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Research Article

Comparative Evaluation of pKSE401 and pHSE401 gRNA Constructs for CRISPR/Cas9-Mediated Resistance to Cotton Leaf Curl Virus

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ABSTRACT

Cotton leaf curl disease (CLCuD), caused by begomoviruses and their betasatellites, is a major threat to cotton production, especially in South Asia, where periodic viral outbreaks continue to affect cotton yields, quality, and livelihoods. The advent of CRISPR/Cas9 gene-editing technology has transformed plant biotechnology, offering efficient, accurate, and programmable methods for combating viral pathogens at the genetic level. Here, the antiviral efficacy of two most widely used CRISPR/Cas9 binary plant expression vectors, pKSE401 and pHSE401, was tested in *Nicotiana benthamiana* against *Cotton leaf curl Kokhran virus* (CLCuKoV) and *Cotton leaf curl Multan betasatellite* (CLCuMuB). The guide-RNAs (gRNAs) were designed to target viral genes that play significant roles in pathogenicity and replication, with pHSE401 encoding a single gRNA and pKSE401 a multiplex of two gRNAs. *Agrobacterium*-mediated transient transformation and viral inoculation experiments revealed that both CRISPR/Cas9 vectors effectively delayed symptom onset and reduced virus titers relative to infected controls. Remarkably, the multiplex pKSE401 system was more effective at suppressing viral infection, achieving about a 90% reduction in viral accumulation compared with a 75% reduction by the single gRNA pHSE401 construct. pKSE401-treated plants showed delayed symptom development, reduced severity, and partial recovery, demonstrating the improved efficiency of multiplex genome editing. The results demonstrate the cutting-edge potential of CRISPR/Cas9 multiplex approaches as next-generation methods for designing sustainable resistance to multifaceted plant virus diseases. This research not only contributes to our understanding of CRISPR-based antiviral response mechanisms but also provides a promising avenue for designing broad-spectrum, sustainable resistance against viral epidemics in cotton and other commercially valuable crops.

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Introduction

Cotton (*Gossypium* spp.) is a globally important fiber crop and a key source of natural fiber, oil, and animal feed. It is critical for sustainable agriculture and economic growth. Pakistan is one of the world's major cotton-producing nations, after India, China, the US, and Brazil (Abbas, 2020). Despite its importance, cotton yields are constrained by a range of biotic and abiotic

factors, leading to an economic crisis, with viral diseases among the largest threats to sustainable cotton production (Sattar et al., 2013). Specifically, cotton leaf curl disease (CLCuD), caused by begomoviruses (family *Geminiviridae*), is one of the most devastating viral pathogens for cotton production, especially in South Asia. Geminiviruses are small, circular, single-stranded DNA viruses that can be either monopartite or bipartite (Mahmood et al., 2022). These viruses encode several proteins that control viral replication, movement, pathogenicity, and host interactions. Monopartite begomoviruses and the DNA-A component of bipartite begomoviruses generally encode six open reading frames (ORFs) - *AV1* and *AV2* on the virion sense and *AC1* (*Rep*), *AC2*, *AC3*, and *AC4* on the complementary sense (Ahmed et al., 2021). The Rep protein (*AC1*) initiates viral DNA replication, while other proteins activate gene expression, enhance DNA replication, and induce symptoms. The intergenic region harbors cis-regulatory elements such as the nonanucleotide motif (TAATATTAC) and iterons, which are crucial for initiating and carrying out viral genome replication (Heyraud et al., 1993; Rojas et al., 2005).

CLCuD is transmitted by whitefly (*Bemisia tabaci*) and has caused significant damage in Pakistan since its first appearance. Although first reported in 1967 in Multan, Pakistan (Hussain and Ali, 1975), the virus triggered an epidemic in the early 1990s, destroying about 1.3 million bales of cotton and severely damaging cotton-growing areas (Farooq et al., 2014). The disease is characterized by curled leaves, thickened veins, enations, and stunted growth, leading to yield and quality losses (Briddon and Markham, 2001). The emergence of new begomovirus recombinants and their associated betasatellites has also complicated disease control and the development of resistant varieties. Conventional methods for CLCuD control, such as traditional breeding and genetic engineering, have proved ineffective in the long term because of the dynamic evolution and variation of begomoviruses (Aragao and Faria, 2009; Zaidi et al., 2016). As a result, there is an urgent need for new, sustainable antiviral approaches.

