

amiR414e-mediated silencing of overlapping *C1/C4* genes confers resistance to Cotton leaf curl Multan virus in *Gossypium hirsutum* cv. HS6

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Research Article

Keywords: RNA silencing, artificial microRNA, *Gossypium hirsutum*, Transformation, Begomovirus, CLCuMuV

Posted Date: December 2nd, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-8249670/v1>

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Additional Declarations: The authors declare no competing interests.

Abstract

Background

Cotton leaf curl disease (CLCuD), caused by monopartite begomovirus in association with satellite molecules, remains a serious threat to cotton production in the Indian subcontinent. Conventional control approaches have shown limited success, whereas RNA interference (RNAi) has proven an effective antiviral approach.

Methods

A synthetic artificial (a) miR414e was engineered to target the overlapping regions of the *C1* and *C4* genes of Cotton leaf curl Multan virus (CLCuMuV). The pre-amiR414e construct was cloned into the pBI121 vector and transformed into *Gossypium hirsutum* cv. HS6. Molecular analyses were conducted using PCR and quantitative real-time PCR (qRT-PCR).

Results

Transgenic cotton plants expressing amiR414e were successfully developed, and the transgene showed expected Mendelian inheritance in the T_1 and T_2 generations. Majority of the plants remained asymptomatic following CLCuMuV inoculation, while a few developed mild symptoms. At 60 days post-inoculation (dpi), PCR assays failed to detect CLCuMuV in all the asymptomatic plants, demonstrating a high degree of resistance in the T_1 and the T_2 lines. Additionally, transgenic (T_2) lines overexpressing amiR414e effectively downregulated *C1* gene transcripts, showing a negative correlation between amiR414e and the *C1* gene accumulation.

Conclusion

In this study, expression of amiR414e in *G. hirsutum* cv. HS6 conferred stable resistance against CLCuMuV, with majority of the plants remaining asymptomatic. These findings demonstrate the potential of amiRNA-mediated gene silencing as a viable strategy for developing CLCuMuV resistant cotton.

Introduction

Cotton (*Gossypium hirsutum* L.) ranks among the world's most important fibre and cash crops globally, cultivated for its textile fibre, edible oil, and paper [1]. Recently, India has emerged as the largest producer of cotton, sustaining the livelihoods of millions of farmers [2]. However, cotton production faces severe biotic constraints, the foremost being cotton leaf curl disease (CLCuD). The disease is caused by one of a monopartite begomoviruses, predominantly Cotton leaf curl Multan virus (CLCuMuV)

along with its associated satellites [3]. Infected plants exhibit characteristic symptoms such as vein thickening, leaf curling, and enation formation, ultimately resulting in significant yield losses and deterioration of fibre quality [4].

CLCuMuV is a whitefly (*Bemisia tabaci*)-transmitted begomovirus belonging to the family *Geminiviridae*. Its genome is monopartite, comprising a circular single-stranded DNA (ssDNA) molecule of approximately 2.7 kb [5]. The virus encodes multiple proteins with distinct functions: Rep (C1) and REn (C3) are involved in replication; C2 acts as a transcriptional activator; C4 and C5 function in host defence suppression; and V1 and V2 are responsible for genome encapsidation and movement within the plant [6, 7].

The Rep protein is a multifunctional, conserved protein essential for viral replication and contains three conserved motifs (I, II, and III) within its N-terminal region. It is also involved in transcriptional regulation, suppression of gene silencing, and interaction with various host factors, making it a potential target for engineering resistance against geminiviruses [8, 9].

These intricate molecular interactions between virus and host underscore the potential of RNA interference (RNAi)-based approaches, particularly artificial microRNAs (amiRNAs), to precisely target crucial viral genes, thereby offering a promising and durable strategy for conferring resistance to CLCuD in cotton, which is the focus of the present study.

Efforts to breed for CLCuD resistance have had limited success, largely because of the scarcity of resistant germplasm and the frequent emergence of resistance breaking CAB (CLCuD-associated begomoviruses) strains [5, 10, 11]. However, RNAi-based approaches have shown efficacy in controlling plant viruses, and in some cases their insect vectors [12–17]. RNAi is a conserved phenomenon in eukaryotes wherein small RNA (sRNA) molecules mediate sequence specific gene silencing at transcriptional or post-transcriptional level [18, 19]. The two major classes of sRNAs are small-interfering RNA (siRNA) and microRNA (miRNA), which share common pathways for biogenesis and mode of action [20]. The siRNAs (21–24 nt.) are generated from long double-stranded RNA (dsRNA) precursors via the action of Dicer like proteins (DCLs) [21]. A major drawback of this strategy is the diversity of generated siRNAs may lead to off-target effects if unintended mRNA are also targeted [22]. On the other hand, plant miRNAs originate from MIR genes and are transcribed as pri-miRNAs. These fold into hairpin structures and are processed by DCLs into mature miRNA duplexes, one strand of which guides the RNA induced silencing complex (RISC) containing Argonaute (AGO) to target mRNA for cleavage or translational repression. [19, 23, 24]. In addition to regulating host development process, *in-silico* analysis has identified plant miRNAs with potential binding sites in begomovirus genomes, associated satellite DNA molecules, and expressed sequence tags (ESTs) of insect vectors [17, 25]. The overexpression of ghr-miR398 and ghr-miR2950 targeting genes of CLCuMuV and its satellite (CLCuMB) has been shown to attenuate CLCuD symptoms [12].

However, not all the miRNAs exhibit antiviral properties owing to limited or tissue-specific expression, the presence of virus silencing suppressors or rapid evolution of viral sequences can evade miRNA

pathway. To overcome these obstacles, artificial miRNA (amiRNA) can be engineered by altering mature miRNA sequences without disrupting their normal biogenesis [26–29].

