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CRISPR/CAS9 IN *GOSSYPIUM HIRSUTUM* (COTTON) COKER 312 FOR CLCUD COTTON LEAF CURL VIRUS DISEASE RESISTANCE MEDIATED BY AGROBACTERIUM

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Abstract

The cotton crop is the largest contributor in the world-wide economy. On the other hand, Gemini-virus is the biggest enemy of the cotton crop, causing CLCuVs infection in the African and Asian regions. It destroys the GDP rate to an alarming point. As biotechnology is serving mankind by introducing many conventional techniques like; specific RNA editing and plant breeding and advance techniques like; ZFNs, CRISPR/Cas9, and TALENs. To produce disease-resistant Coker 312 Cotton a CRISPR/Cas9 technique was used due to its site-specific targeting and efficiency. The targeted Rep and βC1gene play role in CLCuVs replication. An expression vector pHSE-401 containing Cas9 and multiple guided RNAs was cloned. The vector was transformed by excision of hypocotyls of cotton (*Gossypium hirsutum)* type Coker 312 plants and infection delivery was

mediated by EHA-105 Strain agrobacterium. The hypocotyls were grown on MSB regeneration media containing specific antibiotics. Then calluses were cultured in culture media and were planted into soil containing pots and kept under continuous observation. At different growth periods, no symptom of CLCuD was observed in the transgenic plants after 30 days of the growth period. The RNA was extracted at different growth periods and confirmed by PCR. The PCR products were visualized by running it on gel electrophoresis 0.8kb single guide RNA (sgRNA) bands confirmed the CRISPR/Cas9 in cotton Coker 312. Plants containing pHSE-401vector showed fewer virus traits and delay in disease development in 30 days growth period. This proposed project exhibits the desired results with a successful gene editing.

Keywords: CRISPR/Cas9, Cotton Coker 312, 3gRNA, Agrobacterium EHA-105, Multiplex vector, Transformation, Cloning,

INTRODUCTION

To edit undesired genes a lot of molecular tools have been discovered and are in practice (Mohanta *et al*., 2017). A CRISPR is as RNA based clustered regularly interspaced short palindromic repeats with Cas9 enzyme CRISPR-associated proteins. It is an adaptation in the immune system in the prokaryotes: about 50% bacteria and 90% Archaea (Ishino *et al*., 2018). It is complex Ribo-Nucleo-protein in nature and plays a defensing role in bacteria which protects it from the attacking DNAs. It has multiplex characteristics; it can edit many genes simultaneously (Bortesi and Fischer, 2015). CRISPR consists of 20 nucleotides base pairs sequence of gRNAs that is capable to bind to the targeted DNA sequence. Nuclease Cas9 has 2 domains: HNH and RuVc domain, each digest a single-stranded DNA, Cas9 stands as a 2nd component. After protospacer adjacent motif (PAM), 5' NGG Cas9 does cleavage of 3–4 bases (Jinek *et al*., 2012). Due to this activity, it is a major contributor to genome editing techniques. Cas9 nuclease enzyme cleaves the specific DNA site guided by the CRISPR. This technology has been used in a lot of fields including in human gene therapy (Stella and Montoya, 2016). In a molecular lab to perform a CRISPR technique some of the general steps are always followed, (i) PAM sequence identification, (ii) to do sgRNA designing and synthesis, (iii) construction and cloning of