Advances in genome-editing technologies have transformed crop improvement by enabling precise editing of target DNA sequences. Among these technologies, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems (Cas) have proven to be the most efficient, versatile, and cost-effective tools for plant genome engineering. CRISPR/Cas9 uses a customizable guide RNA (gRNA) to direct the Cas9 nuclease to specific genomic sites, where it creates double-stranded breaks (DSBs) that induce site-specific mutagenesis or gene disruption (Ali et al., 2015). The efficiency, simplicity, and versatility of CRISPR/Cas9 have made it a promising technology for developing resistance to plant viruses.

Recent studies have shown that CRISPR/Cas systems effectively suppress geminiviruses, sometimes by targeting viral genomes. For instance, simultaneous targeting of multiple *Cotton leaf curl virus* (CLCuV) strains with CRISPR/Cas9 reduced virus accumulation and increased resistance in *Nicotiana benthamiana* and cotton plants (Mubarik et al., 2021). Likewise, Binyameen et al. (2021) reported that multiplex CRISPR/Cas9 approaches successfully targeted CLCuV by simultaneously targeting multiple conserved viral sequences, thereby reducing the likelihood of viral escape. However, few studies have compared the efficiency of various CRISPR/Cas9 vector systems for antiviral applications. pHSE401 and pKSE401 are well-established plant expression vectors that differ in their capabilities for single- and multiplex gRNA expression, respectively.

In this study, we compared the antiviral efficacy of two CRISPR/Cas9 vector systems, pHSE401 (single gRNA) and pKSE401 (dual gRNA), against CLCuKoV and CLCuMuB in *N. benthamiana*. By targeting key viral genes responsible for replication and pathogenicity, we aimed to determine whether multiplex genome editing is more effective in suppressing viral infection than single-target editing. These findings provide information about the design of improved strategies for engineering resistance to cotton leaf curl disease.

Material and methods

In silico analysis and guide RNA design

CLCuKoV and CLCuMuB nucleotide sequences were obtained from the National Center for Biotechnology Information (NCBI) database. These sequences were analyzed to identify suitable target sites for gRNA design (Khan et al., 2020). SnapGene was used to generate a genomic map of CLCuKoV to visualize its gene organization and select conserved regions. Potential gRNA target sites in key viral genes were selected based on sequence specificity and suitability for CRISPR/Cas9-mediated cleavage (Mubarik et al., 2021).

Construction of CRISPR/Cas9 expression vectors

CRISPR/Cas9 vectors pKSE401 and pHSE401 were obtained from Addgene and engineered to express two gRNAs and one gRNA, respectively. Vectors were designed using established procedures (Khan et al., 2020; Mubarik et al., 2021). For pHSE401, target sequences in the *Rep* (C1) gene were identified using the CRISPRdirect tool (https://crispr.dbcls.jp/), and 20-bp gRNA sequences were selected manually. Complementary oligonucleotides encoding the single-guide RNA (sgRNA) were obtained from Thermo Fisher Scientific and annealed by thermal cycler. The annealed oligonucleotides were inserted into the pHSE401 construct, which contains a gRNA scaffold under a suitable promoter. The plasmids were then transformed into *Escherichia coli* (*E. coli*) (*DH5α*), and positive clones were selected on LB agar plates containing kanamycin. The same approach was used to construct the pKSE401 vector, which includes a maize codon-optimized Cas9 gene and allows expression of two gRNAs under the U6-26 promoter (Khan et al., 2020).

Plasmid isolation and restriction enzyme validation

The alkaline lysis method was used to isolate plasmid DNA from transformed *E. coli* cells (Binyameen et al., 2021). Briefly, a single bacterial colony was inoculated into LB medium containing the appropriate antibiotics and incubated overnight at 37°C with shaking. Plasmid DNA was then isolated from the bacterial culture using freshly prepared alkaline lysis solutions. The integrity and identity of the plasmid constructs were confirmed by *Hind*III restriction digestion. The digestion mixture, containing plasmid DNA, restriction enzyme, buffer, and nuclease-free water, was incubated at 37°C for 20 min. The digestion products were separated on a 1% agarose gel (Binyameen et al., 2021).

Plant material and growth conditions

Peat-moss-sown *N. benthamiana* seeds were grown under controlled conditions in plastic pots. Plants were maintained under a 16-h light/8-h dark photoperiod at 25°C (Mubarik et al., 2021). Seedlings were transplanted into individual pots after 8-10 days. Plants were allowed to develop for an additional three weeks until reaching the appropriate agro-infiltration stage. In this study, 32 plants were selected and divided into four experimental groups (A, B, C, and D) based on the treatment applied, as shown in Table 1.

