



OPEN ACCESS

EDITED BY

Tushar Ranjan,
Bihar Agricultural University, India

REVIEWED BY

Shraddha Bhaskar Sawant,
Bihar Agricultural University, India
Ranjeet Kaur,
Harlal Institute of Management and
Technology, India

*CORRESPONDENCE

Jawaid A. Khan
✉ jkhan1@jmi.ac.in

[†]These authors have contributed equally
to this work

RECEIVED 06 May 2026

REVISED 24 May 2026

ACCEPTED 29 May 2026

PUBLISHED 16 June 2026

CITATION

Khan M, Sharma D, Baig MS, Kuba S
and Khan JA (2026) Artificial miRNA-
based silencing of begomovirus
gene confers resistance to cotton
leaf curl disease in cotton.
Front. Virol. 6:1873471.
doi: 10.3389/fviro.2026.1873471

COPYRIGHT

© 2026 Khan, Sharma, Baig, Kuba and
Khan. This is an open-access article
distributed under the terms of the
[Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/)
(CC BY). The use, distribution or
reproduction in other forums is
permitted, provided the original
author(s) and the copyright owner(s) are
credited and that the original publication
in this journal is cited, in accordance
with accepted academic practice. No
use, distribution or reproduction is
permitted which does not comply with
these terms.

Artificial miRNA-based silencing of begomovirus gene confers resistance to cotton leaf curl disease in cotton

Masnad Khan, Divya Sharma, Mirza Sarwar Baig[†], Shweta Kuba[†]
and Jawaid A. Khan ^{*}

Plant Virus Laboratory, Department of Biosciences, Jamia Millia Islamia, New Delhi, India

Background: Cotton leaf curl disease (CLCuD), caused by a complex of begomoviruses and their associated betasatellites, severely reduces cotton productivity. Conventional breeding approaches have shown limited success due to the rapid evolution of viral populations and the involvement of the long duration breeding cycles. To address this challenge, the present study explores an artificial microRNA (amiRNA)-based strategy to confer durable resistance against CLCuD in *Gossypium hirsutum* cv. HS6.

Methods: A computationally predicted endogenous miR2949a was engineered to generate the artificial miRNA (amiR2949a), which was designed and computationally validated *in silico* to simultaneously target key genes (*C1*, *C3*, *C4*, *V1*, and *V2*) of *Cotton leaf curl Multan virus* (CLCuMuV, a begomovirus), as well as the β C1 gene of the betasatellites collectively associated with CLCuD. The engineered pre-amiR2949a construct was transformed into cotton via *Agrobacterium tumefaciens*-mediated transformation. Putative transgenic plants were subsequently inoculated with viruliferous whiteflies, and resistance was assessed through molecular approaches and phenotypic evaluation.

Results: The transgenic lines exhibited strong resistance to CLCuD showing 71–81% disease suppression, and a complete absence of disease symptoms and virus accumulation up to 60 days post-inoculation (dpi). Quantitative expression analysis of CLCuMuV, although limited to the *C1* gene, revealed a significant reduction (69%–90%) in its transcript levels. This reduction was accompanied by an inverse correlation between amiR2949a accumulation and *C1* expression, indicating efficient amiRNA-mediated silencing of viral gene.

Conclusion: These findings demonstrate that targeted suppression of the viral *C1* gene effectively restricts CLCuMuV accumulation and symptom development in cotton. Furthermore, the study establishes amiRNA-mediated RNA silencing as a robust and scalable strategy for engineering resistance against the cotton-infecting begomovirus complex, with potential applicability to other economically important crops.

KEYWORDS

artificial miRNA, begomovirus, CLCuMuV, cotton, RNA silencing, transformation

1 Introduction

RNA interference (RNAi) is a conserved phenomenon in eukaryotes that regulates gene expression, and acts as a primary antiviral defense response against invading pathogens via small RNA (sRNA) molecules (1). The two major classes of sRNAs i.e. small interfering RNAs (siRNAs) and microRNAs (miRNAs), mediate gene silencing at transcriptional and post-transcriptional levels based on their sequence complementarity with target transcripts. siRNAs are generated following cleavage of double-stranded RNA precursors by the Dicer-like enzymes (DCLs), whereas miRNAs are processed from endogenous MIR gene transcripts through a highly regulated biogenesis pathway involving DCL1 and AGO1, culminating in sequence-specific gene silencing (2, 3).

Cotton (*Gossypium hirsutum* L.) is a key fiber crop of global agricultural importance. However, its productivity is severely compromised by cotton leaf curl disease (CLCuD), a destructive disease induced by begomoviruses associated with satellite components (4–6). Among these, *Cotton leaf curl Multan virus* (CLCuMuV), in association with a betasatellite, predominates across the Indian subcontinent.

CLCuMuV possesses a monopartite, circular, and single-stranded DNA genome of ~2.7 kb. The V1 (coat protein) and V2 (pre-coat protein) are encoded by the virion-sense strand of the genome, while the complementary-sense strand is responsible for encoding proteins which participate in replication (C1/Rep, C3/REn), transcriptional regulation (C2/TrAP), and host defense suppression (C4) (7, 8). The betasatellite markedly enhances disease severity through the expression of β C1 protein, a key pathogenicity determinant that interferes with host RNAi pathway and promotes viral accumulation (9–11). The begomovirus-satellite interaction accelerates virus evolution and complicates the development of durable host resistance.

Breeding for CLCuD resistance has shown limited success due to scarce resistance source and the frequent emergence of resistance-breaking viral strains (12, 13). RNAi-based genetic approaches have therefore been explored as promising alternatives to achieve durable resistance against viruses. However, siRNA-mediated strategies often generate heterogeneous siRNA populations that can cause off-target silencing and raise biosafety concerns (14).

Although, artificial miRNA (amiRNA) technology offers a precise and efficient alternative for targeted gene silencing, its application to simultaneously target multiple begomoviral genes and associated betasatellites in cotton remains insignificantly explored. Further, studies demonstrating amiRNA-mediated suppression of critical virus replication genes in transformed cotton are limited.

