INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–1741/2019/22–3–517–522
DOI: 10.17957/IJAB/15.1094
http://www.fspublishers.org

Full Length Article

CRISPR-Cas9 based Suppression of Cotton Leaf Curl Virus in *Nicotiana benthamina*

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Abstract

Begomoviruses infect many plant species including economically important crops worldwide. Conventional approaches for resistance management have not been successful to tackle the emerging and rapidly evolving plant viruses. The last decade has witnessed an unprecedented progress in plant genome editing technologies. CRISPR-Cas9 based gene editing has been successfully applied to engineer plants with tolerance against plant viruses. In the present study, we successfully demonstrated the use of the CRISPR-Cas9 system to suppress disease symptoms of cotton leaf curl disease (CLCuD) caused by *begomoviruses*. A unique gRNA specific for a consensus DNA sequence of the three most prevalent strains of *Cotton Leaf Curl Virus* (CLCuV) was synthesized. The consensus sequence was located in the Common Region of CLCuV genome. Transient assays of CLCuV infectious clones (CLCuKoV/CLCuMuB) and Cas9-gRNA construct were performed in 3–4 weeks old *Nicotiana benthamiana* plants by agroinfiltration. Virus accumulation in local and systemic leaves was analyzed by semi-qPCR which demonstrated lowering of virus titer by 65–75%. Infiltrated plants showed reduced symptom severity relative to control plants infiltrated with virus only. Double strand break (DSB) in DNA at the target sequence mediated by Cas9-gRNA were confirmed by T7E1 assays showing indel mutations in the CLCuV genome vs. no mutations in control plants. The results demonstrated potential use of the CRISPR-Cas9 system for engineering virus resistance in plants. Furthermore, our results also revealed that by utilizing a consensus sequence of multiple viruses, resistance to multiple virus infections can be engineered by a single gene construct. © 2019 Friends Science Publishers

Keywords: Genome editing; Transient assays; *Begomoviruses*; CLCuV; Virus resistance

Introduction

Plant viruses are pervasive in nature and can severely affect crop productivity around the globe. Significant economic losses have been reported due to vector transmitted viral diseases of plants. *Begomoviruses* infect many crop species and posit a major threat to food security of the world, especially underdeveloped regions (Czosnek *et al.*, 2017). Cotton leaf curl disease (CLCuD) is a well-known and devastating disease of cotton caused by multiple *begomoviruses*. *Begomoviruses* are single stranded DNA viruses which are transmitted by whiteflies. Other major crops infected by *begomoviruses* include common bean, mungbean, blackgram, lima bean, cowpea, soybean, tomato, potato, eggplant, pepper, chilli pepper, melon, watermelon, squash, okra and cassava (Mansoor *et al*., 2006). Improving host plant resistance against viruses is the major approach to deter *begomovirus* transmission.

Durability of plant resistance to viruses is challenged by the rapid evolution of viruses due to mixed infection in a single host. In the past, resistance strategies mainly involved conventional plant breeding and various transgenic approaches including RNA mediated interference and expressing non-pathogen derived antiviral agents or viral proteins. To date, these approaches have had limited success (Aragao and Faria, 2009; Zaidi *et al*., 2016).

Bacteria and archaea harbor a unique immunity system against invading viral or plasmid DNAs called CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR)- CRISPR-associated 9) (Marraffini and Sontheimer, 2010; Bikard *et al*., 2012). The CRISPR-Cas9 system consists of CRISPR loci, guided RNA (gRNA) and Cas9 endonuclease. As soon as any exogenous DNA molecule integrates in the CRISPR loci, it produces specific gRNAs which identify incoming target DNA and directs the Cas9 endonuclease to cleave it by inducing DSB (Hsu *et al*., 2014). Biologist have engineered the components of the CRISPR-Cas9 for utilizing in plant genome engineering experiments to remove or add DNA sequences at desired locations.