***Agrobacterium*-mediated transformation and agro-infiltration**

CRISPR/Cas9 constructs (pKSE401 and pHSE401) were agro-infiltrated into *N. benthamiana* plants (Khan et al., 2020). Transformation was performed using the *Agrobacterium tumefaciens* strain *GV3101*. Cultures of *Agrobacterium* containing the relevant constructs were prepared and infiltrated into the leaf surfaces of plants using the method described by Broghammer et al. (2012).

Molecular confirmation of transgene integration

The cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA from agroinfiltrated plants 21 days after infiltration. Leaf tissues were ground in CTAB extraction buffer, and DNA was purified using standard procedures (Healey et al., 2014). Polymerase chain reaction (PCR) with gene-specific primers was used to verify the presence and expression of the Cas9 transgene (Ashraf et al., 2023). PCR products were analyzed by agarose gel electrophoresis. Table 2 lists the primers used to analyze transgenes.

Table 1. Description of different groups of plants according to their treatment.

Group	Treatment	No. of plants
Group- A	Negative control (Plants inoculated with inoculation buffer)	8
Group- B	Plants co-infiltrated with infectious clones and Cas9-pKSE401 vector	8
Group- C	Plants co-infiltrated with infectious clones and Cas9-pHSE401 vector	8
Group- D	Positive control (Plants infiltrated with infectious clones only)	8

Table 2. List of primers used for transgene analysis.

Vectors	Name of primers	Sequence (5′→ 3′)
pHSE401	Cas9-F (Forward)	CCAGCTGCTGACAAGAAGTACTCGA
	Cas9-R (Reverse)	GCTGCTGCCTGACCAGCG
pKSE401	Cas9-F2 (Forward)	GCAGCTCCCCGAGAAGTACAAG
	Cas9-R2 (Reverse)	CCTTGAATGTGAGGCTGTCATCGT

Virus challenge and infectivity assay / Disease resistance assay

Agroinfiltration of 23-day-old *N. benthamiana* plants with infectious clones of CLCuV, including CLCuKoV and CLCuMuB, also known as KOK and U89, was performed. Cultures of *A. tumefaciens* harboring the viral constructs were grown in LB broth supplemented with antibiotics (50 µg/mL rifampicin, 50 µg/mL kanamycin, and 50 µg/mL gentamicin) and incubated at 28°C with constant shaking. Bacterial cells were centrifuged at 8000 rpm for 10 min and re-suspended in infiltration buffer (Mubarik et al., 2021). The abaxial (lower) surface of plant leaves was infiltrated with the bacterial suspension. Plants inoculated with the virus were maintained under control conditions and monitored for symptom development to assess viral infectivity (Mubarik et al., 2021).

Quantification of viral load by qPCR

Plants were co-infiltrated with infectious viral clones and either the pKSE401 or pHSE401 CRISPR/Cas9 constructs for comparison. For qPCR analysis, four plants per treatment group served as biological replicates, and statistical analyses were performed on these replicates. Results are presented as mean ± SD. Disease progression and symptom development were monitored over three weeks. Viral accumulation in plant tissues was quantified by quantitative PCR (qPCR) as previously described (Khan et al., 2020).

Results

Confirmation of CRISPR/Cas9 vector integrity through restriction digestion

The structure of the CRISPR/Cas9 plant expression vectors pKSE401 and pHSE401 was characterized by plasmid DNA extraction and restriction digestion. *E. coli* transformants were cultured, and plasmid DNA was extracted and purified using alkaline lysis. *Hind*III was used to digest the plasmid DNA and confirm construct integrity. *Hind*III was chosen because it produced a specific fragment of the cloned insert in the vectors. After 20 min of enzymatic digestion at 37°C, the products were run on a 1% agarose gel. Distinct banding patterns confirmed the integrity of both plasmids. The expected digested fragment (1919 bp or ~2 kb) was clearly visible, consistent with the release of the insert, and undigested plasmid DNA at around 16 kb due to the supercoiled structure of the vector. The presence of the expected digested fragment confirmed successful vector construction and the purity of the pKSE401 and pHSE401 vectors for plant transformation assays. This indicated that the vectors were structurally correct and ready for agroinfiltration (Figure 1).

