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Full Length Article

CRISPR/dCas9-mediated Inhibition of Replication of Begomoviruses

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Abstract

CRISPR/Cas9 system with deactivated or dead nuclease domain of Cas9 (known as dCas9) can also be used as a DNA binding protein to target specific DNA sequences. Similar to zinc fingers and transcription activator like effectors (TALEs), dCas9 can be fused with other effector domains for different purposes such as gene activation, gene repression, modification of epigenetic marks etc. Previously, TALEs were used to target and occupy the most conserved nonanucleotide sequence of Cotton Leaf Curl Virus (CLCuV) with promising results. The purpose of this study is to check the efficiency of dCas9 as DNA binding protein targeting the nonanucleotide sequence of CLCuV to inhibit virus replication. *Nicotiana benthamiana* was used to evaluate the efficiency of CRISPR/dCas9 system for viral interference. The resistance against virus infection and suppression of replication of the virus was assessed by PCR, RT-PCR and symptoms development on plant leaves in terms of days' post inoculation (dpi). Expression of the Cas9 and gRNA was quantified using RT-PCR. The results showed partial inhibition of CLCuV replication, lower disease symptoms and virus accumulation as compared to control plants. A comparison of current and previous results using TALEs showed that dCas9 was slightly less efficient to suppress virus replication. Multiplexing of gene editing techniques could be the way forward to engineer virus resistance in plants. © 2019 Friends Science Publishers

Keywords: Begomoviruses; dCas9; Nonanuceotide; Plant virus resistance; TALEs

Introduction

Gene editing technology has transformed the agricultural landscape. The last decade has witnessed a clear shift from conventional genetic engineering to targeted gene editing to develop economically important traits in crop plants. Use of engineered nucleases for genetic improvement has been reported with equal success in plants and animals (Knott and Doudna, 2018). Many engineered nucleases including zinc finger nuclease (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/CRISPR-associated system (CRISPR/Cas) have been used successfully for genome targeting along with specific DNA binding domains/proteins (Qi *et al.*, 2013; Cheng *et al.*, 2015; Xu *et al.*, 2016). The CRIPSR/Cas9 is an antiviral enzyme of the Type II adaptive immune system in prokaryotes (Barrangou *et al.*, 2007; Bhaya *et al.*, 2011). This endogenous enzyme uses two catalytic domains (RuvC and HNH) to introduce double-strand breaks in the target DNA. In natural system, CRISPR/Cas9 is guided to its target sequence by two RNA molecules; CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). Additionally, a protospacer adjacent motif (PAM) region flanking the target site is also required for target recognition which is recognized by the crRNA (Jinek *et al.*, 2012). While designing and cloning of Cas9 protein to target a preselected DNA sequence involves a single guided RNA (sgRNA) according to the availability of PAM region. CRISPR/Cas9 system has been proved as a successful tool for targeted genome engineering in a variety of organisms (Cho *et al.*, 2013; Mali *et al.*, 2013; Doudna and Charpentier, 2014).

A nuclease deactivated Cas9 (dCas9) has been reported by mutating the catalytic amino acids necessary for cleavage of dsDNA (Jinek *et al.*, 2012). Even after mutation of both nuclease domains, the catalytically inactive Cas9 retained the RNA-guided DNA-binding activity. Therefore, this specific DNA binding system can be used for targeting any DNA sequence of choice. Fusion of other effector domains with dCas9 has also

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been reported (Gilbert *et al.*, 2013; Qi *et al.*, 2013; Kearns *et al.*, 2015) with diverse applications in genomics and epigenomics. It has been demonstrated for staining of chromosomal regions for live cell imaging (Chen *et al.*, 2013; Anton *et al.*, 2014; Ma *et al.*, 2015), editing of epigenetic marks (Hilton *et al.*, 2015; Kearns *et al.*, 2015), gene expression modulation (Cheng *et al.*, 2013; Gilbert *et al.*, 2013) and transcriptional blockage (Bikard and Marraffini, 2013; Qi *et al.*, 2013).