Transgenic plants expressing Cas9-gRNA complex designed against a viral DNA can confer resistance when

To cite this paper: Mubarik, M.S., S.H. Khan, B. Sadia and A. Ahmad, 2019. CRISPR-Cas9 based suppression of cotton leaf curl virus in *Nicotiana benthamina*. *Intl. J. Agric. Biol*., 22: 517–522

challenged with the same virus (Baltes *et al*., 2015). The transgenic plants produced so have shown reduced virus replication, delayed symptoms and lower infection severity (Ali *et al*., 2015; Ji *et al*., 2015). These studies show potential of CRISPR-Cas9 based virus interference for developing resistance against *begomoviruses*. Furthermore, CRISPR-Cas9 based gene editing is efficient, cost effective and less laborious as compared to conventional approaches like zinc finger nucleases (ZFNs) and transcription activator-like effectors nucleases (TALENs).

In previous reports, different research groups targeted different coding genes (Viral Rep and coat protein etc.) and non-coding or intergenic regions of five different plant viruses including CLCuV, *Bean Yellow Dwarf Virus* (BeYDV), *Beet Curly Top Virus* (BCTV), *Tomato Yellow Leaf Curl Virus* (TYLCV) and *Merremia Mosaic Virus* (MeMV) (Ali *et al*., 2015; Baltes *et al*., 2015; Ji *et al*., 2015; Ali *et al*., 2016; Khan *et al*., 2018; 2019). These reports have indicated that targeting the coding regions allows virus to escape Cas9 function by sustaining replication and systemic movement of virus molecules (Ali *et al*., 2016). However, targeting the non-coding Common Region, which is mostly conserved across related viruses, could engineer broad-spectrum virus resistance against multiple viruses (Zaidi *et al*., 2016). In the present study, we analyzed multiple sequences and selected a consensus sequence in the non-coding Common Region of the three most prevalent strains of *begomoviruses*, *Cotton Leaf Curl Kokhran Virus* (CLCuKoV*)*, *Cotton Leaf Curl Multan Virus* (CLCuMuV*)* and *Cotton Leaf Curl Burewala Virus* (CLCuBuV).

Materials and Methods

In silico Analysis of Virus DNA and gRNA Selection

DNA sequences of three most prevalent CLCuV strains, CLCuKoV, CLCuMuV and CLCuBuV were retrieved from National Center for Biotechnology Information (NCBI). DNA sequence of non-coding Common Region of all three virus strains were used to find a consensus sequence to design gRNA using online software CRISPR-Multitargeter (http://www.multicrispr.net/).

Synthesis and Annealing of 20 bp-gRNA

The 20-nucleotide target site (also known as protospacer) selected by CRISPR-Multitargeter was used for gRNA oligos synthesis. The 20-nucleotide gRNA target and adapter sequence of *Bsa*I restriction enzyme were synthesized commercially. The desalted oligos for gRNA were annealed to develop double stranded gDNA with overhangs of *Bsa*I to further clone into plant expression vector (pHSE401).

Construction of CRISPR-Cas9 Plant Expression Vector

The plant expression vector containing plant codon optimized Cas9 (pcoCas9) (pHSE401-pcoCas9) (Addgene, U.S.A., Plasmid # 62201) was digested with *Bsa*I restriction enzyme and a DNA fragment of 1554 bp was gel purified. Annealed gDNA oligos and gel purified DNA fragment were ligated using standard T4 DNA Ligase reaction. The presence of 20 nucleotide gDNA sequence was confirmed by sequencing.

Evaluation of the CRISPR-Cas9 based Constructs in *Nicotiana benthamiana*

Plant growth conditions: Wild-type seeds of *N. benthamiana* were grown in small plastic pots containing commercially available germination soil. All pots were kept under 16 h light and 8 h dark photoperiod with appropriate light intensity at 26°C. Seedlings were started to germinate after 5 days and transferred to new plastic pots (one seedling per pot). Three to four weeks old plants (4–5 fully expanded leaves) were used for agroinfiltration based disease resistance assay.