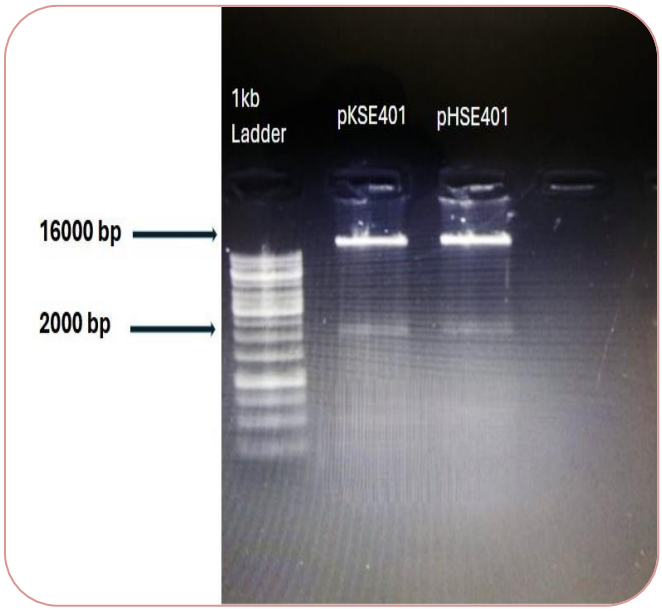

Figure 1. Confirmation of plasmids by *Hind*III restriction digestion. Lane 1 contains a 1 kb ladder. Lane 2 shows pKSE401, and lane 3 shows pHSE401. Digested samples (D_3H_2O 11 µl, buffer 3 µl, enzyme 1 µl, plasmid 5 µl = 20 µl) were run on a 1% agarose gel. The *Hind*III-digested pHSE401 vector shows a band around 2000 bp. The undigested plasmid DNA of pKSE401 and pHSE401 shows a band around 16000 bp.

Establishment of CLCuV infectivity in *N. benthamiana*

To assess pathogenicity and establish the infection model system, 2-3-week-old *N. benthamiana* plants were infiltrated with infectious clones of CLCuKoV and CLCuMuB. Disease symptoms were monitored to confirm virus establishment. Symptoms first appeared approximately 10 days after inoculation (DAI), characterized by leaf curling and slight distortion. Over time, plants exhibited severe disease symptoms, including severe leaf curling, enation, stunting, and tissue degeneration. By 25 DAI, disease symptoms increased significantly, and by 40 DAI, plants heavily infected with the virus complex exhibited near-complete wilting or death. This established the virulence and infectivity of the CLCuKoV/CLCuMuB complex in *N. benthamiana* and confirmed that this plant species is an ideal model system for assessing CRISPR-based antiviral resistance (Figure 2 and 3).

