar (1%) in the beaker (Figure 1) and inoculated with 20 to 25 newly hatched *B. tabaci*. The setup was placed under sterile conditions and monitored daily for a period of six days to count the number of live whiteflies, for calculating percentage mortality.

Table S1: List of primer sequences used for PCR and Real Time PCR experiments.

S. No.	Name of primer	Primer sequence
1	Pre-166 For	5´TGTCACCCAAAAAGGCCAAA3´
	Pre-166 Rev	5´GCTGCAGTCCATGGGAGTTA3´
2	*npt* II For	5´GGAGCGGCGATACCGTAAAGC3´
	npt II Rev	5´GAGGCTATTCGGCTATGACTG3´
3	166 RT For	5´GCTGCAGTCCATGGGAGTTA3´
	166 RT Rev	5´TCTAATCCGTTTTCCCTGCAA3´
4	Actin For	5´GCATAGAGGGAAAGCACAGC3´
	Actin Rev	5´TGGTAGGTATGGGCCAGAAA3´

Note: For: Forward Primer; Rev: Reverse Primer

Experiment was performed in triplicates. Average number of *B. tabaci* on each line was calculated by adding the number of surviving whiteflies on each replicate, divided by the total number of replicates (three in this case). Percentage decline in *B. tabaci* population was calculated by comparing the average of *B. tabaci* survived on each line compared to initial number of *B. tabaci* inoculated on each replicate of that line in total. Percentage mortality of *B. tabaci* on each line was also compared to mortality on the non-transformed leaves, on the same day.

Whole plant bioassay: All the five transgenic *N. tabacum* lines were further evaluated by infesting whole plants with around 200 adult viruliferous *B. tabaci* (whiteflies carrying cotton leaf curl virus) for 15 days in insect-proof cages under greenhouse conditions at 26°C ± 2°C and 60% to 70% relative humidity. Three plants of each transgenic line were taken along with three plants of non-transformed control tobacco. All the plants were of same age (75-days-old). *B. tabaci* were inoculated on plants and covered with transparent cylinders for allowing their settling on the leaves. Once the *B. tabaci* got settled, the plants were transferred to insect-proof cages. Initial *B. tabaci* count on each plant was taken at Day 0. The plants were then regularly kept under observation and number of surviving *B. tabaci* were counted at an interval of 5 days for 15 days. Decline in population of *B. tabaci* on the transgenic lines was compared with that on control plants [23]. Percentage mortality in insect population on transgenic lines, compared to control, was calculated according to Fleming and Retnakaran formula [33].

Statistical analysis: Statistical analysis of the leaf feeding bioassay was performed by One-way ANOVA followed by Duncan's Multiple Range Test (DMRT). Percent mortality of *B. tabaci* was calculated as Mean ± Standard Deviation (SD) and statistically significant variables were analyzed at a confidence level of 95% (P<0.05). The whole plant bioassay was performed by 2-way ANOVA followed by Dunnett's multiple comparison test. The comparison tests were performed against control tobacco plants. The non-transformed control and the transgenic plants were of same age in both the experiments. All values were processed as mean ± SD; P<0.01 was considered significant. Statistical analyses were performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla, California USA, http://www.graphpad.com.

Detection of *Begomovirus* in the transgenic plants: All the transgenic (T_1) *N. tabacum* lines were subjected to Rolling Circle Amplification (RCA)-PCR based detection of cotton leaf curl virus (CLCuV, a *Begomovirus*).

Genomic DNA was isolated from the transgenic *N. tabacum* lines and non-transformed *N. tabacum* plants, post inoculation with viruliferous *B. tabaci*, using DNeasy Plant mini kit, according to manufacturer's instructions. The isolated DNAs were used as template for RCA employing TempliPhi DNA amplification kit according to the manufacturer's instruction (GE healthcare, USA). The RCA products were further subjected for detection of the *Begomovirus via* PCR amplification employing *Begomovirus*-specific primers (F2 for/F2 Rev; [34], (Suppl Table 1). The cycling conditions were: Initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 2 min with final extension of 72°C for 5 min.

Results

Analysis of germination efficiency of transgenic *N. tabacum* seeds

With a view of determining the segregation of the transgene in transgenic (T_1) *N. tabacum* lines, the survivors obtained on the selective media were counted (Table 2). The expected ratio for Mendelian inheritance is 3:1. The *Chi-Square* analysis showed the data was significant with p (χ^2, with df =1, ≥ 0.004) = 1.329.