Use of site-specific DNA binding proteins for suppression of plant viruses has been reported by many researcher (Takenaka *et al*., 2008; Mori *et al*., 2013; Cheng *et al*., 2015). An artificial zinc finger (AZP) protein was used successfully for the inhibition of replication of begomoviruses (Sera, 2005; Koshino-kimora *et al*., 2008). Replication of begomoviruses is mediated by the replication associated AC1 (Rep) protein. Inhibition of Rep protein function by blocking the promoter region of *AC1* gene using site-specific DNA binding proteins has been reported as a promising approach to suppress viral replication. Moreover, the geminivirus conserved region in their stem loop structure, called "nonanucleotide" has also been targeted with artificial DNA binding proteins such as "TALEs" (Cheng *et al*., 2015).

Begomoviruses are posing a big threat to our agriculturally important crops and vegetables (Kenyon *et al.*, 2014; Leke *et al.*, 2015). Many efforts have been made to control begomoviruses infections. Replication in begomoviruses is mediated through rolling-circle mechanism in the nucleus of host cells (Saunders *et al.*, 1991; Stenger *et al.*, 1991). Replication initiator protein (rep) is the key protein involved in the replication of begomoviruses encoded by C1 or rep gene (Hanley-Bowdoin *et al.*, 1999). The common region (CR) contains Rep binding sites in the form of iterons which are responsible for initiation and regulation of replication associated gene expression (Hanley-Bowdoin *et al.*, 1999). It is believed that binding of Rep protein at iterons results a nick in the nonanucleotide region, TAATATT/AC (slash indicates the nicking site) to initiate rolling circle replication (Laufs *et al.*, 1995a; Laufs *et al.*, 1995b; Orozco and Hanley-Bowdoin, 1996; Settlage *et al.*, 1996). Nonanucleotide (TAATATT/AC) is most conserved region among all begomoviruses and betasatellites (Zaidi *et al.*, 2017).

Previously, use of TALEs was reported for suppression of CLCuV by targeting Rep and nonanucleotide regions (Khan *et al.*, 2018). In previous study, it was found that the DNA binding proteins can be used to inhibit the replication of the viruses. So, now we used another type of the DNA binding protein, dCas9, which can also inhibit replication of the virus and ultimately can decrease virus infection as well. The objective of the current study was to investigate the efficiency of dCas9 in suppression of begomoviruses as a binding protein for transcriptional blockage. These results laid a foundation for future usage of dCas9 as DNA binding protein.

Materials and Methods

Designing of gRNA for Cloning into dCas9 Plant Expression Vector (pHSE401)

DNA-A sequence of Cotton Leaf Curl Khokhran Virus (CLCuKV) was retrieved from NCBI. The common region of DNA-A sequence of CLCuKV (about 200 bp) was used to identify potential target sites for dCas9 using freely available CRISPRdirect software. The target site containing nonacucleotide region was selected with respect to PAM region (Fig. 1). Forward and reverse primers were designed according to the target sites as follows;

dT4-F: 5'ATTGGGCCATCCGTTTAATATTAC3'
dT4-R: 5'AAACGTAATATTAAACGGATGGCC3'

DNA bases in red indicate overhangs added for ligation into restricted backbone vector.

Construction of dCas9 Plant Expression Vector (pHSE401-dT4)

Cloning of the gRNA oligoes was achieved through a three step process, *i.e.*, hybridization of the primers, restriction of the dCas9 (backbone vector) and ligation into backbone. For hybridization of primers, 25 μL of each primer was mixed in a 200 μL PCR tube. The reaction was conducted in a thermocycler using following program:

95°C (3 min)+90°C (1 min)+80°C (1 min)+70°C (1 min)+ 60°C (1 min)+50°C (1 min)+40°C (1 min)+30°C (1 min)+ 20°C (1 min)+4°C (∞)

The backbone vector was digested with BsaI and the gel DNA was eluted using GeneJET Gel Extraction Kit (Thermo Scientific). Ligation reaction was run at 22ºC for 4 h using T4 ligase. Ligation product was transformed into *E. coli* DH5α chemically competent cells. To confirm cloning of gRNA into dCas9 backbone, colony PCR was conducted using specific primers;

U6-26-F: 5'TGTCCCAGGATTAGAATGATTAGGC3'
dT4-R: 5'AAACGTAATATTAAACGGATGGCC3'

Plasmid DNA was isolated from the PCR confirmed clones using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and was sequenced before transformation into *Agrobacterium* (GV3101).