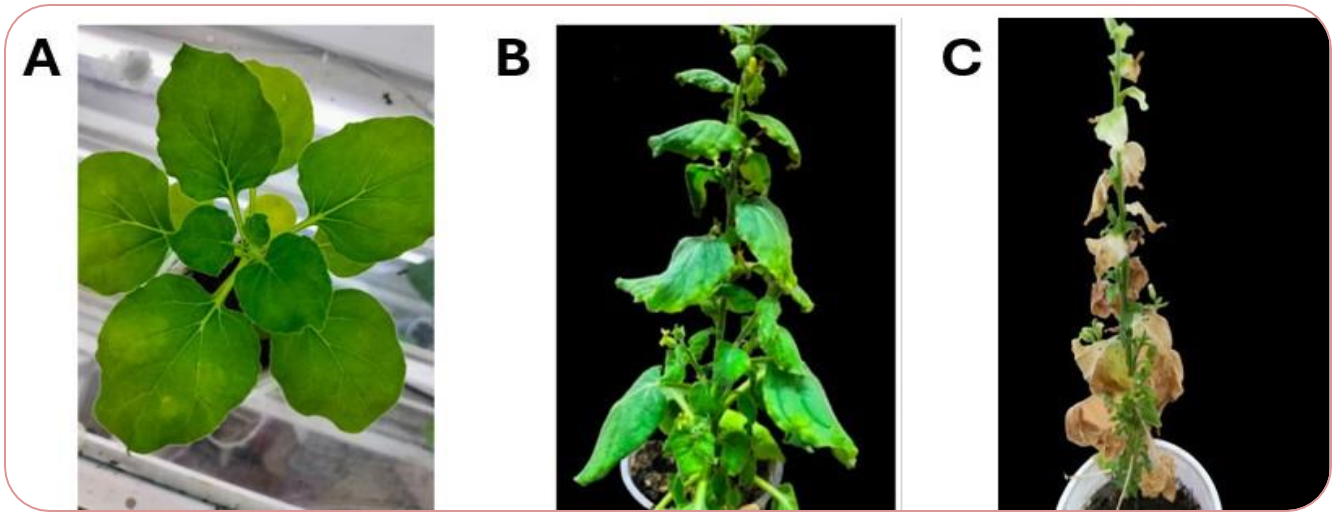

Figure 2. Virus infectivity assay in *N. benthamiana* plants. A) Before inoculation with infectious clones of CLCuV B) After infiltration with infectious clones of CLCuKoV/CLCuMuV (KOK and U89), symptoms appear 10 days after infiltration. C) *N. benthamiana* plant dead after 40 DAI.

Figure 3. Virus infectivity assay in *N. benthamiana* plants. A) Before inoculation with infectious clones of CLCuV (KOK and U89) and gRNA-cloned constructs. B) After co-infiltration of pKSE401-cloned gRNA, symptoms appear 18 DAI. C) After co-infiltration of pHSE401-cloned gRNA, symptoms appear 20 DAI. D) After infiltration of infectious clones (CLCuKoV/CLCuMuV), symptoms appear 10 days after infiltration.

Multiplex gRNA-mediated viral suppression by pKSE401

To assess the antiviral efficiency of single- and multiplex-gRNA systems, we co-infiltrated *N. benthamiana* plants with infectious viral clones and either the pKSE401 construct (two gRNAs) or the pHSE401 construct (a single gRNA).

Plants co-infiltrated with pKSE401 and viral infectious clones exhibited a significantly delayed onset of symptoms compared with plants infected with infectious clones alone. While the controls began showing symptoms at approximately 10 DAI, pKSE401-treated plants showed symptoms at 18-20 DAI. In addition to the delay, symptom severity was reduced. Leaves showed less curling, and plants appeared relatively healthy for longer. Some plants even showed symptom recovery in later stages, indicative of effective viral suppression. This suggests that simultaneous targeting of different viral regions using two gRNAs enhanced antiviral protection (Figure 4).

Single gRNA-mediated viral suppression by pHSE401

Plants infected with the pHSE401 construct also showed delayed symptom onset compared with positive controls. Disease symptoms emerged at 20 DAI, compared with 8-10 DAI in the controls. Although symptom onset was delayed, disease suppression was not as strong as with pKSE401. Plants infiltrated with

pHSE401 displayed viral symptoms later than non-infiltrated plants but with more severe symptoms than those in plants infiltrated with pKSE401. However, some plants exhibited recovery responses, suggesting that single-gRNA targeting of the *Rep* gene also reduced viral pathogenicity (Figure 5).

Figure 4. Multiple gRNA-based virus suppression in the *N. benthamiana* plant. A) *N. benthamiana* plant before co-infiltration with the pKSE401-cloned gRNA vector and infectious clones. B) After 18 days of co-infiltration, the plants show the delayed symptoms of the CLCuV.

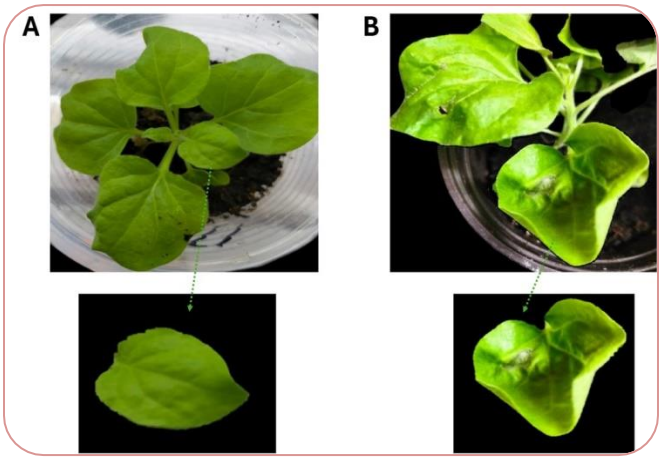

Figure 5. Single gRNA-based virus suppression in the *N. benthamiana* plant. A) *N. benthamiana* plant before co-infiltration with the construct and infectious clones. B) After co-infiltration with the pHSE401-cloned sgRNA vector and infectious clones, CLCuV symptoms appear in *N. benthamiana* after 20 days. These phenotypic comparisons show that both CRISPR/Cas9 systems disrupted the virus; however, using multiplex targets with pKSE401 produced greater and more durable disease suppression.