In the present study, we attempt to address this limitation by engineering transgenic cotton plants expressing a single rationally designed amiRNA directed against the highly conserved C1 gene of CLCuMuV. In addition to experimentally validating suppression of C1, *in silico* analyses predicted that the engineered amiRNA could potentially target multiple begomoviral genes (C1, C3, C4, V1, and V2) as well as the β C1 gene of the associated betasatellite. Using this strategy, we demonstrate that amiRNA-mediated silencing

effectively reduces CLCuMuV accumulation and confers resistance to CLCuD in transgenic cotton (*G. hirsutum* cv. HS6). These findings highlight the potential of amiRNA-based antiviral engineering as a precise, scalable, and potentially broad-spectrum approach for developing durable virus resistance in cotton.

2 Materials and methods

2.1 Designing and construction of amiRNA expression vector

A cotton encoded microRNA, ghr-miR2949a, previously predicted to target overlapping regions of the C1 and C4 genes of *Cotton leaf curl Burewala virus* (CLCuBuV) (15), was selected as the amiRNA backbone (Supplementary Table 2). To enhance complementarity with conserved regions of viral target genes, mismatched base pairs between the endogenous mature miRNA and the viral targets were manually modified to create an efficient amiRNA. Viral target regions were selected based on sequence conservation and predicted target accessibility to maximize silencing efficiency. The engineered amiRNA demonstrated the ability to *in silico* target multiple genes from various cotton-infecting begomoviruses, as well as the β C1 gene of associated betasatellites (Supplementary Table 3). In the *G. hirsutum* pre-miR2949a backbone, the native mature miRNA duplex was manually changed by replacing with the modified amiR2949a-5p (5'-CCUUGUGUACUGGAUGA GCGCG-3') and amiR2949a-3p (5'-UGUGCUCAUCCAGCACACAAGGUA-3') sequences (Supplementary Figure 1). The modified pre-amiR2949a construct was synthesized commercially (GenScript, USA), and *in silico* analysis confirmed the absence of off-targets within the *G. hirsutum* genome. The secondary structure of the pre-amiRNA was predicted by the MFOLD program (<http://www.unafold.org/mfold/applications/rna-folding-form.php>).

The pre-amiR2949a (~335 bp) fragment was cloned into the pUC57 cloning vector and subsequently ligated into the pBI121 vector, downstream of the CaMV 35S promoter, generating pBI121-pre-amiR2949a, which was transformed into *Agrobacterium tumefaciens* strain LBA4404 (16).

2.2 Transformation of *G. hirsutum* cultivar HS6 and plant regeneration

The genetic transformation of *G. hirsutum* was carried out using an *Agrobacterium*-mediated method employing shoot tip explants (16). Following co-cultivation, explants were placed on MS selection medium, regenerated plantlets were rooted, acclimatized in Hoagland solution and, hardened under controlled greenhouse conditions, before transfer to soil.

2.3 Molecular confirmation and segregation analysis

Genomic DNA was extracted from the T₀, T₁, T₂, and NT control plants employing DNeasy Plant Mini Kit (QIAGEN, Germany). PCR-based detection of the *nptII* (*neomycin*

phosphotransferase II) transgene was performed using gene-specific primers (Supplementary Table 1) (17).

Two independent transgenic lines ($T_{0.18}$ and $T_{0.24}$) were self-pollinated to obtain T_1 progeny followed by T_2 generation development. Segregation of the transgene was assessed by PCR-based screening and inheritance patterns were evaluated using the χ^2 test assuming the 3:1 Mendelian ratio ($p \leq 0.05$).

2.4 Virus inoculation and symptom assessment

Resistance of transgenic plants to CLCuMuV was evaluated following viruliferous whiteflies (*B. tabaci*)-mediated inoculation (18). Plants were maintained under greenhouse conditions, and symptom development was recorded at 15-days intervals up to 60 days post-inoculation (dpi). Disease severity was assessed using a 0–4 scoring scale, and resistance percentage was calculated using the formula: Resistance (%) = [(total number of inoculated plants – number of symptomatic plants)/total number of inoculated plants] $\times$ 100. The assessment was carried out as described previously (13).

2.5 Virus detection

Genomic DNA from the inoculated plants and NT controls was used as the template for PCR-mediated CLCuMuV detection using primers specific for the coat protein (CP) gene (Supplementary Table 1) (19).

2.6 RNA isolation, cDNA synthesis and expression analysis

Total RNA was isolated from leaf tissues of transgenic (T_2) and NT control plants (20). cDNA synthesis and polyadenylation were performed employing the Mir-XTM miRNA First-Strand Synthesis Kit (Takara Bio, USA), following a 1 hr incubation at 37 °C. qRT-PCR was performed using the TB Green[®] Premix Ex TaqTM II (Takara Bio, USA) on a LightCycler[®] 96 System (Roche, Switzerland).

Expression of amiR2949a was quantified employing an amiR2949a-specific forward primer and the mRQ 3' reverse primer, with the U6 SnRNA as the internal reference (Supplementary Table 1) (21). *C1* gene expression was analysed using *C1* gene-specific primers, normalized to GAPDH (Supplementary Table 1) (22), and relative gene expression was calculated using the delta-delta Ct method (23). All reactions were carried out in three biological and three technical replicates.

2.7 Statistical analysis

The data were statistically analysed using one way analysis of variance (ANOVA), followed by Tukey's test ($p \leq 0.05$). Results are presented as mean $\pm$ standard deviation (SD), and statistical analyses were performed using OriginPro software (OriginLab, USA; <https://www.originlab.com/OriginProLearning.aspx>).

3 Results

3.1 Construction of amiRNA expression vector

An amiR2949a was rationally designed to simultaneously target key genes (*C1*, *C3*, *C4*, *V1* and *V2*) of cotton-infecting begomoviruses, and the $\beta C1$ gene of associated betasatellites (Figures 1A, B). *In silico* analysis revealed the highest sequence complementarity (77–82%) with the *C1* and *C4* genes across begomoviruses, including CLCuMuV, CLCuBuV, Cotton leaf curl Rajasthan virus (CLCuRV), Cotton leaf curl Kokhran virus (CLCuKoV) and Cotton leaf curl Bangalore virus (CLCuBV) with moderate complementarity (69–73%) to *V2*, *C3*, and $\beta C1$ genes.