Plant Growth Conditions

Tobacco (*Nicotiana benthamiana*) plants were initially grown on MS media (MS salt 4.43 g, sucrose 30%, agar 12 g) in petri plates. After seven days, the nursery was shifted to pots having soil (one plant each pot, 2.5" x3", with 200 g soil). Total 10 pots/10 plants were maintained for each replication and were watered daily. *N. benthamiana* plants were grown in a growth room under controlled conditions at 25+2°C temperature with 16/8-light dark period.

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Virus Infectivity Assay in *N. benthamiana*

The infectivity of the infectious clones was checked in *N. benthamiana* plants through agro-infiltration. Two weeks old plants were infiltrated with *Agrobacterium* having cotton leaf curl Multan beta satellite (CLCuMB) and cotton leaf curl kokhran virus (CLCuKV). Briefly, inoculation culture of *Agrobacterium* was grown to an optical density of 0.6 at 600 nm after growing at 28°C for 48 h with 25 μg/mL of Rifampicin and 50 μg/mL of Kanamycin. *Agrobacterium* cells were spun at 5,000 × g for 15 min at 20°C and re-suspended in 10 m*M* $MgCl_2$ containing 150 μg of acetosyringone per mL. Infiltration medium was left on bench for 3 h. Infiltration was done into the young and fully expanded leaves of *N. benthamiana* plants. After syringe infiltration, the plants were kept under same growth conditions given in Section 3. The plants were observed for symptoms development after 9 days of infiltration. The plant leaf samples were taken at 14 days' post inoculation (dpi) for DNA extraction to perform PCR and qPCR. Same primers were used for PCR and qPCR as given previously (Khan *et al*., 2018).

Evaluation of dCas9 for Virus Suppression in *N. bethamiana*

Plants of *N. benthamiana* were grown in controlled conditions at 25+2°C temperature, 65% relative humidity and 16 h photoperiod. *Agrobecterium tumefaciens* strain GV3101 was transformed with dCas9 construct grown overnight at 28ºC. At 0.5 OD, the bacterial culture was harvested and centrifuged at 5000 rpm for 6 min at room temperature. Pallet was suspended into infiltration medium until 0.5 OD of bacterial suspension and then incubated for 4 h at bench. The resulting *A. tumefaciens* cells were transformed into 3 weeks old leaves of *N. benthamiana* through agro-infiltration method using 1 mL syringe. These leaves were infiltrated at 2 dpi with infectious clones of CLCuKV/CLCuMB. Infiltrated plants were again subjected to the same growth conditions. For molecular analysis, leave samples were collected at 9-21 dpi and immediately immersed into liquid nitrogen for further analysis. Accumulation of CLCuKV was detected by qPCR using Applied Biosystems 7500 Fast Dx Real-Time system according to manufacturer protocol. *PPR* gene of *N. benthamiana* was used as an internal control. Concentration of each DNA sample was adjusted to 20 ng/µL using NanoDrop 2000 (Thermos Scientific). Delay in symptom development was observed and recorded from 9-21 dpi (Fig. 10).

Results

Cloning of gRNA

The gRNA was cloned into pHSE401 dCas9 plant expression vector using designed oligos. Cloning of gRNA into the dCas9 expression vector was confirmed through colony PCR using the same primers; U6-26-F & dT4-R.