In this study, we developed an amiRNA-based strategy to generate transgenic *G. hirsutum* cv. HS6 plants resistant to CLCuD. The transgenic plants express amiR414e designed against the overlapping C1/C4 region of CLCuMuV, and effectively suppress viral accumulation. To our knowledge, this is the first report harnessing amiRNA-based strategy to silence the *C1* transcripts of CLCuMuV in transgenic *G. hirsutum* cv. HS6 plants.

Materials and methods

Development of expression vector

Previously *in-silico* identified miR414e, that exhibits sequence homology with the overlapping regions of *C1/C4* genes of CLCuMuV [25], was modified by replacing mismatched (A, C and U), and gapped (nucleotide 10 and 11 at the 5' end) of miR414e nucleotides with the matching nucleotides to achieve a perfect match with the viral targets. The native *G. hirsutum* pre-miR414e served as the backbone in which the mature miR414e sequence was replaced with the designed amiR414e sequence (5'-TCATCTTCACGTGCTCATCCAGT-3'), henceforth referred to as pre-amiR414e (Supplementary Fig. S1). The pre-amiR414e sequence was repeated in tandem three times and synthesized (GenScript, USA). The 23-nucleotide amiR414e sequence was screened across the cotton genome to ensure absence of off-target matches. The secondary structure of pre-amiR414e was predicted using the MFOLD (<http://www.unafold.org/mfold/applications/rna-folding-form.php>) program with default settings (Supplementary Fig. S2).

The ~ 395 synthetic pre-amiR414e fragment was cloned into the binary vector pBI121, under control of the CaMV 35S promoter and NOS terminator. The developed plant expression vector, henceforth referred to as pBI121-pre-amiR414e, harbouring a pre-amiR414e sequence was mobilized into *Agrobacterium tumefaciens* strain LBA4404 to transform cotton.

Agrobacterium-mediated transformation of *G. hirsutum* cv. HS6

G. hirsutum cv. HS6 explants were transformed with the pBI121-pre-amiR414e vector via *Agrobacterium*-mediated transformation [30]. Surface-sterilized seeds of *G. hirsutum* cv. HS6 were germinated *in vitro* on MS medium (HiMedia, India). Shoot-tip explants were excised and co-cultivated for 480 hr/28⁰ C in the dark with *A. tumefaciens* strain LBA4404 cells harbouring pBI121-pre-amiR414e. After co-cultivation, explants were transferred on the hormone-free selection medium for plantlet regeneration. Regenerated plantlets with well-developed roots and leaves were acclimatized in sterile Hogland medium, then transferred to mixture of soil, sand and peat moss (1:1:1) for hardening in strict greenhouse conditions.

Molecular screening of transgene

The total genomic DNA was isolated from twelve primary transformants (T_0), sixty-five T_1 , and eighty-four T_2 progeny plants as well as non-transformed controls using a plant DNA isolation kit (QIAGEN, Germany). The genomic DNA of each plant served as template for PCR-based detection of amiR414e transgene. The PCR mix consisted of 10 μ L of GoTaq® Green Master Mix, 2X (Promega, USA), 0.5 μ L of each *nptII* forward and reverse primers (5 pmol each), 2 μ L genomic DNA template (~ 100 ng). The thermal conditions included initial denaturation at 94°C for 5 minutes, followed by 32 cycles of 94°C for 40 seconds, 55°C for 30 seconds, and 72°C for 50 seconds, with a final extension at 72°C for 7 minutes. PCR products were resolved on agarose-EtBr gels, and visualized using a gel documentation system (Gel Doc™ XR + Imaging System, BioRad, USA).

Mendelian segregation analysis of transgene

Five-independent T_0 ($T_{0.2}$, $T_{0.9}$, $T_{0.13}$, $T_{0.19}$, $T_{0.20}$) events were selected and self-pollinated to raise T_1 progenies. Likewise, 15–20 seeds from five T_1 lines ($T_{1.2.5}$, $T_{1.9.9}$, $T_{1.13.11}$, $T_{1.19.1}$, $T_{1.20.8}$) were germinated to produce T_2 progeny plants. PCR detection of transgene was performed as described above, and segregation ratios were analysed using the chi-squared test to assess conformity to Mendelian inheritance.

Virus inoculation of plants

To assess the level of CLCuMuV resistance in transgenic cotton plants, they were inoculated with viruliferous whiteflies (*B. tabaci*) carrying CLCuMuV, as essentially described previously [31]. Inoculated plants were maintained in a controlled glasshouse and monitored every 15 days over a 60-day period for symptom development.

Detection of CLCuMuV in transgenic plants

Genomic DNA was extracted from transgenic and NT-control plants at 60 days post-inoculation (dpi). PCR was performed using coat protein (CP) gene specific primers for CLCuMuV [14].

RNA isolation and cDNA synthesis

Total RNA was isolated from 100 mg leaf tissue of transgenic (T_2) and NT plants [32]. The RNA was simultaneously poly (A) tailed and reverse transcribed to cDNA using the Mir-X™ miRNA First-Strand Synthesis Kit (Takara Bio, USA). The reaction was incubated at 37°C for 1 hour, then stored at -20°C, until further analysis.

amiRNA detection and quantification by poly (A) tailing-based qRT-PCR

Quantitative real-time PCR (qRT-PCR) was conducted to detect and quantify amiR414e expression using TB Green® Premix Ex Taq™ II SYBR Green chemistry (Takara Bio, USA) on a LightCycler® 96 System (Roche, Switzerland). An amiR414e-specific forward primer was designed with five unpaired nucleotides at its 3' end (Supplementary Table S1) to avoid pairing with the amiR414e sequence during PCR. The kit-supplied primer (mRQ3') was used as the reverse primer.