sgRNAs, (Kirthi *et al*., 2004) construct introduction inside the targeted cells leads to screening, selection. Just basic facilities are required in the labs to edit genes of different organisms by the CRISPR technique. That's why this technology is in practice by the scientist around the world to make the plants and many other organisms' disease resistant. Geminiviridae family, genus Begomovirus is a challenge for cotton crops in Asia. Begomovirus strains are the main cause of different types of diseases in the cotton crop with the α and β satellite molecules. Begomoviruses are responsible for the cotton leaf curl virus disease infection in cotton plants. It contains ssDNA viruses which are small in size and contain 1 mono-partite A-DNA sometime 2 bi-partite A-DNA and B-DNA genomes bearing 2800 base pair nucleotides (Czosnek *et al*., 2017). There is a separate open reading frame (ORF) for each component in both directions (Zaidi *et al*., 2016). A-DNA contains 6 ORFs 4 are complementary to each other (AC1/C1, AC2/C2, AC3/C3, and AC4/C4) 2 are in Virion pattern (A1V1/V, and A2V2/V2). The six A-DNA genes specificity is as follows, proteins associated with replication are encoded by the gene C1 commonly called Rep gene sequence. The REn (replication-enhancer) encoded the C2 gene sequence, the TraP (transcriptional-activator protein) encoded C3 gene sequence, and C4 proteins are encoded C4 gene sequence. The B-DNA ORF's encodes the gene BC1 for movement-protein (Jiang *et al*., 2014), and BV1 for nuclear-shuttle-protein (Ha et al., 2008). It has a conserved region called a common-region or CR located on A-DNA and B-DNA strands. It contains a conserved nucleotide structure hairpin (TAATATT/AC). In hairpin at 5'end 5–7 nucleotides of Iteron plasmid act as a binding site for the proteins of the Rep gene. The Begomoviruses are pathogenic in nature and contain α and β satellites that have self-replication capability. The viral infection spread by the β-C1 protein that is encoded by the β satellite (Chatterji *et al*., 2000). The major transmitter of this virus is whitefly; it acts as a vector of the Begomoviruses (Wei et al., 2017). While feeding on an infected phloem sap fluid whitefly carries the virions with it and then these virions reach to the gut, then proceed toward the hemolymph, then towards the salivary glands and transmit the virion inside the next healthy cotton plant during feeding on sap (Czosnek et al., 2017). CLCuD infection is a dominant cotton plant infection in Pakistan, some regions of Africa and in India as well (Tahir et al., 2011; Kirthi et al., 2004). It is the biggest enemy of cotton crop with a great loss of economy (Mansoor et al., 1999). Recently this infectious disease also been reported in the Philippines and the South-Eastern region of China (Masood and Briddon, 2018). The Symptoms of this disease are curling of upper and lower

surfaces of the leaves and swelling in the veins (Briddon and Markhan, 2000). During severe infection conditions outgrowth forms on the surface of the leaf and suppresses the plant growth. The level of infection varies depending on the plant age and type (Sattar *et al*., 2013). Incidence of CLCuD causes bigger loss in crop yield ranging from 10–70% (Rahman *et al*., 2017). CLCuV epidemic was reported with many mono-partite viruses in 1991-1992 in Pakistan (Saeed *et al.*, 2015) and in 1993 in India. In recent studies many CLCuV variants with a greater potential of the outbreak are reported in the Asian subcontinent (Qadir *et al*., 2019). For about 10 years many researchers are working to produce CLCuV resistant plants. CLCuVs is curable by using different tools one of them is the CRISPR/Cas9. The objective to carry out this study is to examine the level of expression of the pHSE401 vector containing the Cas9 nucleases and sgRNAs. The study intended to transform the vector in Coker312 Cotton and to analyze the symptoms and signs of disease by evaluating the presence of the virus.

MATERIAL AND METHODS

Viral genome analysis

The cotton leaf curl virus (CLCuV) Viral genome sequence was collected from the NCBI (https://www.ncbi.nlm.nih.gov/).

Vector construction

A CRISPR design was used to analyze the targeted Rep-gene (Replication gene) sequence for the pHSE401construct (Ali *et al*., 2016). By following the defined criteria in the non-coding region selection of target site containing 20bp was performed (Baltes *et al*., 2015). The expression vector pHSE401 having a sgRNA, DNA-oligos framework and promoter was designed and constructed manually and cloned. In a PCR tube, sgRNA and distilled water in a specific given amount were added. The applied conditions for this PCR reaction were; temperature 90 to +20°C/1min. Moreover, to anneal the DNA whole mixture was kept for incubation for 5 minutes in an incubator at 90°C, and for 20 minutes at 20°C gradually.

The annealed oligos were analyzed by gel electrophoresis run containing 2.5% agarose and was stored at -20°C temperature in the refrigerator. The vector pHSE401 and annealed oligos were ligated. Then this mixture was put on incubation for about 2–3 hours at temperature 25°C. After incubation *Escherichia coli* was transformed by this ligated mixture. To select the transformed

colonies, the transformed *Escherichia coli* was grown on kanamycin antibiotic containing LB (Luria-Bertani) plates.

Confirmation of gRNAs

To make sure the presence of transformed material into the DH5α cells colony PCR was performed. The used primers are given in Table 1.