The mature amiR2949a duplex was manually replaced with the modified amiR2949a-5p and amiR2949a-3p duplex sequences within the pre-amiR2949a backbone. Secondary structure prediction confirmed correct folding of the precursor (Supplementary Figure 2). The synthetic pre-amiR2949a was cloned in pBI121

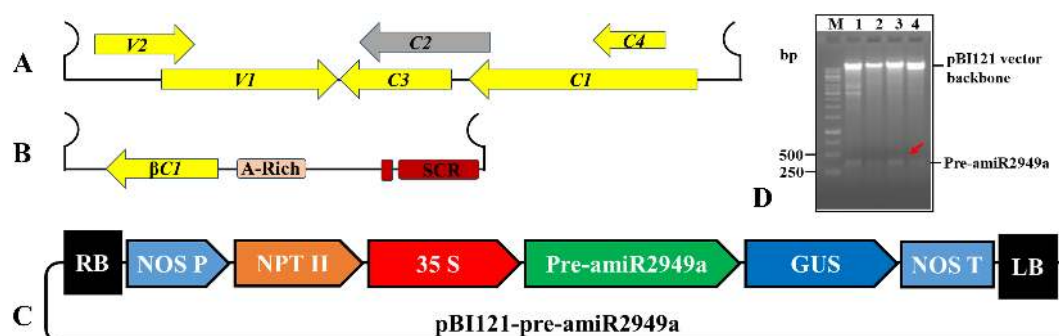


FIGURE 1

Construction of the amiRNA expression vector. Predicted amiR2949a target sites in (A) begomoviruses (B) betasatellites ORF(s). Target regions are highlighted in yellow (C) Schematic representation of the amiRNA expression cassette containing the sequence driven by CaMV 35S promoter. (D) Restriction analysis of amiRNA expression vector confirming integration of the pre-amiR2949a cassette (~335 bp). Lane M; 1 kb DNA ladder.

(pBI121-pre-amiR2949a) plasmid and subsequently transformed into *A. tumefaciens* strain LBA4404 (Figures 1C, D).

3.2 Transformation, transgene detection and inheritance analysis

Genetic transformation (Figures 2A–H) yielded six primary transformants (T_0) of *G. hirsutum*, of which five were PCR positive for the amiRNA transgene (~700 bp; Figure 2I). Two asymptomatic lines ($T_{0.18}$ and $T_{0.24}$), which showed no detectable virus accumulation at 60 dpi, were advanced for further analysis (Figure 2J).

Stable inheritance of the transgene was confirmed in T_1 (19/25) and T_2 (28/38) progeny (Supplementary Figures 3, 4). Segregation followed a Mendelian 3:1 ratio in both generations (Table 1) indicating predicted single-locus integration (24).

3.3 Evaluation of resistance to CLCuD

Non-transgenic plants developed typical CLCuD symptoms following inoculation with viruliferous whiteflies. However, most transgenic (T_1 and T_2) plants remained asymptomatic throughout the 60-days observation period (Table 2; Supplementary Figure 5).

PCR analysis confirmed the absence of CLCuMuV in asymptomatic transgenic plants, whereas mildly symptomatic transgenics and all the NT control showed virus accumulation (Figure 3; Supplementary Figure 6). Notably, viral DNA levels in symptomatic transgenic plants were significantly lower than those in the NT controls. On the basis of symptom severity scoring, transgenic lines displayed overall resistance levels of 75–81% (T_1) and 71–78% (T_2).

3.4 Overexpression of amiRNA suppress C1 expression

The qRT-PCR analysis revealed variable expression levels of amiR2949a among the tested transgenic (T_2) lines, with the highest expression detected in $T_{2.18.4.19}$ (Figure 4A). Correspondingly, C1 transcript levels were markedly reduced in all transgenic lines compared to NT controls, with maximal suppression observed in $T_{2.18.4.19}$ (Figure 4B).

A strong inverse correlation between amiR2949a and C1 expression indicates effective amiRNA-mediated silencing of the viral replication gene, thus contributing to reduced virus accumulation and enhanced CLCuD resistance.

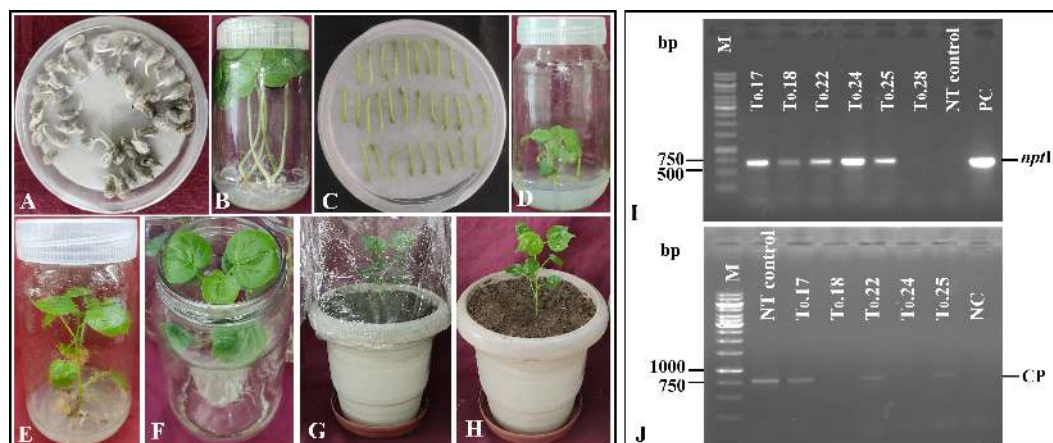


FIGURE 2

Agrobacterium-mediated transformation and screening of primary transformants of *G. hirsutum* cv. HS6. (A) *In vitro* germinated seeds. (B) Seedlings cultivated on MS medium. (C) Shoot-tip explants following *Agrobacterium* co-cultivation. (D, E) Two weeks old and four weeks old regenerated plantlets on selection medium, respectively. (F) Acclimatization in Hoagland solution. (G) Primary hardening under humidity conditions. (H) Fully hardened plants maintained under controlled glasshouse conditions. PCR-based detection of (I) *nptII* transgene (J) CLCuMuV coat protein (CP) gene. M, 1 kb DNA ladder; NT control, non-transgenic control plant; PC, Positive control (pBI121 plasmid DNA); NC, Negative control (uninoculated plant genomic DNA).