The qRT-PCR reaction comprised 5 µL of 2X TB Green Premix Ex Taq II master mix, 1 µL cDNA (0.5 ng), and 0.2 µL each of amiR414e/U6 gene-specific primers (0.2 µM). Cycling conditions included an initial denaturation at 95°C for 30 seconds, followed by 45 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and 72°C for 20 seconds.

PCR amplicons (~ 80 bp) were gel purified, cloned into a TA vector (Real Biotech Corporation, RBC, Taiwan), and sequenced to verify correct processing of amiR414e. Expression levels were normalized to U6 snRNA using U6 gene-specific primers [33]. All the reactions were performed in triplicate, experiment repeated three times and data were analysed using the $2^{-\Delta\Delta C_t}$ method [34].

Expression analysis of CLCuMuV-encoded C1 gene

Total RNA was isolated from the transgenic (T_2) and control plants at 60 dpi, and cDNA synthesized as described above. Expression of *C1* transcripts was quantified employing *C1*-specific primers (Supplementary Table S1) using TB Green Premix EX Taq II (Takara Bio, USA) on a LightCycler® 96 System (Roche, Switzerland) as described above. The qRT-PCR setup and conditions were similar to those described in the previous section, except that 2 ng of cDNA and 0.4 µM each of forward and reverse primers were used. The *GAPDH* gene (accession XM_041105532.1) served as the internal control. Each reaction was performed in triplicate, and the experiment was repeated three times. Relative expression was calculated using $2^{-\Delta\Delta C_t}$ method [34].

Statistical analysis

All results are presented as means based on independent experiments, with the number of replicates specified in figure legends. Differences in amiR414e expression, *C1* transcript level, and plant height at 60 dpi between transgenic and control plants were assessed by ANOVA followed by Tukey's test ($p < 0.05$). Distinct letters indicate statistically significant differences among transgenic and control plants. Error bars represent the mean $\pm$ standard deviation (SD). All statistical analyses were conducted using Origin software (<https://www.originlab.com/OriginProLearning.aspx>).

Results

Designing of amiRNA vector, transformation, and molecular analysis of cotton plants

The amiR414e was designed (Fig. 1A) to target the overlapping regions of C1 (motif II) and C4 of CLCuMuV (Fig. 1C), and replaced the native miR414e sequence in the pre-miR414e backbone to generate pre-amiR414e. The predicted secondary structure of pre-amiR414e indicated proper folding, enabling mature amiRNAs biogenesis (Supplementary Fig. S2). Following sequence verification, the synthetic pre-amiR414e was cloned into the pBI121 vector, producing pBI121-pre-amiR414e (Fig. 1B and D) that was successfully transformed into *A. tumefaciens* strain LBA4404.

G. hirsutum cultivar HS6 was transformed through *Agrobacterium* carrying pBI121-pre-amiR414e cassette, and regenerated on MS medium (Fig. 2).

A total of twelve putative T_0 transformants were obtained, of which ten independent transgenic (T_0) lines confirmed the presence of amiR414e transgene (Fig. 3A). Following inoculation of T_0 lines with viruliferous whiteflies, five lines ($T_{0.2}$, $T_{0.9}$, $T_{0.13}$, $T_{0.19}$, and $T_{0.20}$) remained asymptomatic and tested negative for CLCuMuV by PCR (Fig. 3B) at 60 dpi. These lines were selected to produce T_1 and T_2 progenies for further evaluation.

The stable inheritance of the transgene in the progeny was confirmed by PCR. In the T_1 generation, 51 out of 65 plants were PCR positive for 750 bp amplicons corresponding to the amiR414e transgene (Supplementary Fig. S3). In the T_2 generation, 64 of 84 plants were positive (Supplementary Fig. S4). These results establish stable transgene inheritance through both generations T_1 (Supplementary Fig. S5) and T_2 (Supplementary Fig. S6).

Segregation analysis (based on transgene presence confirmed by PCR) revealed that both T_1 (Table 1) and T_2 (Table 2) populations followed a 3:1 Mendelian ratio (confirmed by chi-square tests), indicating single-locus insertion and stable inheritance.

Table 1
Inheritance pattern of transgene in T₁ progeny of *G. hirsutum* cv. HS6 transgenic lines.

Transgenic lines	Total plants	Observed ratio		Expected ratio		χ^2 value
		Transgene present	Transgene absent	Transgene present	Transgene absent	
T ₁ .2	14	11	3	10.5	3.5	0.095
T ₁ .9	12	9	3	9	3	0.000
T ₁ .13	14	11	3	10.5	3.5	0.095
T ₁ .19	12	10	2	9	3	0.444
T ₁ .20	13	10	3	9.75	3.25	0.025
df = 1, p = 0.05, χ^2 tabulated value 3.84						

Table 2
Inheritance pattern of transgene in T₂ progeny of *G. hirsutum* cv. HS6 transgenic lines.

Transgenic lines	Total plants	Observed ratio		Expected ratio		χ^2 value
		Transgene present	Transgene absent	Transgene present	Transgene absent	
T ₂ .2.5	18	14	4	13.5	4.5	0.074
T ₂ .9.9	13	10	3	9.75	3.25	0.025
T ₂ .13.11	18	14	4	13.5	4.5	0.074
T ₂ .19.1	20	15	5	15	5	0.000
T ₂ .20.8	15	11	4	11.25	3.75	0.022
df = 1, p = 0.05, χ^2 tabulated value 3.84						

Symptom scoring, virus detection and agronomic trait analysis

By 15 dpi, the NT control began to show early symptoms (vein thickening), whereas no CLCuD symptoms were observed in the transgenic (T₁ and T₂) plants. At 30 and 45 dpi, characteristic symptoms of CLCuD appeared in the control plants, while only a few transgenics exhibited mild symptoms. By 60 dpi, control plants showed severe symptoms (leaf curling, enation formation, stunted growth), whereas most of the transgenics (Table 3 and Table 4; Fig. 4A-C; Supplementary Fig. S7) plants remained symptom free.