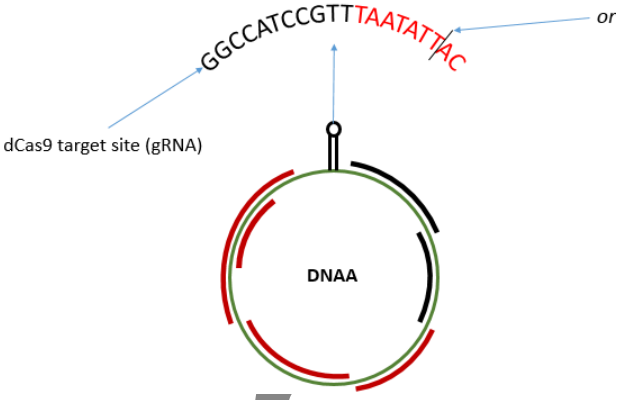

Fig. 1: Selection of target site in Begomovirus DNA A. Nonanucleotide sequence (shown in red) was selected for targeting with dCas9. A sequence of 20 nucleotides was selected as per requirement using CRISPRdirect, a freely available online software. *Ori* is the origin of replication of virus which is located within nonanucleotide region

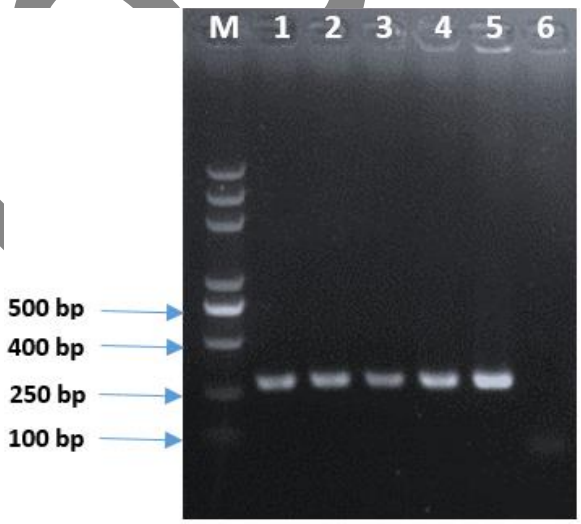

Fig. 2: Confirmation of gRNA clones. Colony PCR was electrophoresed to confirm cloning of 20 nt gRNA in dCas9 vector. The PCR product was run on 1.2% agarose gel. Lane M contains DNA ladder. Lane 1-5 contains expected clone with amplification of around 280 bp. Lane 6 contains negative control

The result of colony PCR is given in Fig. 2. Positive clones showed DNA amplification of about 280 bp. Plasmid was sequenced using specific primers to check the presence and orientation of the cloned gRNA sequence in the vector. Sequencing results are given in Fig. 3a and b. The map of the dCas9 expression vector, pHSE401 (dT4), with cloned gRNA is given in Fig. 4. dCas9 fused with NLS (nuclear localization signal) was expressed under 2x35S promoter and E9ter terminator. While gRNA was under U6-26 promoter and terminator.

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ATCTTCATTCTTAAGATATGAAGATAATCTTCAAAAGGCCCCTGGGAATCT
GAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACAATAGTATTTCT
TATATAGGCCCATTTAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTT
AGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGA
TTGGGCCATCCGTTTAATATTACGTTTTAGAGCTAGAAATAGCAAGTTAAA
ATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
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ACACTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAG
GGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGAT
TCATTGCCCAGCTATCTGTCATTTATTGGGAAATAGTGGAAAAGGAAGTG
GCTCCTACAAATGCATCATTGCGATAA