Molecular confirmation of Cas9 transgene presence in agroinfiltrated plants

To confirm delivery and expression of CRISPR/Cas9 constructs in infiltrated plants, DNA was extracted from transformed *N. benthamiana* leaves 21 days after infiltration and used as a template for PCR with Cas9-specific primers. PCR produced bands of approximately 1000 bp in plants infiltrated with the pKSE401 and pHSE401 constructs, confirming the presence of the expected Cas9 band in all transformed plants. This was not observed in negative controls. These findings confirmed successful transient Cas9 expression in infiltrated tissues and demonstrated that the observed antiviral responses were linked to CRISPR/Cas9 delivery (Figure 6).

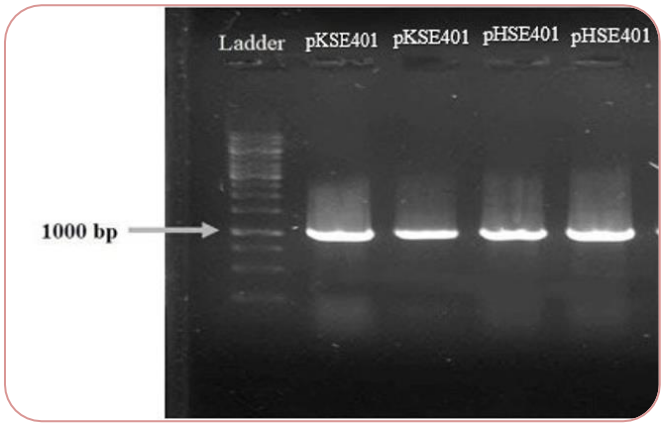

Figure 6. PCR screening of transgenic *N. benthamiana* plants. PCR products were run on 1.5% agarose gel. Specific Cas9 forward and reverse primers were used to assess Cas9 expression. A 1 kb DNA ladder is shown in the first lane. Lanes 2-3 show Cas9 amplicons (1000 bp) from the pKSE401 plasmid, and lanes 4-5 show Cas9 bands at 1000 bp from the pHSE401.

Quantitative analysis of viral accumulation through qPCR

Molecular analysis of viral suppression was performed by qPCR using DNA from infiltrated plants. Viral expression was calculated using the 2^-(ΔΔCt) method, with virus-only plants serving as the control (relative expression = 1). qPCR results showed a significant decrease in viral accumulation in plants infiltrated with CRISPR/Cas9 constructs. Plants co-infiltrated with pHSE401 showed about a 75% reduction in viral accumulation compared to control plants, with fold expression ranging from 0.38 to 0.40 (average).

However, plants treated with pKSE401 showed higher antiviral activity, with an estimated 90% reduction in viral accumulation (fold expression values: 0.21-0.24). Overall, pKSE401 exhibited the lowest viral load among all treatment groups, indicating the highest antiviral efficacy. The reduction in fold expression in pKSE401-treated plants positively influenced the onset of disease symptoms and the recovery of plant health. On the other hand, although pHSE401 considerably suppressed the virus, it was less effective than the multiplex construct. These quantitative results strongly suggest that multiplex targeting with two gRNAs is more efficient than single-gRNA targeting for reducing CLCuV. This indicates that simultaneous targeting of the *Rep* and *βC1* genes suppresses viral replication more effectively than targeting either gene alone (Table 3; Figure 7).

Table 3. Fold gene expression values for determination of virus accumulation in each infected plant.

Sample	Ct value	Average of Ct Value	ΔCt value	Fold gene expression (2^-(ΔΔCt))
pKSE401-1	0.21	0.227	2.12	0.23
pKSE401-2	0.23			
pKSE401-3	0.24			
pKSE401-4	0.23			
pHSE401-1	0.39	0.39	1.35	0.39
pHSE401-2	0.38			
pHSE401-3	0.4			
pHSE401-4	0.39			
Control	1	1	0	0
Control	1			
Control	1			
Control	1			