Table 3

Symptom severity scoring in transgenic (T_1) lines of *G. hirsutum* cv. HS6 expressing amiR414e and non-transgenic control plants following CLCuMuV inoculation.

Transgenic lines	Symptomatic plants/Inoculated plants			Symptom severity*	Resistance (%)
	30 dpi	45 dpi	60 dpi		
T ₁ .2	0/11	0/11	1/11	2	90.90
T ₁ .9	1/9	1/9	1/9	2	88.88
T ₁ .13	1/11	1/11	2/11	1–2	81.81
T ₁ .19	1/10	1/10	1/10	2	90.00
T ₁ .20	1/10	2/10	2/10	1–2	80.00
NT	7/10	8/10	10/10	4	0.00
dpi: days post inoculation					

*Symptom severity evaluation based on scale described by Nazeer et al. (2014) [35].

Table 4

Symptom severity scoring in transgenic (T_2) lines of *G. hirsutum* cv. HS6 expressing amiR414e and non-transgenic control plants following CLCuMuV inoculation.

Transgenic lines	Symptomatic plants/Inoculated plants			Symptom severity*	Resistance (%)
	30 dpi	45 dpi	60 dpi		
T ₂ .2.5	1/14	1/14	5/14	1–2	64.28
T ₂ .9.9	0/10	1/10	4/10	2	60.00
T ₂ .13.11	1/14	2/14	5/14	2	64.28
T ₂ .19.1	3/15	6/15	6/15	2	60.00
T ₂ .20.8	0/11	1/11	3/11	1–2	72.72
NT	6/10	10/10	10/10	4	0.00
dpi: days post inoculation					

*Symptom severity evaluation based on scale described by Nazeer et al. (2014) [35].

Interestingly, in the T_1 generation, transgenic plants displayed resistance ranging from 80%-90%, while in T_2 , resistance ranged from 60–72%, with symptom severity scores between 0 and 2 as described by

Nazeer et al. (2014) [35]. These findings clearly demonstrate improved resistance in transgenic lines compared to non-transgenic controls.

In T_1 , PCR tests on 51 transgenic plants showed that 44 asymptomatic plants were virus negative, while seven transgenics showing mild symptoms, and controls tested positive (Supplementary Fig. S8). In T_2 , 41 of 64 transgenics remained asymptomatic, 23 symptomatic (mild symptoms) transgenics and controls were PCR positive (Supplementary Fig. S9). Importantly, the symptomatic (T_2) plants yielded a significantly lower-intensity virus DNA fragment, suggesting reduced viral accumulation.

The impact of amiR414e expression on agronomic traits of transgenic cotton plants was evaluated at 60 dpi. Remarkably, no phenotypic abnormalities were observed in any transgenic generations (T_0 , T_1 , and T_2) compared to NT control plants, indicating that amiR414e does not target endogenous *G. hirsutum* cv. HS6 genes.

At 60 dpi, measurements of plant height in ten T_2 plants per line indicated a statistically significant height increase (16.31% to 24.91%) compared to controls (Fig. 4D). Flower buds and bolls development in transgenic proceeded normally, while infected controls showed markedly reduced boll size (Supplementary Fig. S10).

Over-expression of amiRNA suppresses C1 transcript levels

Using poly (A)-tailing qRT-PCR, amiR414e was detected in all five transgenic T_2 lines (T_2 .2.5.15, T_2 .9.9.6, T_2 .13.11.18, T_2 .19.1.20 and T_2 .20.8.4), with highest expression in T_2 .13.11.18 followed by T_2 .19.1.20, T_2 .9.9.6, T_2 .2.5.15 and T_2 .20.8.4 lines (Fig. 5A).

Sequencing of the processed amiR414e from (T_2 .13.11.18) line showed a 21-nucleotide mature form (rather than the designed 23-nucleotide) with a poly (A) tail near its 3' end (Fig. 5B), likely reflecting DCL1-mediated processing of the pre-amiRNA [36].

When C1 transcript levels were quantified by qRT-PCR at 60 dpi, all ami414e expressing lines showed reduced C1 expression compared to controls. The T_2 .13.11.18 line exhibited the lowest suppression followed by other lines in descending expression order (Fig. 5C).

Corelation between amiR414e and C1 expression

A negative correlation emerged between amiR414e expression and C1 transcript levels. Transgenic lines with higher expression (Fig. 5A) consistently showed a significant reduction in C1 suppression (Fig. 5C). This correlation supports the conclusion that amiR414e directs RNA-induced silencing complex (RISC)-mediated cleavage of C1 mRNA, thereby contributing to enhanced resistance against CLCuMuV infection.

Discussion

MicroRNAs can contribute to immunity by modulating host defence genes or directly targeting genes of invading pathogens [37, 38]. In this work, we focused on ghr-miR414e, which potentially binds the overlapping regions of C1/C4 in CLCuMuV. We hypothesised that converting miR414e into an artificial miRNA414e (amiR414e) and overexpressing it in *G. hirsutum* could confer protection against CLCuD.

The *C1* gene is highly conserved and essential for viral replication, while *C4* entirely embedded in *C1* in a different ORF acts as a silencing suppressor [39, 40]. We selected five T₀ transgenic plants that displayed no visible CLCuD symptoms or detectable CLCuMuV even eight-week post inoculation. Their progeny exhibited stable 3:1 Mendelian inheritance pattern, suggesting stable integration of the amiR414e cassette. This was statistically validated using a chi-square test, in agreement with previous reports [14, 41].