Integration of transgene in transgenic lines

Five plants from each transgenic (T_1) *N. tabacum* line, obtained on selection medium, were randomly selected to confirm the inheritance of ghr-MIR166b cassette in the progeny. Genomic DNA isolated from all the five transgenic lines and was subjected to detection of *npt* II gene *via* PCR. An expected amplicon of ~750 bp was seen among all the transgenic (Figure 2).

Semi qRT-PCR of transgenic lines

The semi-quantitative RT-PCR analysis of expression of ghr-MIR166b in the Transgenic (T_1) lines showed different levels of the transcript in all the lines and non-transformed control tobacco. While, the line NT-5 showed maximum level of transgene expression,

Figure 1: Leaf feeding bioassay of *Bemisia tabaci* (a) *B. tabaci* collected in an Eppendorf (b) leaf of transgenic (T_1) *Nicotiana tabacum* placed on 1% agar and inoculated with *B. tabaci* (c) the setup placed under sterile conditions for six days.

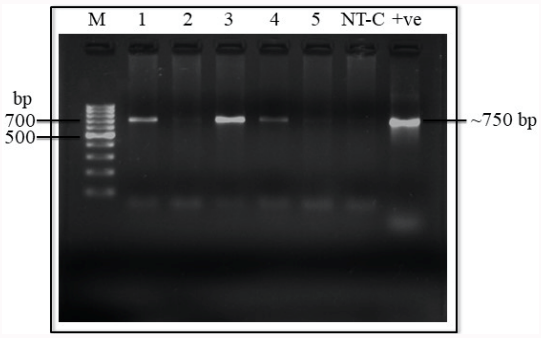

Figure 2: PCR based amplification of *npt* II gene in transgenic (T_1) *Nicotiana tabacum* cv. Xanthi lines transformed with pBI121-ghr-MIR166b construct. Lane: 1 to 5 denotes genomic DNA isolated from 5 transgenic (T_1) NT-5 plants. Positive amplification signals (~750 bp) were seen in lanes: 1, 2, 3, 4 and 5, however no amplification signal was seen in NT-C. M is 100 bp Gene Ruler (Fermantas, USA), +ve = positive control pBI121 plasmid; NT-C= non-transformed *N. tabacum* cv. Xanthi; bp=base pair.

Table 1: Mortality of *Bemisia tabaci* on leaves of non-transformed and Transgenic (T_1) *Nicotiana tabacum* lines (NT-1, -2, -3, -4, -5) following leaf feeding assay.

Nicotiana tabacum lines	Day 3	Day 6
NT-C	16.73 ± 5.32^{ab}	27.54 ± 5.88^{b}
NT-1	23.7 ± 4.24^{b}	44.43 ± 7.49^{c}
NT-2	28.95 ± 1.59^{b}	57.98 ± 1.32^{cd}
NT-3	28.03 ± 1.27^{b}	46.56 ± 5.62^{c}
NT-4	41.96 ± 9.05^{c}	76.18 ± 8.27^{de}
NT-5	51.89 ± 6.62^{c}	80.31 ± 5.10^{e}

Note: Percent mortality of *B. tabaci* feeding on transgenic leaves of tobacco was calculated as Mean ± SD and analyzed by One-Way ANOVA ($p<0.05$). Means followed by same letter in the same column are not significantly different by Duncan's multiple range tests at 5%.

line NT-1 revealed a minimum transgene expression as evident from intensity of PCR amplicons. Whereas, expression of Actin gene (internal control) was almost similar in all the lines and non-transformed control (Figure 3).

Northern blot analysis of transgenic plants

All the transgenic and non-transformed *N. tabacum* lines showed hybridization signals with the DIG-labelled DNA probe carrying ghr-MIR166b sequence. The differential intensity of hybridizing signals reflected the corresponding titer of ghr-miR166b in the respective lines. The line NT-5 displayed strongest signals, thereby suggesting the highest expression of ghr-miR166b in this line. Interestingly, line NT-1 showed ghr-miR166b expression slightly higher than those non-transformed *N. tabacum* (Figure 4).