TABLE 1 Mendelian inheritance of the transgene in *G. hirsutum* cv. HS6.

Transgenic lines	Total plants	Observed ratio		Expected ratio		χ^2 value
		Transgene present	Transgene absent	Transgene present	Transgene absent	
$T_{1.18}$	11	8	3	8.25	2.75	0.030
$T_{1.24}$	14	11	3	10.5	3.5	0.095
$T_{2.18.4}$	19	14	5	14.25	4.75	0.017
$T_{2.24.7}$	19	14	5	14.25	4.75	0.017

Chi-square analysis showed no significant difference from the expected 3:1 ratio ($df = 1$, $p > 0.05$).

TABLE 2 Assessment of symptom severity in transgenic *G. hirsutum* lines following whitefly-mediated inoculation of CLCuMuV.

Transgenic lines	Symptomatic plants/Inoculated plants			Symptom severity*	Resistance (%)
	30 dpi	45 dpi	60 dpi		
T ₁ .18	2/8	2/8	2/8	2	75.00
T ₁ .24	0/11	1/11	2/11	2	81.81
NT	6/10	10/10	10/10	4	0.00
T ₂ .18.4	0/14	3/14	3/14	2	78.57
T ₂ .24.7	1/14	4/14	4/14	1-2	71.42
NT	7/10	9/10	10/10	4	0.00

Disease severity scoring was done as described previously (13). *0: asymptomatic; 1: mild symptoms; 2: moderate symptoms; 3: severe symptoms; 4: highly severe symptoms. dpi, days post inoculation; NT, non-transformed control.

3.5 Phenotypic assessment

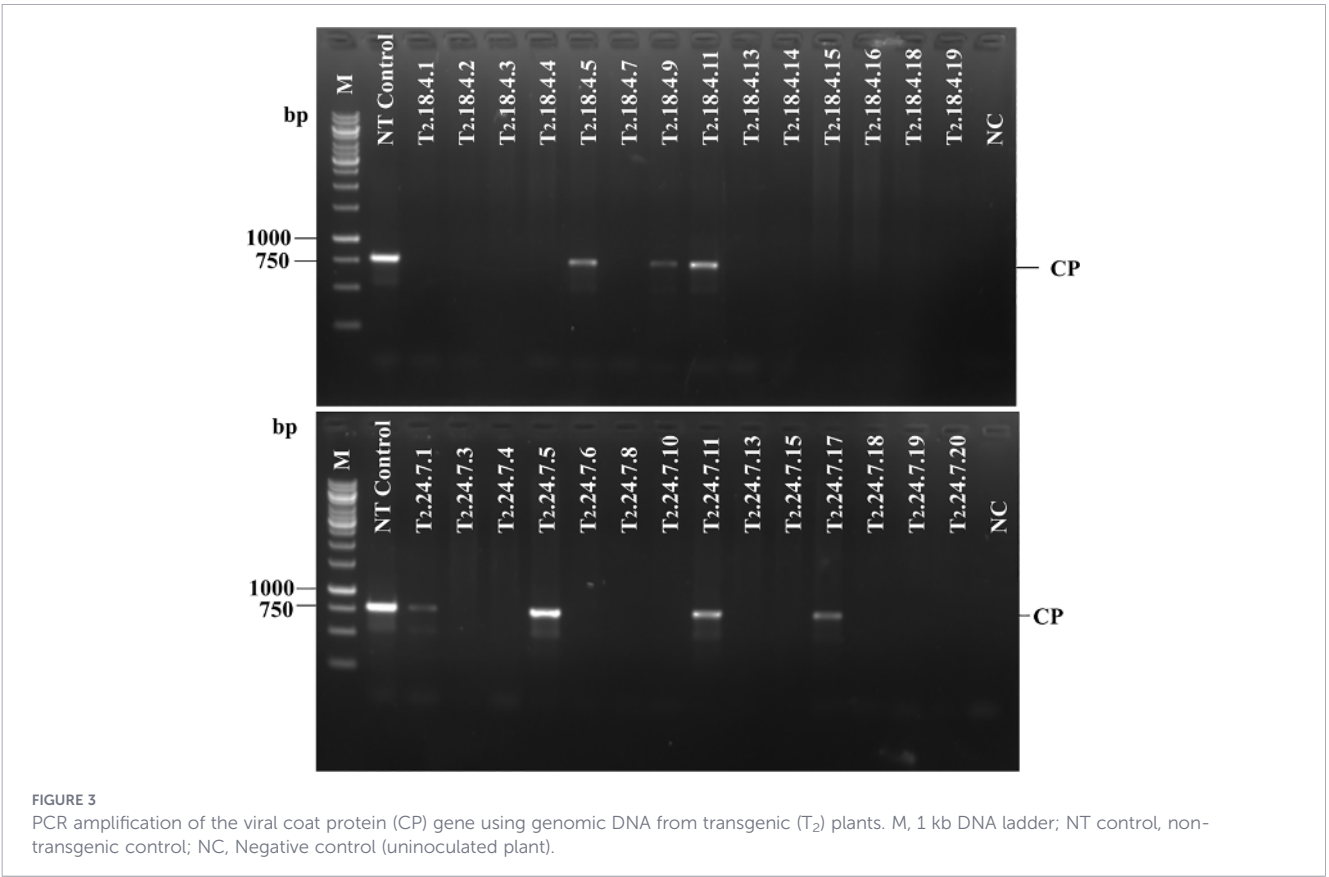
Transgenic lines displayed no detectable developmental abnormalities through the T₂ generation (Figures 4C–E, 5A). A significant increase in plant height (16%–24%) was observed in transgenic T₂ lines relative to NT controls (Figure 5B). Flowering and boll development remained normal in all transgenic plants, even though virus-infected NT plants exhibited reduced boll size (Supplementary Figure 7).