Most transgenics remained symptom free and virus negative, even two weeks post inoculation. Thus, advocating the efficacy of amiR414e to silence the *C1* gene. Conversely, the virus could be detected at significantly lower quantity in few lines that exhibited mild symptoms. This may be attributed to partial suppression of the *C1* transcripts due to lower expression of amiR414e. In contrast, control plants developed severe disease symptoms. Similar findings have been reported in *Nicotiana benthamiana*, where transient expression of amiRNAs targeting *C1* gene of Tomato yellow leaf curl virus Oman strain conferred resistance [42].

Variation observed in amiR414e expression levels among different lines may not be surprising. It could be due to differences in pre-amiRNA structure, processing efficiency or site effects during *Agrobacterium* transformation [43, 44]. A negative correlation observed between amiR414e expression and CLCuMuV accumulation is consistent with previous RNAi studies [28]. An overexpression of amiR414e effectively suppressed virus accumulation by downregulating *C1* gene expression. The native amiR414e sequence, comprising 21 nucleotides, was detected in the transgenic line, likely generated through DCL1-mediated processing of the pre-amiR [45].

Although the designed amiR414e sequence was predicted *in-silico* to have no off targets in *G. hirsutum*, it was not validated, which is a limitation. Interestingly, high amiR414e levels not only suppressed CLCuMuV infection, but also seemed to enhance agronomic traits, such as plant height, while maintaining normal boll development.

The resistance observed may be described as tolerance to CLCuMuV infection, wherein a low viral load is compromised without manifesting symptoms. In regions where fully immune cultivars are unavailable, tolerance may be valuable as it mitigates disease severity while sustaining growth [13, 46]. However, the presence of the virus in the tolerant plants coupled with the genetic variability within the begomoviruses responsible for CLCuD may pose a risk for emergence of new virus strains [47, 48].

Conclusion

This study demonstrates that amiRNA-mediated silencing of *C1* gene can confer effective resistance against CLCuMuV. *G. hirsutum* plants expressing amiR414e showed Mendelian inheritance of the transgene until the T₂ generation, and exhibited resistance against CLCuMuV, while most plants remaining asymptomatic. The observed downregulation of the *C1* gene is inversely correlated with amiR414e expression, further confirmed the effectiveness of amiR414e in suppressing virus accumulation. These findings suggest that an amiRNA approach holds promise for conferring resistance against whitefly-transmitted CLCuMuV, and potentially other related begomovirus species.

Declarations

Acknowledgements

University Grant Commission (Govt. of India) is acknowledged for the fellowships (JRF and SRF) to MK [F. No. 16-9(June 2018)/2019(NET-CSIR)] to carry out this work, and MS Baig for help with amiR414e designing.

Author contributions

MK: Data curation, Investigation, Methodology, Validation, Writing – original draft. JAK: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Supervision, Visualization, Writing – review & revising original draft

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability

The data generated and analysed in the current study are provided in the supplementary material.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

Not applicable.

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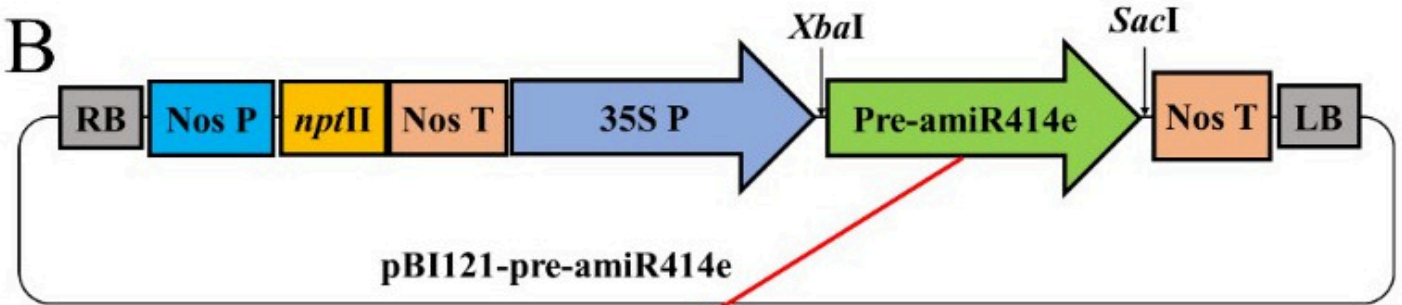
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Figures

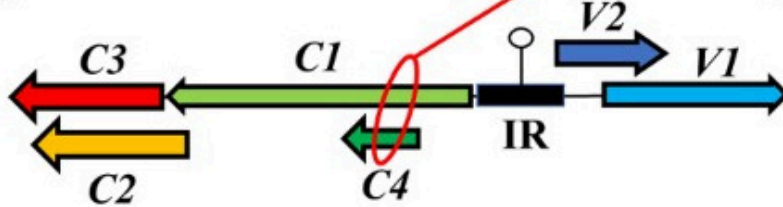
A

Accession number	ΔG (kcal/mol)	miRanda Score	Complem entarities %	Binding position	miRNA-Target Pairs
C1 gene					
DQ191160	-22.73	161	78.95	903-925	21 1 Query: 3' ccuCCUACU<u>ACT</u>--A<u>UUCUA</u>u 5' Ref: 5' actGGATGAGCACGTGAAGATGa 3'
C4 gene					
DQ191160	-22.73	161	78.95	271-293	21 1 Query: 3' ccuCCUACU<u>ACT</u>--A<u>UUCUA</u>u 5' Ref: 5' actGGATGAGCACGTGAAGATGa 3'