Leaf feeding assay of transgenic lines

The leaf feeding assay of *B. tabaci* on transgenic lines demonstrated

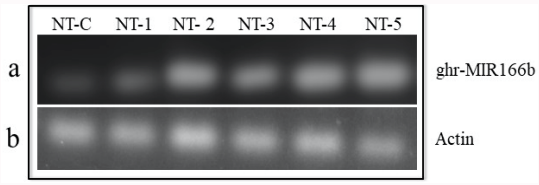

Figure 3: Reverse Transcriptase PCR amplification of ghr-MIR166b transcript in a non-transformed control (NT-C) and transgenic (T_1) *Nicotiana tabacum* cv. Xanthi lines NT-1, -2, -3, -4, -5. b Actin gene was used as internal control.

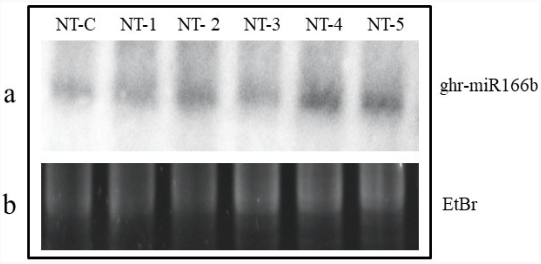

Figure 4: Detection of mature miRNA through northern hybridization. Small (s) RNA was isolated from non-transformed control (NT-C) and transgenic (T_1) *Nicotiana tabacum* cv. Xanthi lines NT-1, -2, -3, -4, -5. a: sRNA resolved through Polyacrylamide Gel Electrophoresis; b: transferred onto Hybond N+ and hybridized with DIG-labeled-antisense ghr-MIR166b probe. M: Ultra-low range DNA ladder (Fermantas, USA) was used for estimation of size.

significant reduction in *B. tabaci* population, post feeding. Comparatively, higher numbers of live *B. tabaci* were observed on non-transformed leaf whereas transgenic leaf showed dead *B. tabaci* (Figure 5). Rate of mortality was recorded on each transgenic line and non-transformed control leaves at 3rd and 6th days post-feeding (Table 1). Transformed line NT-5 showed a maximum reduction of about 80% in *B. tabaci* population while transformed line NT-1 showed minimum as compared to non-transformed tobacco.

Whole plant assay of transgenic *N. tabacum* lines

Transgenic (T_1) *N. tabacum* lines showed reduction in percentage survival of insect population, whereas non-transformed *N. tabacum* plants had an increase in *B. tabaci* population. The data on insect survival was significant following Dunnett's multiple comparison test comparing mean survival of *B. tabaci* on three plants each of transgenic line and non-transformed control tobacco.

Results of whole plant assay were in accordance with that of leaf disc assay. Transgenic line NT-1 showed ~37% mortality (Figure 6, Day 15, bar), while line NT-5 recorded ~77% mortality (Figure 6, Day 15, bar).

Detection of *Begomovirus* in the transgenic lines

Transgenic lines NT-2, -3, -4, -5 did not reveal any amplification

Table 2: Inheritance of the transgene in the T_1 progeny of the transgenic *Nicotiana tabacum* cv. Xanthi lines.

Nicotiana tabacum cv. Xanthi line	Number of seeds placed	Number of seeds germinated (O_i)	E_i	$(O_i-E_i)^2/E_i$
NT-1	27	20	20.25	0.003
NT-2	22	16	16.5	0.015
NT-3	25	19	18.25	0.031
NT-4	28	20	21	1
NT-5	27	21	20.25	0.277
$\chi^2 = \Sigma(O_i-E_i)^2/E_i$				1.329

The percent germination rate of transformed seeds of *N. tabacum* cv. Xanthi lines (NT-1, -2, -3, -4, -5) followed the expected 3:1 ratio of Mendelian inheritance [43]. The Chi-Square analysis showed the data was significant with p (χ^2, with df=1, ≥ 0.004) = 1.329

Figure 5: Leaf feeding bioassay of *Bemisia tabaci* (a) live *B. tabaci* on non-transformed *Nicotiana tabacum* leaf. (b) Leaf of transgenic (T_1) NT-5 showing dead *B. tabaci*, on 6th day post feeding.