4 Discussion

RNAi operates as a homology-dependent, sequence specific pathway that modulates gene silencing, and constitutes a major

antiviral defense pathway in plants. Among different sRNA classes, miRNAs are central to plant immunity by regulating host defense genes, and in some cases directly targeting viral transcripts (25). The efficacy of RNAi-mediated resistance is often influenced by the degree of complementarity between sRNA and their targets, as well as by the functional relevance and positional context of the targeted viral gene (26–28).

In this investigation, an amiRNA-mediated approach was implemented to combat CLCuD infection. Transgenic *G. hirsutum* cv. HS6 plants were engineered to express a single amiRNA designed and *in silico* predicted to potentially target multiple essential viral genes of the begomovirus and the *βC1* gene of associated betasatellites. Unlike conventional siRNA approaches, which often generate heterogenous siRNA populations with variable efficacy and a risk of off-target effects (26, 29), amiRNAs are processed from endogenous miRNA



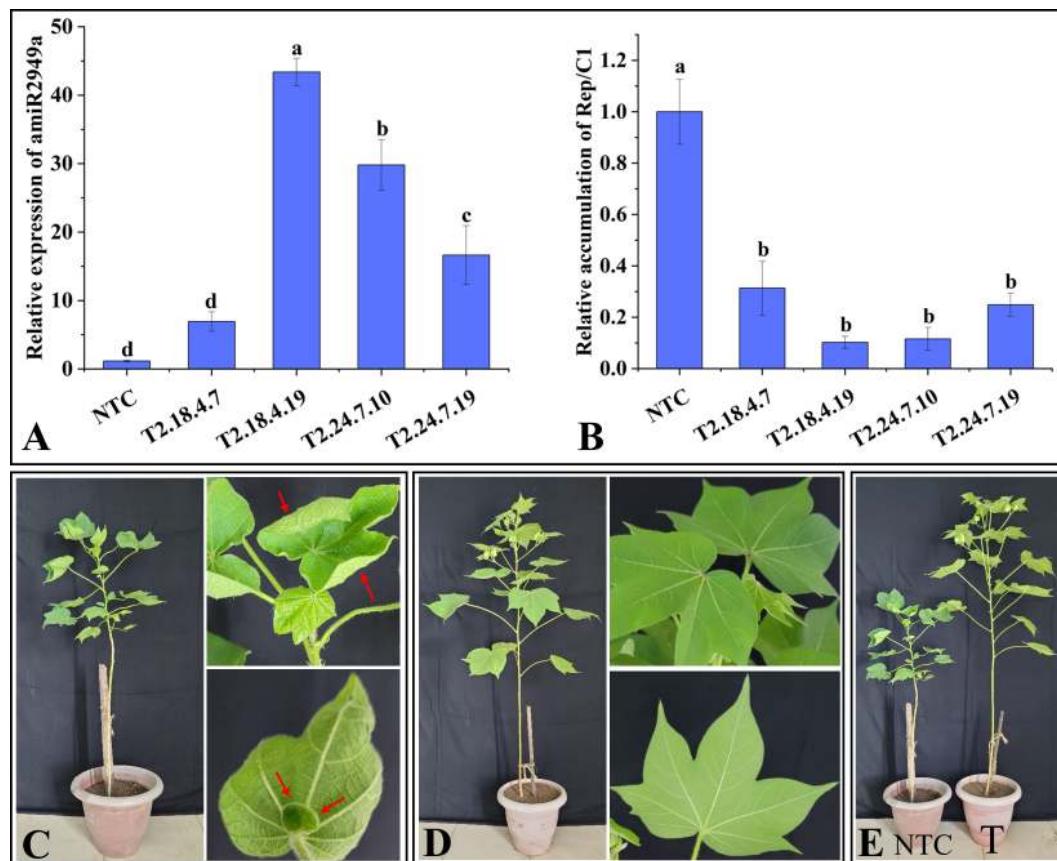


FIGURE 4

Expression and symptom severity assessment (A) Relative expression levels of amiR2949a (B) Relative expression of C1 gene. The data are presented as means $\pm$ SD (three biological and three technical replicates; $n=3$). Significant differences were analysed using the Tukey's test ($p \leq 0.05$) and distinct alphabets (a, ab, b, bc and c) are used to denote statistically significant variations among the experimental groups. (C) Non transgenic (NT) control plant showing typical CLCuD symptoms including upward leaf curling in top view of leaves (upper panel) and enation on the abaxial leaf surface (lower panel). (D) Transgenic T₂ plant exhibiting absence of symptoms in top view of leaves (upper panel) and abaxial surface of leaf (lower panel). (E) Symptomatic NT control (left) and asymptomatic transgenic (right) plant.