B



C



D

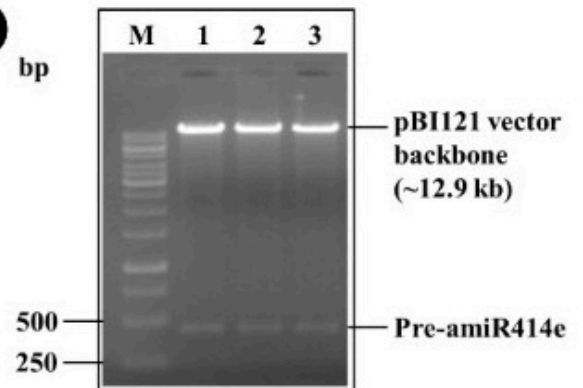


Figure 1

Designing and construction of expression vector. (A) Binding sites of *G. hirsutum*miR414e in *C1* and *C4* genes of CLCuMuV predicted by miRanda. Matches (|), gaps (-), seed region in red, central region (10–12 nt) in green. (B) pBI121-pre-amiR414e vector showing the pre-amiR414e cassette cloned between *Bam*HI and *Sac*I. (C) CLCuMuV genome depicting amiR414e target sites in the *C1* and *C4*genes (red circles). (D) Confirmation of pBI121-pre-amiR414e clones by restriction digestion. M:1 kb DNA marker; lanes 1-4: digested fragments.

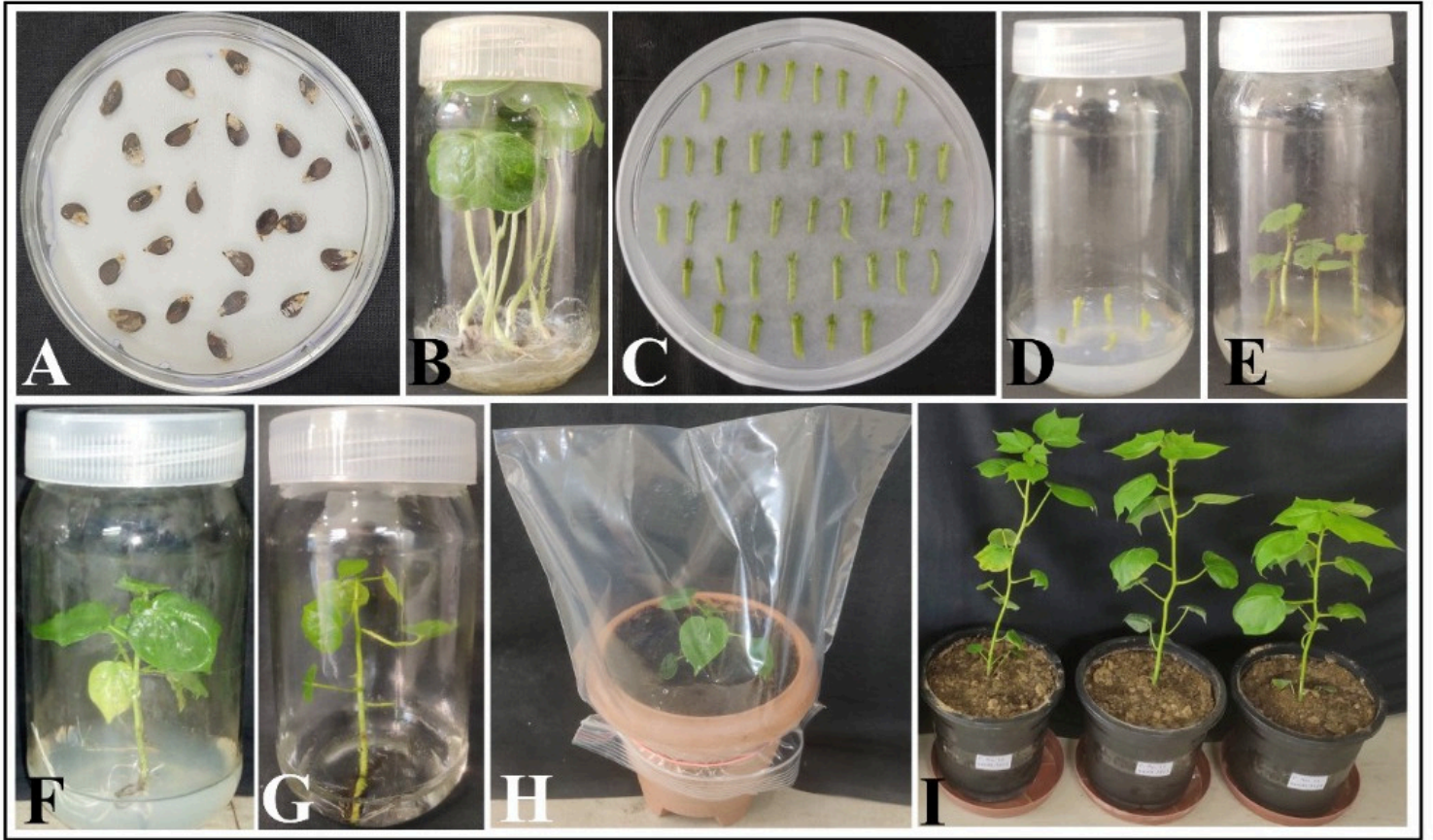


Figure 2

Agrobacterium-mediated transformation and regeneration of *G. hirsutum* cv. HS6. (A- B) Surface sterilized seeds and seedlings on MS medium. (C-D) Shoot tip explants during co-cultivation and selection. (E-G) Regenerated and rooted plantlets. (H-I) Hardened plantlets grown under controlled conditions in glasshouse.