Disease Resistance Assay

To evaluate the efficacy of the CRISPR-Cas9 constructs for virus suppression in plants, infectious clones having CLCuKoV and Cotton Leaf Curl Multan Beta-Satellite (CLCuMuB) and Cas9-gRNA were co-infiltrated in the *N. benthamiana* leaves. The *Agrobacterium tumefaciens* strain EHA-105 was used for agroinfiltration. First, EHA-105 containing pHSE401-CR-gRNA vector and CLCuKoV/CLCuMuB infectious clone vector were grown in LB medium containing respective antibiotics. A well grown *Agrobacteria* were harvested by centrifugation and diluted in infiltration media (10 m*M* $MgCl_2$, 10 m*M* MES pH 5.6 and 150 μM acetosyringone). A fully expanded leaves of three to four weeks old *N. benthamiana* plants were chosen and inoculated with pHSE401-CR-gRNA infiltration solution using needleless syringe. Two days later, infectious clones of CLCuKoV/CLCuMuB were infiltrated into the same leaves. The positive control plants infiltrated with CLCuKoV/CLCuMuB infectious vector only. Virus symptoms were recorded 10 days after infiltration (10 dai). Virus titer was measured from local and systemic leaves by semi-quantitative PCR (sqPCR) using virus specific primers. Actin gene primers were used as a normalizer (internal control) in sqPCR reactions.

T7EI Mutation Detection Assay

To determine efficacy of CRISPR-Cas9 system against CLCuV, we confirmed occurrence of mutations due to DSB repair process through the non-homologous end joining (NHEJ) pathway. Genomic DNA was isolated from infiltrated leaves at 12 dai and used as a template for PCR amplification in gRNA target region. PCR amplicons were denatured, renatured, and treated with T7EI to validate the DSB in the viral genome.

Results

Construction of Cas9-gRNA Construct to Target Common Region of virus DNA

When the non-coding conserved region of the three most prevalent CLCuV strains (CLCuBuV, CLCuKoV and CLCuMuV) was input into the CRISPR-Multitargeter software, a unique 20 nucleotide sequence was identified as a potential target for gRNA (Fig. 1). The resulting nucleotides sequence was cloned into plant expression vector along with Cas9 (pHSE401-CR-gRNA). DNA sequencing of the resulting vector confirmed the integrity of gRNA (Fig. 2).

Infectivity Assay

Plants infiltrated with infectious clones of CLCuKoV/CLCuMuB showed virus symptoms on 10–14 dai. The control plants infiltrated with Cas9-gRNA vector, did not show any disease symptoms (Fig. 3a). Severe disease symptoms were recorded at 18 dai. The symptoms appeared in irregular fashion including leaf curling (upward and downward), stunted growth and vein thickening (Fig. 3b). PCR using virus DNA specific primers amplified virus in symptomatic plants while control plants did not show any amplification of viral DNA.

Evaluation of pHSE401-CR-gRNA Construct for Virus Suppression

When three to four weeks old *N. benthamiana* plants were infiltrated with pHSE401-CR-gRNA and CLCuKoV/CLCuMuB infectious clone, a significant delay in symptoms development was observed. Symptoms started appearing on 15–20 dai in the plants co-infiltrated with pHSE401-CR-gRNA and CLCuKoV/CLCuMuB infectious clone as compared to control plant infiltrated with virus only. Virus titer measured using sqPCR was also found lower both in local and systemic leaves. Together with low virus titer, delayed virus symptoms and lower symptoms severity, results indicated suppression of CLCuV due to CRISPR-Cas9 activity (Table 1). All plants infiltrated with infectious clones alone showed typical leaf curl disease symptoms (Fig. 4a). Co-infiltrated plants with pHSE401-CR-gRNA and CLCuKoV/CLCuMuB infectious clone showed mild disease symptoms. Delayed virus symptoms also indicated a lower virus titer and mild disease symptoms (Fig. 4b). Moreover, it was also observed that the plants showing delayed symptoms development and lower disease symptoms also recovered symptoms between 22–28 dai. Control plants infiltrated with Cas9-gRNA alone showed no virus symptoms (Fig. 4c).