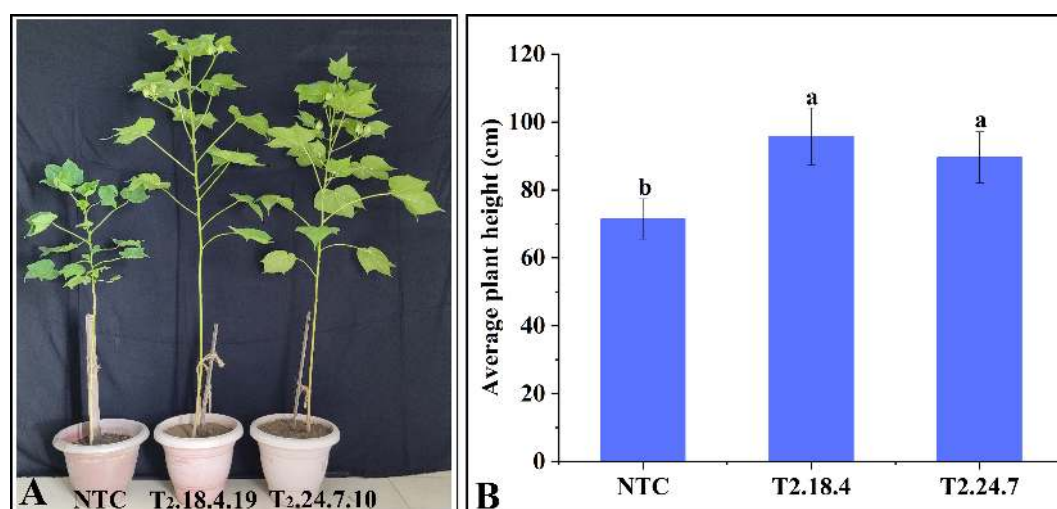


FIGURE 5

Phenotypic assessment of transgenic plants. (A) Representative transgenic (T₂) plant showing no phenotypic abnormalities compared to NT controls (B). Average height of plants (10 plants per line). The significance of differences in average height between transgenic and NTC was determined using the Tukey's test ($p \leq 0.05$). Bars represent the mean $\pm$ SD of transgenic and NT controls ($n=10$). Distinct letters (a, b) indicating significant differences.

precursors to produce a single, precisely designed 21-nucleotide RNA species (22). This feature ensures highly specific gene silencing while minimizing unintended host gene targeting and recombination risks. Moreover, amiRNA precursors lack extensive double-stranded RNA regions, thus reducing the chances of transgene silencing and enhancing stability of expression (14, 22).

In this study, the developed cotton lines exhibited a high level of resistance to CLCuMuV infection, remained asymptomatic and largely free of detectable virus accumulation up to eight weeks after inoculation. A shortcoming of this study is that T-DNA integration and transgene copy number were not directly validated through Southern blot. Instead, PCR-based segregation analysis of the transgene was performed in T₁ and T₂ progeny. The segregation patterns observed in all independent transgenic lines were consistent with a predicted single-locus insertion. Although this approach is commonly used to infer single-locus integration events, it cannot definitively exclude tandem or closely linked multiple T-DNA insertions (24, 30–32). Furthermore, stable Mendelian inheritance of the amiRNA transgene in the T₁ and T₂ generations supports the genetic stability of this resistance trait, consistent with earlier reports employing RNAi-based antiviral strategies in cotton and other crops (19, 33).

To elucidate the basis of CLCuD resistance, we focused on the viral C1 gene (motif II) encoding the replication associated protein (Rep), and exhibits high sequence complementarity with amiR2949a across multiple begomovirus species. The C1 protein, being highly conserved and multifunctional, is essential for rolling-circle replication of the viral genome (7). Transgenic T₂ plants expressing amiR2949a exhibited a significant reduction in C1 transcript levels, accompanied by a strong inverse correlation between amiRNA presence and C1 expression. These findings provide indirect but compelling evidence of amiRNA-mediated silencing of the viral replication gene and offer a convincing explanation for the observed reduction in virus accumulation and disease severity.

Although amiR2949a was rationally designed and predicted *in silico* to target multiple viral genes, transcriptional validation in this study was confined to the C1 gene. Moreover, this study was primarily intended as a proof-of-concept demonstration of amiR2949a efficacy rather than an exhaustive analysis of all predicted targets. Expanding expression analyses to all predicted targets (C3, C4, V1, V2, and β C1) would require additional experiments that were beyond the scope and available resources for this initial report. To still support the intended multi-target design, the authors have included *in silico* amiRNA-target pairing analyses showing the predicted binding interactions with C3, C4, V1, V2 and β C1, which are consistent with the proposed multi-target suppression mechanism. However, the significant reduction in viral accumulation and symptom severity supports its potential for multi-gene silencing.

Additionally, experimental validation of amiRNA-guided cleavage was not performed and remains a limitation of the present study. The targeting of C1 and the overlapping C4 coding region contributes to the broad-spectrum efficacy of this amiRNA, as C1 gene is functionally indispensable and conserved among the begomovirus species. Furthermore, several previous studies have inferred amiRNA specificity indirectly through reductions in target transcript levels and the development of resistance phenotypes, without performing cleavage site analyses (30, 34–39).

While some studies have reported inconsistent correlation between sRNA accumulation and virus resistance (30, 31), our data demonstrate a clear and consistent relationship between amiRNA expression, suppression of an essential viral gene, and enhanced resistance (24, 39–41). Notably, resistant transgenic plants did not exhibit abnormal phenotypic traits in the absence of virus infection, indicating no obvious developmental defects attributable to amiR2949a expression under greenhouse conditions. Following virus inoculation, transgenic lines exhibited significant (16–24%) increase in plant height relative to infected non-transgenic controls, consistent with effective suppression of begomovirus-induced stunting. Together, these observations support the interpretation that improved growth reflects reduced viral accumulation rather than pleiotropic effects of the amiRNA. Importantly, transgenic plants exhibited normal phenotypic development, increased plant height and normal boll development compared to infected non-transgenic controls. This suggests that amiR2949a does not adversely affect endogenous cotton gene expression, and that reduced viral titer contributes to improved growth of the plants. The absence of visible pleiotropic effects underscores the specificity and biosafety of the amiRNA-based approach. Collectively, these findings suggest that an early and efficient suppression of the C1 gene restricts CLCuMuV replication, thereby limiting CLCuD progression. While greenhouse results are promising, multi-location field trials under natural infection pressure are essential to assess the durability, stability and agronomic performance of amiR2949a-expressing cotton lines. Such trials are critical to assess durability and agronomic performance under diverse environmental and epidemiological conditions. However, several challenges limit the immediate implementation of field evaluations, including variable environmental conditions, the presence of diverse and recombinant begomovirus strains and whitefly vector populations, and the occurrence of mixed infections. Further, regulatory approvals, the availability of containment-compliant field sites, and logistical and funding constraints further hinder multi-location trials. Therefore, comprehensive field evaluation remains a prioritized next step for validating the greenhouse proof-of-concept results.