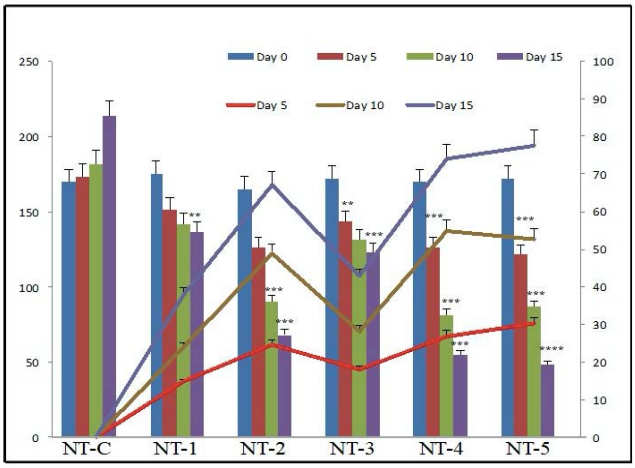

Figure 6: The number of *Bemisia tabaci* survived and % mortality of the insect was plotted for all the five transgenic (T_1) Nicotiana tabacum lines (NT-1, -2, -3, -4, -5). Bars = number of *B. tabaci*; lines = % mortality compared to control on 5, 10 and 15 days. Experiment was performed with three plants of each transgenic *N. tabacum* cv. Xanthi line and non-transformed control tobacco. Statistical analysis was performed by 2-way ANOVA and Dunnett's multiple comparison test. Statistical analyses were performed using GraphPad Prism 6.01 software. All values were processed as mean ± SD; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ was considered as significant difference in all the experiments.

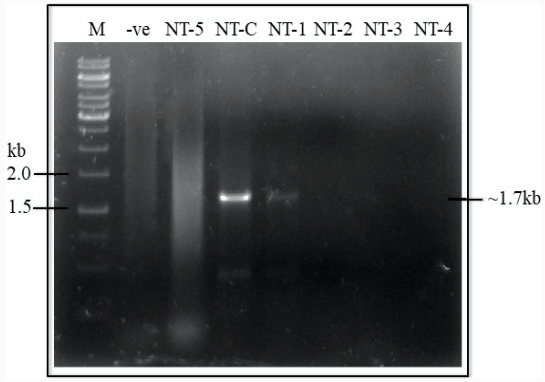

Figure 7: Rolling Circle Amplification- PCR based detection of Cotton leaf curl virus segment (encoding coat, precoat and replicase genes; size ~1.7 kb) in Transgenic (T_1) *Nicotiana tabacum* cv. Xanthi lines (NT-1, -2, -3, -4, -5). Total genomic DNA was isolated from systemic leaf of the transgenic *N. tabacum* plants NT-1, -2, -3, -4, -5, non-transformed tobacco and subjected to RCA-PCR.

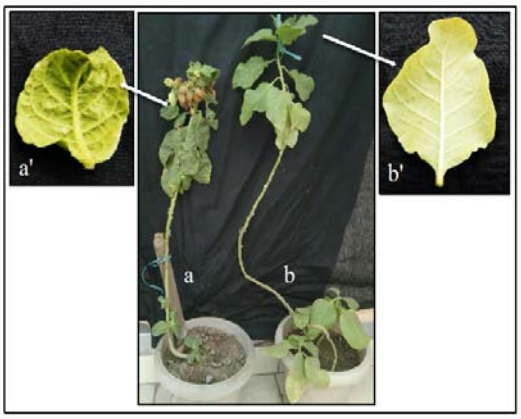

Figure 8: Development of Cotton Leaf Curl Disease (CLCuD) symptoms in non-transformed *Nicotiana tabacum* cv. Xanthi plant after inoculation with viruliferous *Bemisia tabaci*. Enlarged view of a leaf from non-transformed plant showing CLCuD symptoms of leaf curling and vein thickening (a') and transgenic (T_1) NT-5 showing normal phenotype (b'). Four-month-old plants infested with viruliferous *B. tabaci* are shown.

of the CLCuV. Whereas, NT-1 and non-transformed control tobacco plant yielded DNA fragment of ~1.7 kb, indicating the presence of *Begomovirus* in Transgenic (T_1) NT-1 and non-transformed *N. tabacum* plants (Figure 7). Moreover, the transgenic plants remained healthy while non-transformed plants developed characteristic symptoms of Cotton Leaf Curl Disease (CLCuD, Figure 8).

Discussion

Plants producing desirable traits have been a boon of the advanced genetic engineering technologies. RNAi being the most popular and effective strategy to control the expression of desirable genes of the host as well as the infecting pests and pathogens. The RNAi molecules, either in the form of double stranded RNA, intron hairpin RNA or miRNA, have delivered tremendous opportunities for producing plants with desired characters and efficient strength against the devastating pests and pathogens causing enormous losses in the production of the agricultural crops [4,35].