Fig. 3a: Sequencing results of gRNA

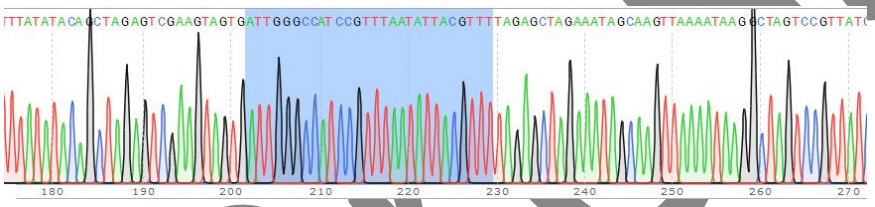

Fig. 3b: DNA sequence (28bp highlighted) showing confirmation of 20 nt of gRNA along with 4 bp overhangs on both ends for cloning into plant expression vector

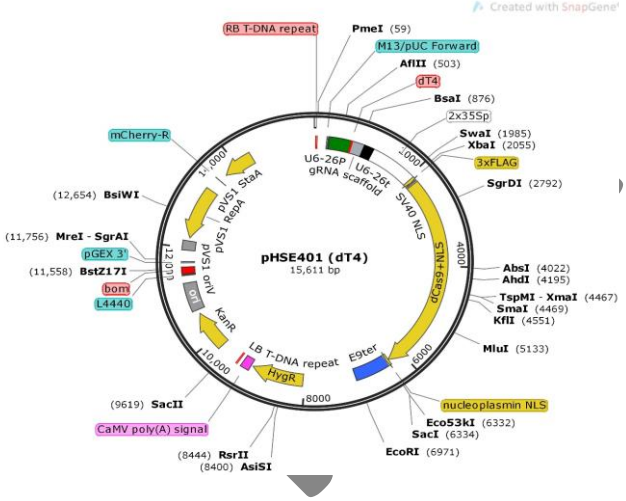

Fig. 4: Map of dCas9 expression vector, pHSE401 (dT4), with dT4 gRNA

Expression Analysis of gRNA-dCas9 in *N. benthamiana* Leaves

For expression analysis of gRNA-dCas9 in agro-infiltrated leaves, DNA was quantified at 2 dpi through qPCR. The relative expression of the sgRNA and dCas9 was adequate and the results of qPCR are given in the Fig. 5 and 6 respectively.

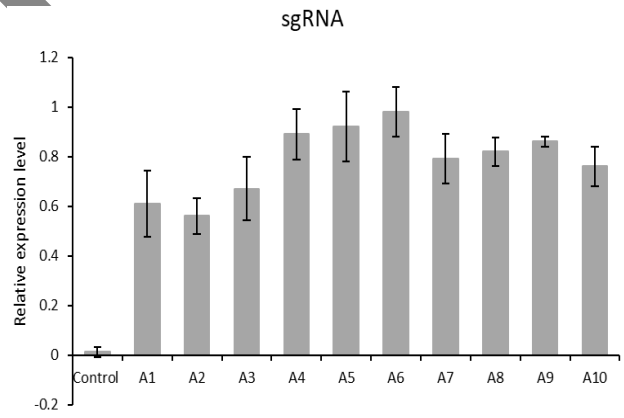

Fig. 5: qPCR results for expression level of sgRNA. Relative expression level of sgRNA in plant leaves is given in Fig. bars (A1-A10) relative to the control

Infectivity Assay

Infectious clones (CLCuKV/CLCuMB) infiltrated leaves were taken at 14 dpi to detect the presence of virus through PCR and compared with symptom development (Fig. 6). Ten out of 10 virus infected plants showed strong disease symptoms and presence of virus (Fig. 7 and Table 1). Results of PCR showed that all plants accumulated CLCuKV/CLCuMB in the systemic leaves showing disease symptoms (Table 1).