5 Conclusion

In conclusion, this study establishes that an amiRNA targeting the C1 gene of CLCuMuV confers effective resistance to CLCuD in cotton. Expression of amiR2949a led to a significant suppression of viral C1 transcripts, reduced CLCuMuV accumulation, and improved CLCuD resistance without causing any detectable adverse effect on plant growth or development. The observed inverse relationship between amiRNA expression and viral accumulation signifies the mechanistic basis of the resistance phenotype. Overall, this study establishes amiRNA-mediated silencing as a precise, durable and scalable approach for engineering broad-spectrum virus resistance in cotton. Furthermore, it provides a strong foundation for the future integration of amiRNA-based antiviral approaches into cotton improvement and breeding programs.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Author contributions

MK: Data curation, Investigation, Validation, Writing – original draft, Methodology. DS: Writing – review & editing, Methodology. MB: Writing – review & editing, Methodology. SK: Writing – review & editing, Methodology. JK: Conceptualization, Formal analysis, Resources, Supervision, Writing – original draft, Writing – review & editing, Methodology.

Funding

The author(s) declared that financial support was not received for this work and/or its publication.

Acknowledgments

MK and DS acknowledge University Grants Commission (UGC, Govt. of India) and Council of Scientific and Industrial Research (CSIR, Govt. of India) for JRF and SRF fellowships, respectively. SK and MSB acknowledge the UGC (Govt. of India) for non-net PhD fellowships.

References

- Hannon GJ. RNA interference. *Nature*. (2002) 418:244–51. doi: 10.1038/418244a
- Brodersen P, Voinnet O. The diversity of RNA silencing pathways in plants. *Trends Genet.* (2006) 22:268–80. doi: 10.1016/j.tig.2006.03.003
- Pashkovskiy PP, Ryazansky SS. Biogenesis, evolution, and functions of plant microRNAs. *Biochem Moscow*. (2013) 78:627–37. doi: 10.1134/S0006297913060084
- Qadir R, Khan ZA, Monga D, Khan JA. Diversity and recombination analysis of cotton leaf curl multan virus: a highly emerging begomovirus in northern India. *BMC Genomics*. (2019) 20:274. doi: 10.1186/s12864-019-5640-2
- Sattar MN, Kvarnheden A, Saeed M, Briddon RW. Cotton leaf curl disease – an emerging threat to cotton production worldwide. *J Gen Virol.* (2013) 94:695–710. doi: 10.1099/vir.0.049627-0
- Mahmood MA, Ahmed N, Hussain A, Naqvi RZ, Amin I, Mansoor S. Dominance of cotton leaf curl multan virus-rajasthan strain associated with third epidemic of cotton leaf curl disease in Pakistan. *Sci Rep.* (2024) 14:13532. doi: 10.1038/s41598-024-63211-8
- Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Crit Rev Biochem Mol Biol.* (2000) 35:105–40. doi: 10.1080/07352689991309162
- Sharma D, Khan M, Khan JA. Genome editing-based strategies to combat geminiviruses: CRISPR/Cas9 and emerging high-fidelity tools. *Arch Microbiol.* (2026) 208:343. doi: 10.1007/s00203-026-04891-w
- Cui X, Tao X, Xie Y, Fauquet CM, Zhou X. A DNA β associated with Tomato Yellow Leaf Curl China Virus is required for symptom induction. *J Virol.* (2004) 78:13966–74. doi: 10.1128/JVI.78.24.13966-13974.2004
- Gnanasekaran P, Kishorekumar R, Bhattacharyya D, Vinoth Kumar R, Chakraborty S. Multifaceted role of geminivirus associated betasatellite in pathogenesis. *Mol Plant Pathol.* (2019) 20:1019–33. doi: 10.1111/mpp.12800
- Saeed M, Behjatnia SAA, Mansoor S, Zafar Y, Hasnain S, Rezaian MA. A single complementary-sense transcript of a geminiviral DNA β satellite is determinant of pathogenicity. *MPMI.* (2005) 18:7–14. doi: 10.1094/MPMI-18-0007
- Mansoor S, Amin I, Iram S, Hussain M, Zafar Y, Malik KA, et al. Breakdown of resistance in cotton to cotton leaf curl disease in Pakistan. *Plant Pathol.* (2003) 52:784–. doi: 10.1111/j.1365-3059.2003.00893.x
- Nazeer W, Tipu AL, Ahmad S, Mahmood K, Mahmood A, Zhou B. Evaluation of cotton leaf curl virus resistance in bc1, bc2, and bc3 progenies from an interspecific cross between *Gossypium arboreum* and *Gossypium hirsutum*. *PLoS One.* (2014) 9:e111861. doi: 10.1371/journal.pone.0111861
- García JA, Simón-Mateo C. A micropunch against plant viruses. *Nat Biotechnol.* (2006) 24:1358–9. doi: 10.1038/nbt1106-1358
- Shweta, Akhter Y, Khan JA. Genome wide identification of cotton (*Gossypium hirsutum*)-encoded microRNA targets against cotton leaf curl burewala virus. *Gene.* (2018) 638:60–5. doi: 10.1016/j.gene.2017.09.061

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declared that generative AI was not used in the creation of this manuscript.

Any alternative text (alt text) provided alongside figures in this article has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fviro.2026.1873471/full#supplementary-material>