Table 1: Primers to construct the vector

Vector	Primer
pHSE401	U626F: TGTCCCAGGATTAGAATGATTAGGC dT4-R: AAACGTAATATTAAACGGATGGCC

Agrobacterium tumefaciens mediated cotton transformation

To transform the plasmid vector, gv3101 *Agrobacterium-tumefacien* stock solution was prepared and transformed by electroporation. Further this was selected on LB agar selection media containing plates at 28°C for overnight. PCR was done for screening of the transformed colonies.

Plant materials

Coker 312 cotton seeds initially were placed in Petri dishes containing MS-media (sucrose-30%, MS-salt 4.43g, and agar 12g) and Murashige. After 1 week plants were shifted in pots containing 250g soil for each plant. For each replicate, 10 plants/10 pots were kept and were monitored and watered regularly. The optimized growth conditions were 8 hours dark and 16 hours light period at temperature 25°C.

Level of expression of Cas9 and gRNAs analysis by qPCR

The *Agrobacterium* culture for inoculation was grown on selection media containing kanamycin 50µg/mL and rifampicin 25µg/mL for 48 hours at 28°C temperature to an OD of 0.5 at wavelength 600 nm. Cells of *Agrobacterium* were centrifuged at 5000 × g rpm at temperature 20°C for 15 min $MgCl_2$ (10 mM) was taken containing acetosyringone (150µg/mL) for resuspension. Infiltrate media was kept for 3 hours at room temperature, medium and the culture

were transformed into the sterilized cotton hypocotyls of 6-10 mm length and were used for *Agrobacterium*-mediated transformation as explants. The hypostyles were grown into callus under continuous observation on selection-media containing plates. Transgenic callus was further cultured and then transferred into the soil containing pots after 7 days of culture. Then by using TRIzol, RNA from the leaves was extracted. To synthesis, the cDNA with RNA-1μg a qPCR-(+gDNA-wiper) HiScript Q-RT Super-Mix kit by China was used according to given instruction by the manufacturer.

To check the expression level of the vector by applying qPCR a specially designed kit by a USA company Thermofisher named as Applied-Biosystems-7500-Fast-Dx-Real-Time-system with the given detailed SOP was used.

As an internal site controller, the pentatricopeptide repeat-containing protein (PPR) gene Accession 030098.1 (20775085; 20778395) Chromosome number 25 of *Gossypium hirsutum* type Coker312 was used with forward and reverse primers. The concentration of 20μg/μL DNA was taken by NanoDrop-2000.

Grouping of experimental Plants

Gossypium hirsutum type Coker 312 plants were agro-infiltrated with the Cotton leaf curl virus (CLCuV) infectious clones (Leuzinger *et al*., 2013).

A total of 30 *Gossypium hirsutum* type Coker 312 plants were grown in 3 different groups A-C. Group A plants were with pHSE401 without virus and group B plants were positive control having virus CLCuV without sgRNA/Cas9. Group C plants were with infectious clone and pHSE401. The plant leaf DNA was extracted after 14 days of inoculation to perform the qPCR and PCR with the same primers mentioned above.

Statistical analysis

To execute the qPCR results statistically the standard error means (SEM) of the values were calculated.

RESULTS

The plasmid vector cloning into E. coli

The plasmid Cas9 expression vector pHSE401map and cloned gRNA is represented in Figure 1. Cloning of sgRNA was confirmed by performing colony PCR with U-26-F and dt4 primers. The *E. coli* clones having a gene of interest gave 900bp or 0.9kb band size represented in Figure 2. The PCR product was run on the gel electrophoresis containing 2.5% agarose. Furthermore, the orientation and presence of the gRNA in the plasmid vector is revealed by sequencing the genome with specific primers.