Quantitative and Semi-quantitative Expression of Cas9-gRNA Construct

For expression analysis, leaf RNA isolated at 2–3 dai was used to synthesize cDNA using dT and gRNA specific primers and the resulting cDNA was used for PCR with Cas9 and gRNA specific primers. The results revealed expected amplification products indicating the expression of Cas9 and gRNA (Fig. 5). On the other hand, the qPCR results showed variable expression of both Cas9 and gRNA

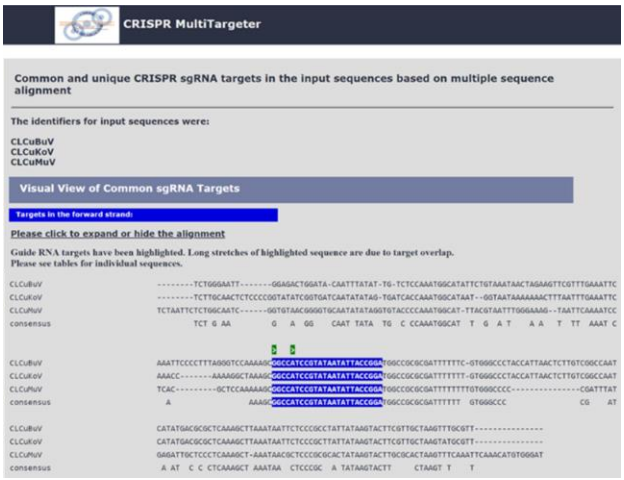

Fig. 1: Consensus nucleotide sequences of CLCuV Common Region. CLCuBuV, CLCuKoV and CLCuMuV sequence were subjected to CRISPR-Multitargeter. Common and unique gRNA target sequence is represented by blue highlighted nucleotides

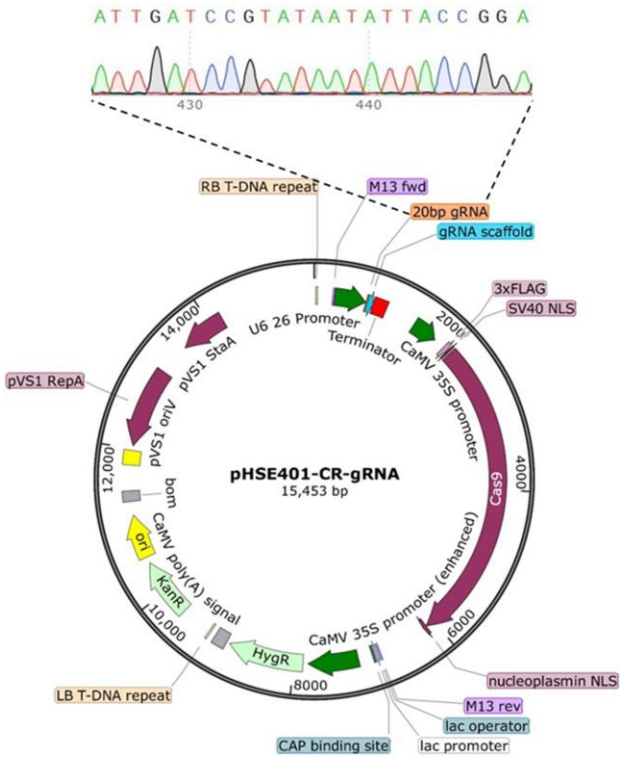

Fig. 2: Architecture of CRISPR-Cas9 based plant expression vector pHSE401-CR-gRNA. Sequencing results of 20-bp gRNA sequence are represented by black arrow lines

Table 1: Evaluation of Cas9-gRNA construct for the suppression of virus

Infiltration Solution	Experiment Number	No. of Plants	Infected Plants	Symptoms Level	Symptomatic Plants%	Delayed Symptoms (No. of Days)	Plants Recovered at 28 dai	Molecular Confirmation	
								PCR	qPCR
Virus Only	1	10	10	Severe	100	-	No	+10/10	1
	2	10	10	Severe	100	-	No	+10/10	1
	3	10	10	Severe	100	-	No	+10/10	1
Cas9-gRNA	1	10	3	Mild	30	6-8	Yes	+08/10	0.40
+ Virus	2	10	4	Mild	40	6-8	Yes	+09/10	0.37
	3	10	4	Mild	40	6-8	Yes	+09/10	0.31
Control	In all experiments	05	NA	NA	NA	NA	NA	NA	NA