Table 1: Evaluation of dCas9 for suppression of CLCuKV in *N. benthamiana*

Inoculum	Experiment No.	No. of plants	Symptomatic plants	% symptomatic plants	Symptoms severity	Delay in symptoms (No. of days)	Plants recovered at 30 dpi	Diagnostics	
								PCR	qPCR
Non-inoculated plants	In all experiments	2	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CLCuKV/CLCuMB	1	10	10	100	Severe	-	No	+10/10	1
	2	10	10	100	Severe	-	No	+10/10	1
	3	10	10	100	Severe	-	No	+10/10	1
dCas9+CLCuKV/CLCuMB	1	10	4/10	40	Mild	2-4	Yes	+8/10	0.38
	2	10	3/10	30	Mild	2-4	Yes	+6/10	0.34
	3	10	5/10	50	Mild	2-4	Yes	+9/10	0.42

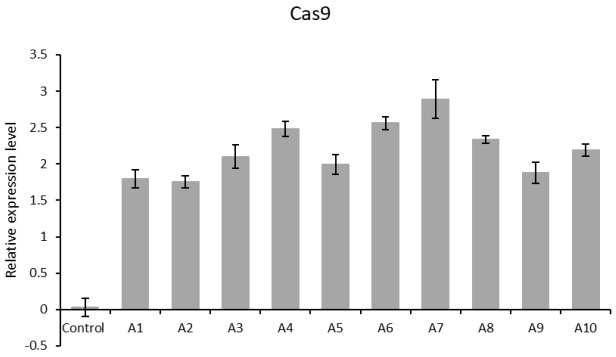

Fig. 6: qPCR results for expression level of Cas9. Relative expression level of Cas9 in plant leaves is given in Fig. bars (A1-A10) relative to the control

Fig. 7: Infectivity assay of infectious clones in *N. benthamiana* plants. Symptoms were developed at 12 dpi. A: Control plant without virus infiltration. B: CLCuKV/CLCuMB infiltrated plants at 14 dpi

Evaluation of dCas9 for Virus Suppression

To evaluate the potential of dCas9 for virus suppression, *N. benthamiana* plants were co-infiltrated with gRNA-dCas9 and infectious clone of CLCuKV/CLCuMB. Symptoms were observed on control plants at 9-14 dpi infiltrated with infectious clone only. A delay of 2-3 days in symptoms development in the co-infiltrated plants with gRNA-dCas9 and CLCuKV/CLCuMB was observed. Expression of gRNA-dCas9 showed suppression of virus proliferation in terms of delay in symptoms, mild and attenuated symptoms, and low virus accumulation (Table 1). All experiments were conducted in three replications.

Determination of Virus Accumulation by qPCR

Accumulation of virus under transient expression of dCas9 was analyzed in *N. benthamiana*. A decrease in virus symptoms, in terms of leaf curling and vein thickening, was evident due to dCas9 as shown in Fig. 8. The results of qPCR analysis indicated low titer of virus (0.2-0.4 compared to 1 of control) due to dCas9 and low accumulation of the virus in systemic leaves. Co-infiltrated plants with dCas9 and CLCuKV/CLCuMB showed decrease in the virus titer up to 60-80% (Fig. 9).

Delay in Symptoms Development

Symptoms of cotton leaf curl disease in control plants started to appear from 9-14 day with equal frequency. The dCas9 and CLCuKV/CLCuMB co-infiltrated plants showed symptoms at 12-16 dpi. (Fig. 10). Moreover, it was found that lower level of infection was associated with the delay in the symptoms. In contrast to control plants, it was also observed that the dCas9 co-infiltrated plants recovered from infection at maturity stage (data not shown).

Discussion

For this study, nonanucleotide region was chosen due to its critical importance on the basis of these previous studies (Fig. 1). Targeting nonanucleotide or origin of replication (ori) for inhibition of replication and viral infection may produce a broad spectrum resistance against geminiviruses. Expression of Cas9 and gRNA in plant leaves was quantified through RT-PCR (Fig. 5 and 6). Similar expression quantification for Cas9 and gRNA was given by Ji *et al*. (2015). Adequate expression of Cas9 protein and gRNA is very crucial for the activity of CRISPR/Cas-gRNA system. In case of infectivity of the infectious clones in *N. benthamiana* plants, the symptoms were observed in all control plants (Fig. 7 and Table 1). These results are similar to Khan *et al*. (2018) in which severe symptoms were found with 100% symptomatic plants. With respect to the symptom appearance, it was found that severe symptoms were appeared at 12 dpi which is in line with results of previous study in which the symptoms were appeared in 10-13 dpi (Khan *et al*., 2018).