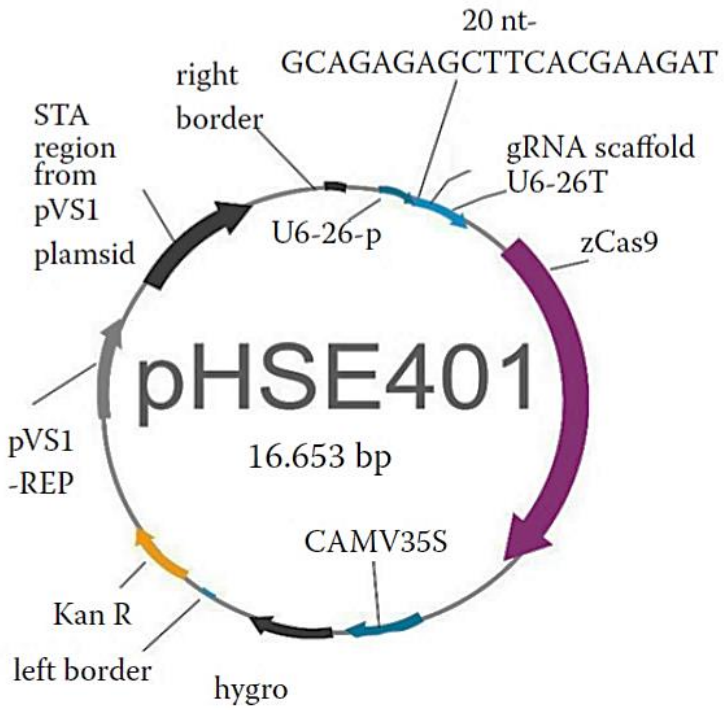

Figure 1: Map of pHSE401 Vector

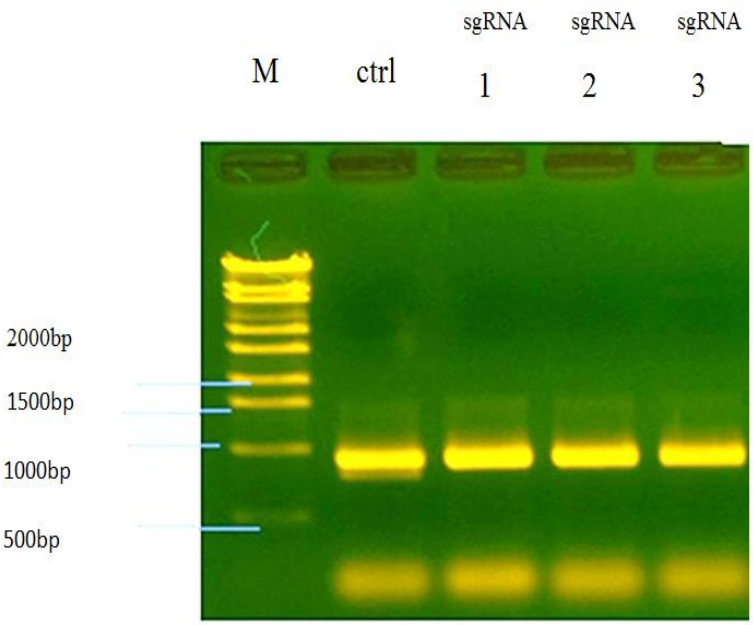

Figure 2: pHSE401 vector with gRNA clone's product by colony PCR and detected the presence of gRNA. Ladder is denoted by M and positive clones are represented by the lanes 1-3.

Analysis of Expression level of gRNA/Cas9

The qPCR was performed to evaluate the level of expression of sgRNA/Cas9 after 1 week time period of growth of plants shown in Figure 3A, B.