Movement of RNAi molecules not only within an organism but also into the interacting pests and pathogens have opened a notable acreage of Cross-Kingdom RNAi [36]. Significant protection against fungi, bacteria, viruses and pests have been generated employing host encoded small RNA molecules, opening domains for modern crop protection [15,37-39].

Plant Mediated- RNAi (PM-RNAi) molecules, such as dsRNA [40-42], artificial miRNA/miRNA [1,23] against insects provide efficient crop protection and proves its supremacy over chemical pesticides.

In this study, seeds of transgenic *N. tabacum* lines were placed on Kanamycin supplemented medium promoting the selective growth of transgenic seeds. Mendelian ratio (the expected germination ratio of 3:1) was calculated for each *N. tabacum* line. The percent germination rate of transgenic seeds of *N. tabacum* lines followed the expected 3:1 ratio of Mendelian inheritance [43] and statistically tested significant by *Chi Squ*are test.

The transgenic lines were further confirmed for the presence and integration of ghr-miR166b cassette. RT-PCR analysis of the Transgenic (T_1) *N. tabacum* lines was consistent with the quantitative real time-PCR-based results of the Transgenic (T_0) lines [23]. Though

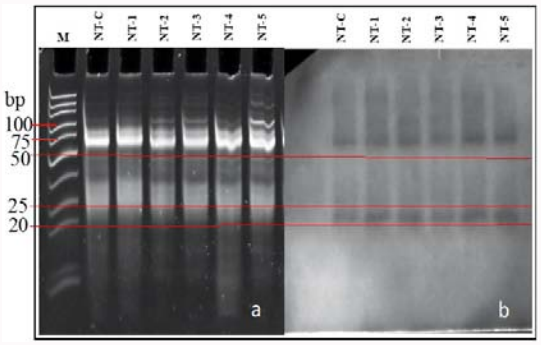

Figure 4': (For Reference).

transgenic lines NT-1 and NT-3 demonstrated a lower expression of the transgene, it was much higher in lines NT-2, -4, -5 as compared to non-transformed *N. tabacum* plants of the same age. Among various lines, line NT-5-showed highest expression and NT-1 showed lowest expression, which was slightly higher than that of the non-transformed plants (Figure 3).

The ghr-MIR166b seems to be processed efficiently in the transgenic *N. tabacum* plants. Although there were slight differences between the backbones of miRNA166b in both *N. tabacum* and *G. hirsutum*, the mature miRNA sequence and the RISC binding regions are similar in the both the plants, which leads to efficient processing of ghr-MIR166b into mature ghr-miR166b in the tobacco plants.

The processing of ghr-MIR166b to mature ghr-miR166b in the transgenic lines was confirmed following northern hybridization of small RNAs. The intensity of hybridization signals, corresponding to mature ghr-miR166b was different in all the transgenic lines. A high intensity hybridization signal was seen in the transgenic line NT-5. Interestingly, corresponding to highest expression of ghr-miR166b, highest whitefly mortality was achieved on the transgenic *N. tabacum* line NT-5, while in NT-1 (30%) showed minimum decline in the *B. tabaci* population.

Further, the transgenic lines were checked for the presence of whitefly-transmitted *Begomovirus*. The *Begomovirus* symptoms were absent in all the transgenic lines. The RCA failed to detect viral DNA in lines NT-2, -3, -4, -5. This could be attributed to the ability of ghr-miR166b to target replicase *(C1)* of CLCuV [30]. However, begomoviral DNA was detected in line NT-1 and non-transformed control, this may be due to the lesser expression of ghr-miR166b in these lines. Moreover, non-transformed control exhibited characteristic CLCuD symptoms induced by the *Begomovirus* whereas no symptoms were seen in NT-1.

Conclusively, ghr-miR166b possesses the capability of downregulating mitochondrial ATP synthase gene of *B. tabaci* in both tobacco and cotton plants. Thus, advocating the potential of host miRNAs in targeting the vital genes of infesting pests *via* cross-kingdom gene silencing. Furthermore, *in silico* analysis may provide a repertoire of essential target genes and the RNAi molecules, silencing those genes. Tobacco plants serves as a brilliant model system for producing and validating the *in-silico* findings *via* transgenics, thereby reducing the labor-intensive tissue culture experiments in recalcitrant plants. PM RNAi is now considered as a promising strategy for insect herbivory opening the possibility of next generation pest management.

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