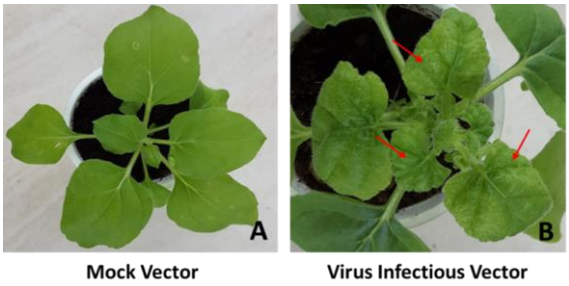

Fig. 3: Virus infectivity assays at 12 dai. Control plant infiltrated with mock vector only (**A**). Plant infiltrated with CLCuV infectious clones, red arrows indicate symptoms of virus infection (**B**)

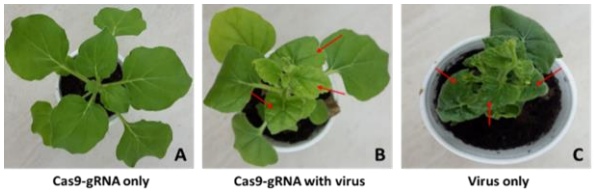

Fig. 4: Cas9-gRNA based CLCuV suppression in *N. benthamiana* plants at 18 dai. Red arrows indicate different levels of virus infection. Virus symptoms in plants with Cas9-gRNA challenged with virus (**B**), Virus symptoms in plants infiltrated with virus alone (**C**), control plants infiltrated with Cas9-gRNA construct alone (**A**)

relative to reaction normalizer actin gene of *N. benthamiana* (Fig. 6).

Virus Titer Determination by qPCR

Plants co-infiltrated with Cas9-gRNA construct and virus infectious clones showed less and delayed disease symptoms. The virus accumulation in local and systemic leaves analyzed by sqPCR analysis showed a decrease in virus accumulation by 65–75% in co-infiltrated plants as compared to plants inoculated with virus alone (Fig. 7). Furthermore, Virus titer was also found reduced in systemic leaves infiltrated with Cas9-gRNA construct when compared to control plants (Virus only) (Fig. 8).

T7 Endonuclease-I Assay

To investigate whether decreased virus accumulation is due to DSB in CLCuV genome created by Cas9-gRNA complex

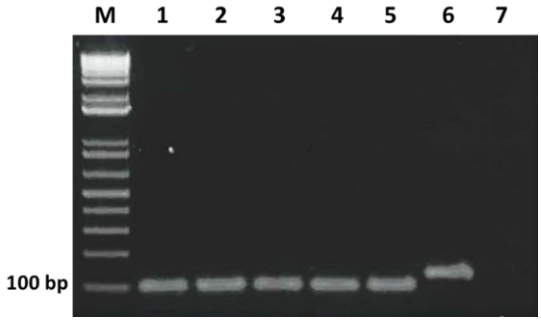

Fig. 5: Semi-quantitative PCR of Cas9 and gRNA. 1 kb plus DNA ladder (M). Cas9 and gRNA amplified fragments (96 bp) using real-time primers (Lane 1-5). Amplification of 135 bp of Actin gene (Positive control) (Lane 6). Negative control (Lane 7)

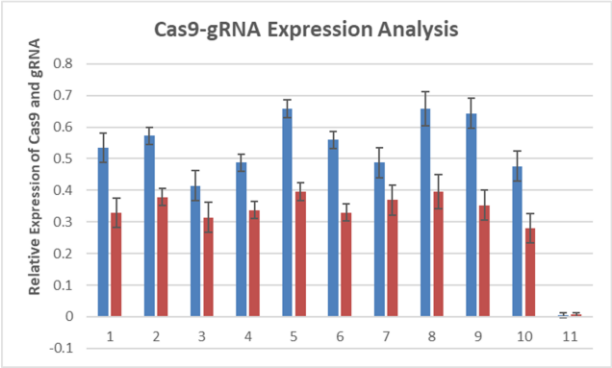

Fig. 6: Expression analysis of Cas9-gRNA construct. Blue bars indicate relative expression of Cas9 and red bars are for relative expression of gRNA (Lane 1-10). Cas9-gRNA expression in control plant (Lane 11). Actin gene of *N. benthamiana* was used as an internal control and reaction normalizer

or not, the T7EI assays were performed to validate DSB. The T7E1 assays revealed that pHSE401-CR-gRNA vector induced DSB in the CLCuV genome while no DSBs were found in DNA samples inoculated with virus alone (Fig. 9).