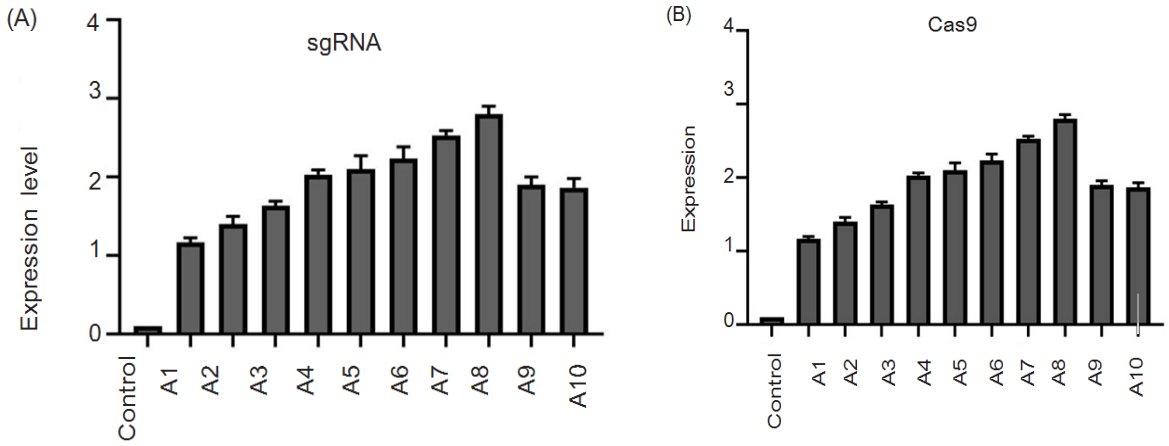

Figure 3: (A) level of expression of sgRNA-pHSE401 resulted by qPCR after 1 week of plants growth and Sample A8 expresses 2.9 expression levels, which was the highest expression level relative to control 0.1. **(B)** level of expression of Cas9-pHSE401 after 1 week of plants growth represents the maximum expression level in A8 sample in relation with the 0.1 control value

Viral infectivity assay

The plants of Group A (with no virus) were without any symptoms or signs of the disease. The plants of control group B (with the virus only) expressed disease symptoms at 1-2nd week. The group C plants show mild expression of symptoms of the infection and recovered at the 30th day of growth period (Figure.4). These experiments were done in three replicates as mentioned in Table 2.

Figure 4: *Agrobacterium* mediated transformation (a) hypocotyls transformation (b-c) callus growth stages (d) embryogenesis in somatic cells (e) regeneration of plant culture (f) regeneration of plants in nutrients (g-j) plant groups A-C grown in green house.

Table 2: Plants group experiments in replicates to express CLCuV.

Group	No. of plants	Symptomatic plants	Symptomatic plants (%)	Severity of disease symptoms	Delay in symptoms (days)	Recovery of plants 30
A	10	0	0	-	-	-
B	10	10/10	100	Severe	-	-
C	10	1/10	10	No/mild	7–8	Yes

Virus accumulation determination by qPCR

The virus accumulation expression of the pHSE401 vector was analyzed in plants at 15 days of the growth period. The qPCR result shows a very low accumulation of viruses with a range of 0.3-0.6 concerning the control group and at 20 days plant growth period 0.2-0.3 range of virus accumulation was observed. This shows that sgRNA and Cas9 system is actively performing its deletion and repair mechanism action (Figure 5 A, B).

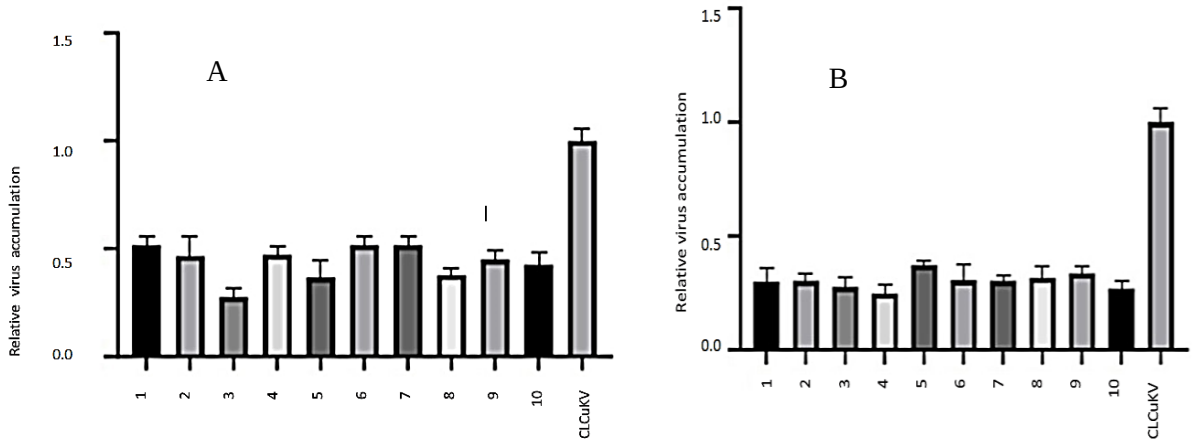

Figure 5: The qPCR values expressing the accumulation of virus with a reference virus and pHSE401 vector in ten plants. A very low accumulation of virus range 0.3-0.6 on 15th day of growth was observed.