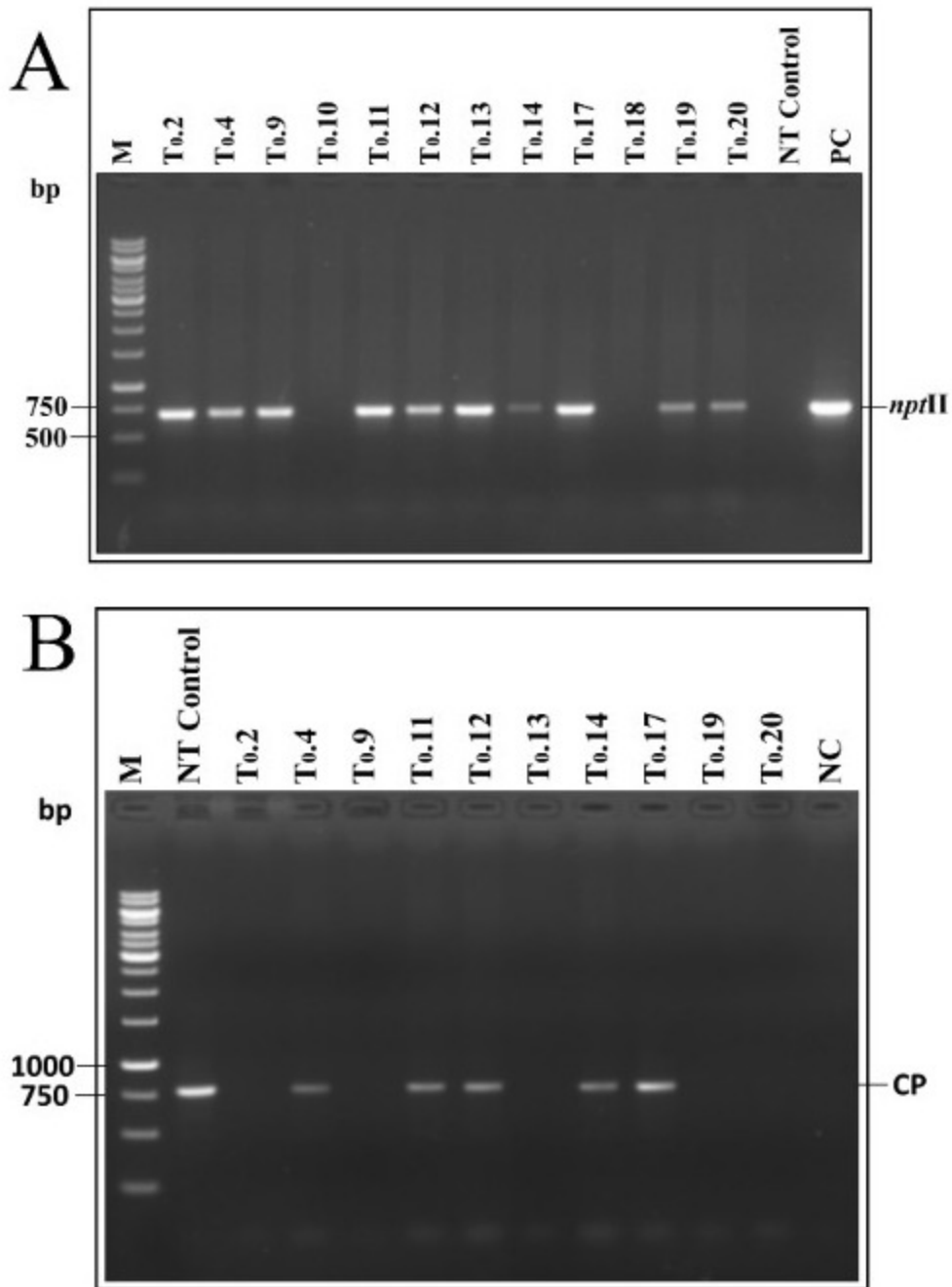


Figure 3

PCR-confirmation of transgene and viral gene in T_0 plants. (A) amplification of amiR414e transgene. (B) Amplification of CLCuMuV coat protein (CP) gene. M: 1 kb DNA marker, NT: non-transgenic control, PC: positive control; NC: Negative control.