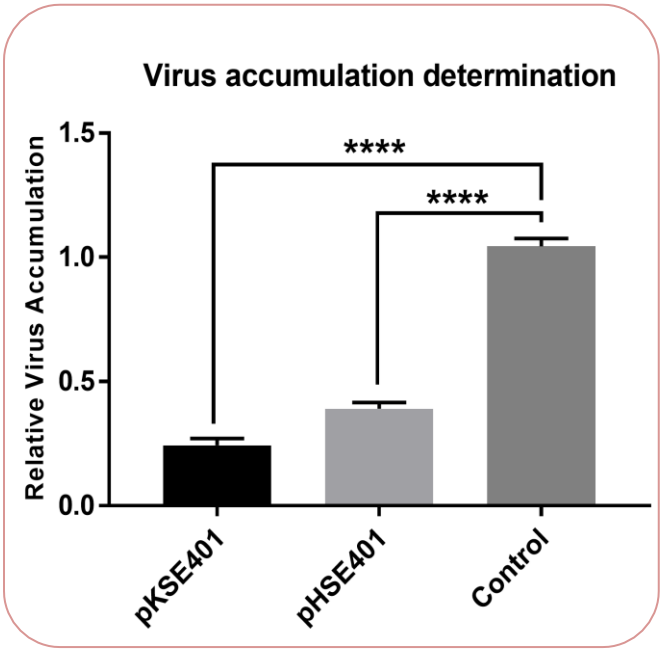

Figure 7. Virus titer analysis by qPCR. This graph shows virus accumulation in plants co-infiltrated with a pKSE401-based gRNA construct and CLCuKoV/CLCuMuB, in plants co-infiltrated with a pHSE401-based gRNA construct and CLCuKoV/CLCuMuB, and in plants infiltrated with virus only (control). Each bar represents a replicate of 4 plants.

Overall comparative performance of single versus multiplex CRISPR/Cas9 systems

Phenotypic, PCR, and qPCR analyses showed that the multiplex CRISPR/Cas9 system (pKSE401) was more effective than the single-gRNA system (pHSE401) at delaying CLCuV infection. These findings collectively demonstrate the superior antiviral efficacy of multiplex CRISPR/Cas9 targeting over single-target approaches in the development of resistance to CLCuV. Table 4 shows the comparative evaluation of both Cas9 constructs.

Discussion

Cotton, also known as white gold, is a globally significant economic crop because it provides fiber, oil, and protein (Monga and Sain, 2021). Nevertheless, cotton yields in Pakistan have declined markedly over the past couple of decades, largely due to CLCuD. Begomoviruses in the family *Geminiviridae* cause this disease, and it is transmitted by whitefly (Rybicki and Fouquet, 1998). The prevalence of CLCuD has led to substantial yield losses and poor fiber quality, thereby affecting the agricultural economy (Farooq et al., 2014). Previous genome-editing techniques, such as zinc finger nucleases (ZFNs) and

transcription activator-like effector nucleases (TALENs), have been investigated to develop viral resistance in plants. ZFNs have suppressed viral replication by targeting conserved sites, such as the Rep-binding site in TYLCV and BSCTV (Sera, 2005; Chen et al., 2014). Likewise, the potential of TALENs was demonstrated by targeting the *AC1* region of begomoviruses (Cheng et al., 2015; Khan et al., 2018). Despite these innovations, off-target effects, limited targeting flexibility, and the intricate process of vector construction have constrained these technologies (Puchta, 2017; Tang et al., 2017), thereby limiting their widespread use. CRISPR/Cas systems have revitalized the field of plant genome editing by making it simple, efficient, and programmable (Chen et al., 2019; El-Mounadi et al., 2020). Specifically, CRISPR/Cas9 has been extensively used to confer resistance to plant viruses by inducing double-stranded breaks (DSBs) in viral genomes, thereby reducing viral accumulation (Ali et al., 2015; Ji et al., 2015). Other CRISPR systems, such as Cas12a (Cpf1), have also been investigated more recently as antiviral tools. For example, Ashraf et al. (2023) have demonstrated the effective use of CRISPR/Cas12a to target CLCuMuV and highlighted its efficacy and specific PAM requirements, thereby expanding the available target viral sequences. This implies that various CRISPR nucleases can be used strategically to boost antiviral responses and broaden resistance spectra.

Table 4. Evaluation of the Cas9 constructs to suppress CLCuKoV in *N.benthamiana* plants.