Discussion

In a rapidly growing global population, food security is one of the foremost current global challenges. Every year, vector transmitted viral diseases of crop plants are estimated to reduce 10 to 15% global crop yields (Mahy and Van-

Regenmortel, 2008). *Begomoviruses* cause devastating losses in crops every year and threaten global food security (Navas-Castillo *et al*., 2011). No doubt, plants with improved resistance are the most sustainable approach to overcome insect transmitted viral diseases. Crop management approaches, such as control of insect vectors with pesticides and removal of infected plants by continuous visual inspection, have been found ineffective, costly and time consuming.

Conventional breeding strategies have been exhaustively used to develop resistance against *begomoviruses* (Zaidi *et al*., 2016). However, these strategies are not effective due to rapid evolution of *begomoviruses* by mutation and recombination (Hanley-Bowdoin *et al*., 2013). These challenges require new approaches that can generate durable resistance. Newly emerged site-specific genome engineering tools offer new opportunities to engineer the genetic architecture of crop plants. Modern genome engineering tools like ZFNs and TALENs are based on DNA binding proteins joined to a DNA nuclease domain (FokI) (Khan *et al*., 2017). A major limiting factor of these tools is the designing and engineering of new constructs every time it is to be used for each target.

The CRISPR-Cas9 system has recently emerged as a simple, efficient and precise genome engineering tool for the development of genetically improved crop plants (Khatodia *et al*., 2016; Mubarik *et al*., 2016; Schiml and Puchta, 2016). It was first introduced into plants in 2013. Since then, it has rapidly been adopted by the scientific community as an efficient genome editing tool in plant research.

Various research groups have reported engineered virus resistance in plants using CRISPR-Cas9 system based on targeting of coding and non-coding virus DNA sequences with different efficiencies (Ali *et al*., 2015; Baltes *et al*., 2015; Ji *et al*., 2015; Ali *et al*., 2016; Chandrasekaran *et al*., 2016; Pyott *et al*., 2016). Studies so far, have shown decreased virus titer and delayed symptom development. It has been reported that targeting coding regions of viral DNA could result development of virus variants capable of duplicating and systemic movement. However, studies involving non-coding regions as the target sites have shown better results due to lower virus titer (Ali *et al*., 2016).

Our results have validated the above findings. Plants infiltrated with Cas9-gRNA construct and virus showed reduced and delayed symptoms. Quantification of viral DNA titer in control and experimental plants showed that targeting of non-coding Common Region of CLCuV with CRISPR-Cas9 approach was successful to inhibit virus. Decreased virus titer was also found in systemic leaves showing lower ability of virus to spread. Expression analysis of both Cas9 and gRNA in the transiently transformed *N. benthamiana* leaf tissues validated the CRISPR-Cas9 activity *in vivo*. Being a conserved region, it is concluded that targeting non-coding region could be a potential approach for the development of broad-spectrum resistance against *begomoviruses*. Ali *et al.* (2016) also reported similar results by targeting the non-coding viral DNA sequences to inhibit replication, systemic infection and generation of viral variants.

The short genome of *begomoviruses* contain specific DNA sequences necessary for different cellular functions inside host plants (viral replication, host infection and host defense systems). For targeted viral DNA interference, focus has been on sequences that provide efficient and better interference. The CRISPR-Cas9 system targeting the non-coding region could be a better choice to inhibit or prevent replication by cleaving the regulatory sequences of *begomoviruses*. Moreover, by screening more target sites, it is possible to identify efficient gRNA target sites which are common to multiple viral genomes. Simultaneous targeting of multiple regions of virus genome would hinder the functionality of NHEJ DNA repair mechanism as chopped viral DNA fragments would ultimately be degraded.

Conclusion

Our results corroborate earlier findings and validate the importance of gRNA target site selection when developing CRISPR-Cas9 constructs for durable resistance to enhance crop productivity.

Acknowledgement

This work has been supported by grant from Higher Education Commission (HEC), Pakistan. We are grateful to Shahid Mansoor and Imran Amin from National Institute for Biotechnology and Genetic Engineering (NIBGE), Pakistan for providing CLCuV infectious clones.

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[Received 27 Nov 2018; Accepted 12 Apr 2019; Published (online) 12 Jul 2019]