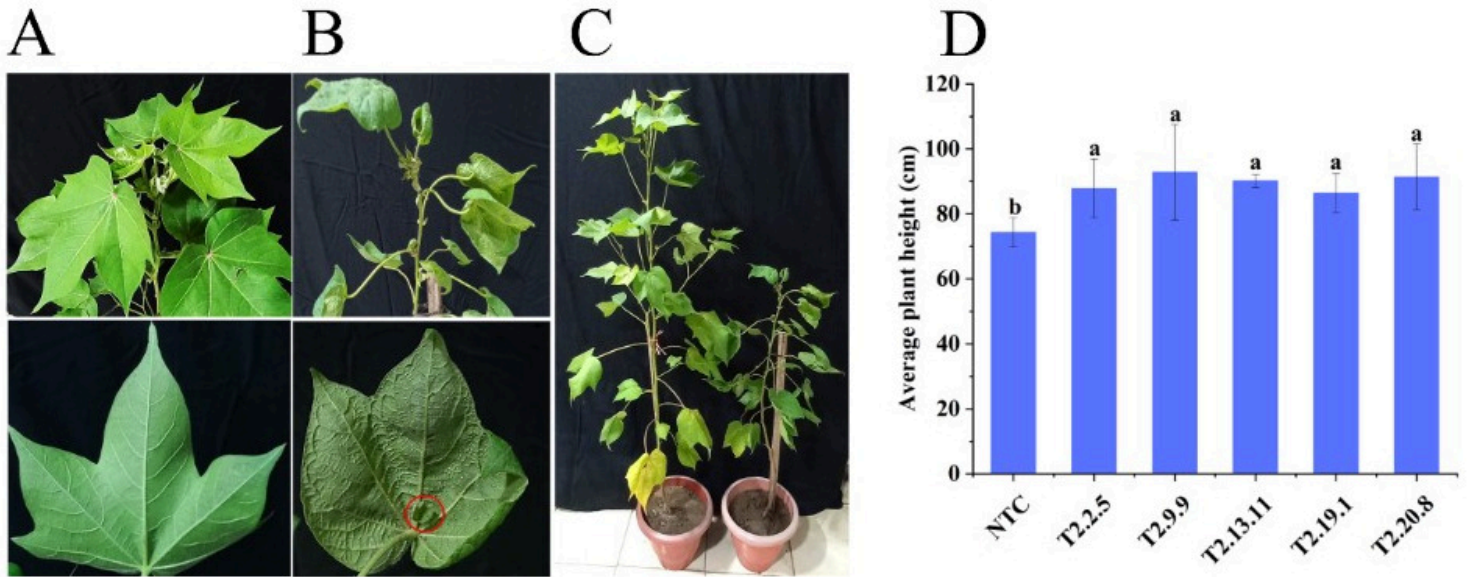


Figure 4

Disease symptoms and plant height in transgenic T_2 lines post-inoculation (dpi). (A-B) Leaves of transgenic and NT plants showing absence and presence of CLCuD symptoms, respectively. (C) Comparison of transgenic (left) and non-transgenic (NT) (right) plants. (D) Average plant height means ($\pm$ SD, $n=10$). Different letters indicate significant differences (Tukey test at $p \leq 0.05$).

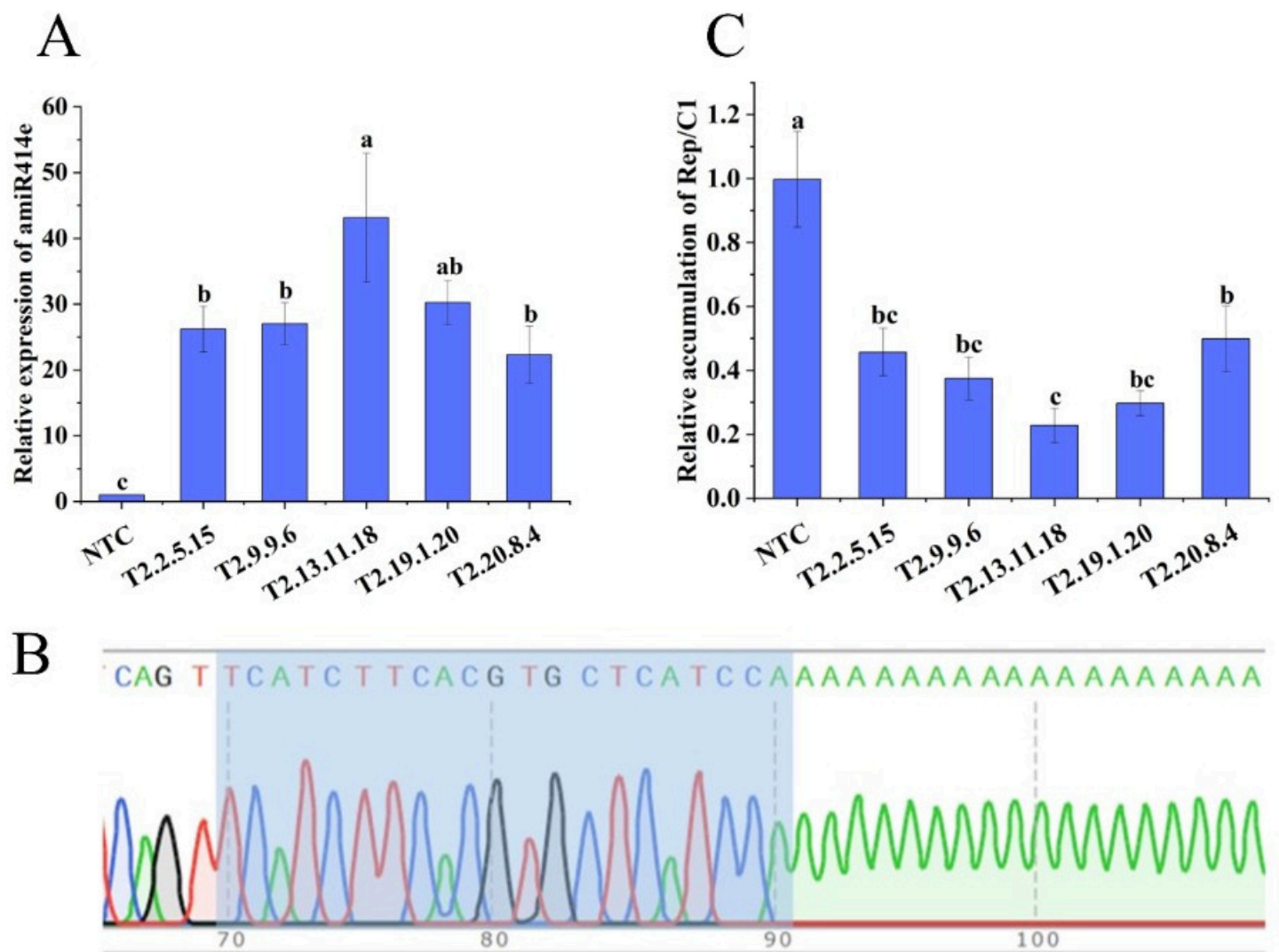


Figure 5

Expression of amiR414e and *C1* gene (A) Relative expression of amiR414e in transgenic T₂ plants of *G. hirsutum* cv. HS6 via poly-A- tailing-based qRT-PCR (mean $\pm$ SD, n=3) Different letters indicate significant variation ($p \leq 0.05$). (B) Nucleotide sequence variation of mature amiR414e (blue highlight). (C) Relative expression of *C1* gene in transgenic and NTC plants at 60 dpi (mean $\pm$ SD, n=3; Tukey's test, $p \leq 0.05$).

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