16. Khan M, Sharma D, Ara H. Transformation in gossypium hirsutum cv. hs6: a path to efficient phytohormone- free protocol overcoming transformation hurdles. *Jour Pl Sci Res.* (2024) 40:1–8.
17. Wamiq G, Khan JA. Overexpression of ghr-mir166b generates resistance against bemisia tabaci infestation in gossypium hirsutum plants. *Planta.* (2018) 247:1175–89. doi: 10.1007/s00425-018-2852-7
18. Khan JA, Ahmad J. Diagnosis, monitoring and transmission characteristics of cotton leaf curl virus. *Curr Sci.* (2005) 88:1803–9.
19. Baig MS, Akhtar S, Khan JA. Engineering tolerance to clud in transgenic gossypium hirsutum cv. hs6 expressing cotton leaf curl multan virus-c4 intron hairpin. *Sci Rep.* (2021) 11:14172. doi: 10.1038/s41598-021-93502-3
20. Zhao L, Ding Q, Zeng J, Wang F, Zhang J, Fan S, et al. An improved ctab–ammonium acetate method for total rna isolation from cotton. *Phytochem Anal.* (2012) 23:647–50. doi: 10.1002/pca.2368
21. Liu Q, Li Y, Xu K, Li D, Hu H, Zhou F, et al. Clay nanosheet-mediated delivery of recombinant plasmids expressing artificial mirnas via leaf spray to prevent infection by plant dna viruses. *Hortic Res.* (2020) 7:179. doi: 10.1038/s41438-020-00400-2
22. Khan M, Khan JA. Amir414e-mediated silencing confers resistance to cotton leaf curl multan virus in cotton. *Mol Biol Rep.* (2026) 53:380. doi: 10.1007/s11033-026-11534-2
23. Schmittgen TD, Livak KJ. Analyzing real-time pcr data by the comparative ct method. *Nat Protoc.* (2008) 3:1101–8. doi: 10.1038/nprot.2008.73
24. Kung Y, Lin S, Huang Y, Chen T, Harish SS, Chua N, et al. Multiple artificial micrnas targeting conserved motifs of the replicase gene confer robust transgenic resistance to negative-sense single-stranded rna plant virus. *Mol Plant Pathol.* (2012) 13:303–17. doi: 10.1111/j.1364-3703.2011.00747.x
25. Luo C, Bashir NH, Li Z, Liu C, Shi Y, Chu H. Plant micrnas regulate the defense response against pathogens. *Front Microbiol.* (2024) 15:1434798. doi: 10.3389/fmicb.2024.1434798
26. Song Y-Z, Han Q-J, Jiang F, Sun R-Z, Fan Z-H, Zhu C-X, et al. Effects of the sequence characteristics of mirnas on multi-viral resistance mediated by single amirnas in transgenic tobacco. *Plant Physiol Biochem.* (2014) 77:90–8. doi: 10.1016/j.plaphy.2014.01.008
27. Duan C-G, Wang C-H, Fang R-X, Guo H-S. Artificial micrnas highly accessible to targets confer efficient virus resistance in plants. *J Virol.* (2008) 82:11084–95. doi: 10.1128/JVI.01377-08
28. Zhang L, Xie X, Song Y, Jiang F, Zhu C, Wen F. Viral resistance mediated by shrna depends on the sequence similarity and mismatched sites between the target sequence and sirna. *Biol Plant.* (2013) 57:547–54. doi: 10.1007/s10535-013-0314-4
29. Chen J, Peng Y, Zhang H, Wang K, Zhao C, Zhu G, et al. Off-target effects of rnai correlate with the mismatch rate between dsrna and non-target mrna. *RNA Biol.* (2021) 18:1747–59. doi: 10.1080/15476286.2020.1868680
30. Kis A, Tholt G, Ivanics M, Várallyay É, Jenes B, Havelda Z. Polycistronic artificial mirna -mediated resistance to Wheat dwarf virus in barley is highly efficient at low temperature. *Mol Plant Pathol.* (2016) 17:427–37. doi: 10.1111/mpp.12291
31. Faisal M, Abdel-Salam EM, Alatar AA. Artificial microrna-based rna interference and specific gene silencing for developing insect resistance in solanum lycopersicum. *Agronomy.* (2021) 11:136. doi: 10.3390/agronomy11010136
32. Warthmann N, Chen H, Ossowski S, Weigel D, Hervé P. Highly specific gene silencing by artificial mirnas in rice. *PLoS One.* (2008) 3:e1829. doi: 10.1371/journal.pone.0001829
33. Ossowski S, Schwab R, Weigel D. Gene silencing in plants using artificial micrnas and other small rnas. *Plant J.* (2008) 53:674–90. doi: 10.1111/j.1365-313X.2007.03328.x
34. Al-Roshdi MR, Ammara U, Khan J, Al-Sadi AM, Shahid MS. Artificial microrna-mediated resistance against Oman strain of tomato yellow leaf curl virus. *Front Plant Sci.* (2023) 14:1164921. doi: 10.3389/fpls.2023.1164921
35. Khalid A, Zhang X, Ji H, Yasir M, Farooq T, Dai X, et al. Large artificial microrna cluster genes confer effective resistance against multiple tomato yellow leaf curl viruses in transgenic tomato. *Plants.* (2023) 12:2179. doi: 10.3390/plants12112179
36. Fahim M, Millar AA, Wood CC, Larkin PJ. Resistance to Wheat streak mosaic virus generated by expression of an artificial polycistronic microrna in wheat. *Plant Biotechnol J.* (2012) 10:150–63. doi: 10.1111/j.1467-7652.2011.00647.x
37. Ali I, Amin I, Briddon RW, Mansoor S. Artificial microrna-mediated resistance against the monopartite begomovirus cotton leaf curl burewala virus. *Virol J.* (2013) 10:231. doi: 10.1186/1743-422X-10-231
38. Mitter N, Zhai Y, Bai AX, Chua K, Eid S, Constantin M, et al. Evaluation and identification of candidate genes for artificial microrna-mediated resistance to tomato spotted wilt virus. *Virus Res.* (2016) 211:151–8. doi: 10.1016/j.virusres.2015.10.003
39. Liang C, Hao J, Li J, Baker B, Luo L. Artificial microrna-mediated resistance to cucumber green mottle mosaic virus in nicotiana benthamiana. *Planta.* (2019) 250:1591–601. doi: 10.1007/s00425-019-03252-w
40. Sharma N, Prasad M. Silencing ac1 of tomato leaf curl virus using artificial microrna confers resistance to leaf curl disease in transgenic tomato. *Plant Cell Reps.* (2020) 39:1565–79. doi: 10.1007/s00299-020-02584-2
41. Qu J, Ye J, Fang R. Artificial microrna-mediated virus resistance in plants. *J Virol.* (2007) 81:6690–9. doi: 10.1128/JVI.02457-06