DISCUSSION

Cotton crop is an important crop in every aspect, it's a cash crop due to its fiber, oil-seeds, textile

industry, and food properties (Long *et al*., 2018). Pakistan, India, China, Turkey, Brazil, and Africa are the agricultural land countries and are the largest producer of the cotton crop. These countries export their cotton worldwide and hence are largest exporters. Although the cotton crop is of prime importance, unfortunately, it's facing a bigger loss due to pandemic of cotton leaf curl disease. Till now a few researches have been done on its genome (Zhang *et al*., 2016). With the advent of proteomics, genomics, and transcriptomics the biotechnological tools played a greater role to improve the cotton crop genes. RNAi or RNA interference is also a well-known tool in the genetics for disruption of the viruses' replication process, but due to the activity of off-targeting, it's not a successful tool. So the CRISPR can disrupt viral DNA replication and base targeting as well (Wang *et al*., 2018). It is a commonly cultivated crop with a 2.5 Gb genome size. It's also called an allotetraploid-species (AtDt) (Li *et al*., 2015). Recently cotton crop attracts the intention of the entire research groups due to a lot of environmental and pathogenic effects.

These researches have improved the functional genomics of the cotton plants. The discovery of CRISPR/Cas9 left behind the functional genomics, due to its remarkable characteristics and efficiency of self-repairing (Yuan *et al*., 2015). Now to improve the cotton genome CRISPR technique becomes in practice due to its site-specific targeting activity. Now CRISPR emerging techniques are the main focus of the researchers as the cotton species contain many homologs alleles and fewer SNPs (Zhang *et al*., 2015).

CRISPR/Cas9 is also one of them which can make the plant free of disease (Jiang *et al*., 2014). It's an efficient tool rather than Zinc Finger Nucleases (ZFNs) and effector nucleases TALENs (Fu *et al*., 2013). To knock out the DNA plant viruses CRISPR/Cas9 is the focus of every researcher due to its efficiency and ease of working. However, the resistance of the virus against the insertion (CRISPR/Cas9) has also been reported in many studies (Ali *et al*., 2016).

This study was done to generate the CLCuV virus-resistant transgenic *Gossypium hirsutum* type Coker 312 plants by targeting the beta-satellite (βC1) gene and Rep gene. A pHSE401vector having sgRNA and a Cas9 system to target the Rep and βC1 gene was constructed according to the above mentioned procedure. The hygromycin gene present in this vector proved its transformational reliability in against selective agents. As this vector

delivered gRNA against the Rep-conserved region of CLCuV, pHSE-401-Rep was transformed to initiate the project of controlling CLCuV (Aslam et al., 2022). The expression level was measured by qPCR represented in the results section. To integrate the Cas9 and sgRNA in the model plants transformation process was mediated by *Agrobacterium* by following the above mentioned procedure in the methodology section and the model plants were kept under observation. In this study, we observed a remarkable delay in disease and recovery after 30 days which expresses the attack of CLCuV and response of CRISPR/Cas9 against the viral DNA. The data and results of this study represent the similarity in mechanism of action of CRISPR/Cas9 as expressed in various studies. The viral essay after 15 days plant growth shows the 0.3-0.6 virus range concerning the control group plants. The viral essay after 20 days plant growth shows 0.2-0.4 virus range in correlation with the control group plants. This phenomenon of recovery shows that the sgRNA and Cas9 system is performing its targeting deletion and repair mechanism of action very actively. It was seen that, mild signs of disease were self-cured within 30 days of the growth period in the CRISPR/Cas9 transgenic plants.

This study revealed a remarkable removal of viral genome by giving the healthy plant growth. Type of mutation analysis was not studied as that was not the part of our project and hence it has been suggested to sequence the transgenic genome by performing the Sanger sequencing to analyze the type of mutation. This study relies on qPCR measurement to analyze the level of expression of sgRNA and Cas9 in different groups at different growth times after inoculation.

CONCLUSION

Genome editing is a potent molecular tool. One of the most influential tools is CRISPR/Cas9 due to its site specific deletion and repair mechanism, but it requires precise measures to get the desirable results. It made the genome editing as easier and efficient than ever before. One of its applications is given in this manuscript to cure the Cotton Leaf Curl Disease (CLCuD). The Begomovirus strain is responsible for the disease. The CRISPR/Cas9 multiplex successfully edited the viral genome to a remarkable extent. A major change in plant growth efficiency and recovery was seen. Further study in this field will lead to the successful and stable application of

this tool to eradicate many other plant diseases.

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