Parameter	pHSE401 (single gRNA)	pKSE401 (multiple gRNAs)
Delayed symptoms (No. of days)	3-5	2-4
Symptom severity	Mild	Very mild
Recovery (viral suppression)	Yes (20 dai)	Yes (18 dai)
Virus accumulation (fold expression)	Moderate	Lower
Antiviral efficiency	75%	90%

In this work, we compared the relative efficacy of two CRISPR/Cas9 vectors, pKSE401 and pHSE401, in conferring resistance to CLCuV in *N. benthamiana*. The pKSE401 construct was designed to express two gRNAs targeting the *Rep* (C1) and *βC1* genes, whereas pHSE401 expressed a single gRNA targeting the *Rep* gene. These genes were chosen because they play significant roles in viral replication and pathogenicity. Similar CRISPR/Cas9 vectors have previously been used for antiviral applications in plants (Khan et al., 2020; Mubarik et al., 2021), supporting the rationale of the present study. CRISPR constructs and viral infectious clones were introduced via agroinfiltration, a proven method for transient gene expression in *N. benthamiana* (Chen et al., 2014). Plants inoculated with viral clones alone developed severe symptoms within 10 days, whereas co-infiltrated plants showed delayed and milder symptoms. Notably, plants treated with the pKSE401 construct exhibited a greater delay and partial recovery than those treated with pHSE401. These phenotypic findings were supported by qPCR results showing a considerable decrease in viral accumulation in CRISPR-treated plants. These results are in line with other studies showing that CRISPR/Cas mediates viral interference (Ji et al., 2015).

One key finding of the research is the high efficiency of multiplex genome targeting. The pKSE401, which includes two gRNAs, led to a greater decrease in viral titer than the single-gRNA system (pHSE401). This shows that antiviral activity improves when multiple viral genomic regions are targeted simultaneously. Multiplex targeting increases the likelihood of disruptive mutations or deletions in the viral genome, thereby reducing replication. This has been observed in studies reporting that multiplex CRISPR/Cas9 systems conferred strong resistance to CLCuD-associated viruses (Binyameen et al., 2021; Mubarik et al., 2021).

A key issue with CRISPR-based antiviral approaches is the potential for viral escape mutants to arise through error-prone non-homologous end joining (NHEJ) repair pathways. Targeting a single genomic region can lead to the accumulation of mutations, enabling the virus to regain functionality (Ali et al., 2016). Conversely, multiplex targeting greatly reduces the likelihood of such escape by concurrently disrupting multiple key sites, causing larger deletions or permanent genomic damage. Moreover, combining various CRISPR systems, including Cas9 and Cas12a, may enhance resistance by targeting multiple PAM sequences and reducing viral

resistance. The lower viral accumulation in this study confirms the efficiency of CRISPR-based antiviral approaches. These data are consistent with previous reports of effective silencing of geminiviruses with CRISPR/Cas systems (Ali et al., 2015; Ji et al., 2015). Moreover, multiplex CRISPR methodologies can prevent the emergence of replication-competent viral strains, thereby supporting their use in long-term resistance generation (Iqbal et al., 2016).

In general, the improved activity of the pKSE401 vector underscores the importance of multiplex genome editing for achieving effective, durable resistance to viruses. The ability to target multiple coding regions simultaneously increases the likelihood of complete disruption of the viral genome. Furthermore, novel applications of alternative nucleases, such as Cas12a, open new possibilities for broadening the range of targets and enhancing resistance. Overall, these findings emphasize that CRISPR-based technologies are powerful tools for developing broad-spectrum, sustainable resistance to plant viruses.

Conclusion

CRISPR-based genome editing is a revolutionary approach to controlling plant viral diseases because it enables precise, effective targeting of viral genomes. Strategies that target multiple viral regions simultaneously confer stronger resistance and reduce the likelihood of viral escape, making them more reliable than single-target approaches. These advances underscore the potential of CRISPR systems as powerful tools for developing long-term resistance in crops. Future studies should focus on improving gRNA design, identifying highly conserved viral and regulatory regions, and using more sophisticated nucleases, such as Cas12a/Cas14, to expand editing capabilities. Practical applications will require developing stable, heritable resistance in economically important crops and validating them in the field. In addition, CRISPR could be combined with other methods, such as RNA interference and traditional breeding, to further enhance resistance. CRISPR technology has enormous potential to achieve broad-spectrum, sustainable viral resistance, which would increase crop yields and support sustainable food security.

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Authors' Contributions

AA, NM, and AAH conceived and designed the study. FY and SA conducted the experiments and drafted the manuscript. AA, NM, and BA analyzed the data. All authors reviewed and approved the final version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger
SDG 9: Industry, Innovation and Infrastructure
SDG 12: Responsible Consumption and Production

Policy Addressed

The study supports policies promoting responsible application of precision genome-editing technologies in crop protection, with emphasis on biosafety, regulatory assessment, sustainable disease management, and development of virus-resistant cotton cultivars to strengthen agricultural productivity and resilience.

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