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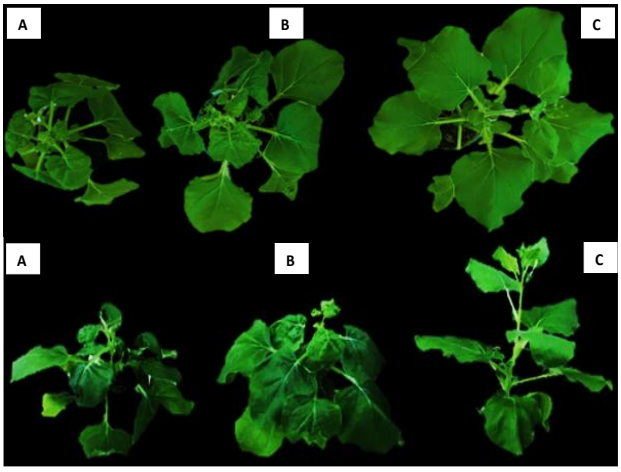

Fig. 8: Suppression of virus infection by dCas9. A. CLCuKV/CLCuMB, B. dCas9+CLCuKV/CLCuMB, C. Mock vector. Severe symptoms were observed in the plants infiltrated with virus only (A). Partial suppression of virus was observed in the co-infiltrated plants (B). While no symptoms were observed in the plants without virus (C)

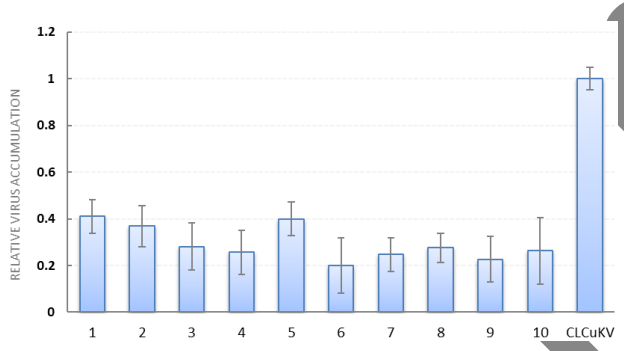

Fig. 9: qPCR results for virus accumulation. CLCuKV was used as reference. CLCuKV graph bar is showing relative virus accumulation in the reference. Graph bars from 1 to 10 are showing relative virus accumulation in the samples. The Fig. is showing low virus accumulation in the samples (0.2-0.4) relative to reference (1) in plants infiltrated with dCas9

In this study, we found delay in symptom's development, attenuated viral symptoms and low titer of the virus given in (Fig. 7, 8, 9 and 10). In the past, other DNA binding proteins has also been used for this purpose. Previously, Zinc finger and TALE proteins has been used for suppression of begomoviruses especially through inhibition of replication (Mino *et al.*, 2006; Cheng *et al*., 2015; Khan *et al*., 2018). Rep protein binding site was blocked by using AZPs to suppress virus infection (84%) in tomato (Sera, 2005) while we found 60-80% decrease in virus infection (Fig. 9). Rep protein aids in initiation of replication by producing a nick in the nonanucleotide region (Heyraud *et al.*, 1993). Our results are also in line with previously study in which we found attenuated virus infection and delay in symptoms using TALEs (Khan *et al*., 2018).

In another report, Chen *et al*. (2014) used artificial zinc finger nuclease (AZFNs) targeting conserved motifs of rep gene in begomoviruses. They found that the target site in the rep gene of the virus was cleaved and disrupted by nuclease activity resulting in inhibition of replication and resistance against viruses. Apart from DNA binding proteins, RNAi technology has also been used to knock down CLCuKV/CLCuMB (Ahmad *et al.*, 2017). But concerns about off-target activity of RNAi based approach has made its utilization limited (Carthew and Sontheimer, 2009; Khan *et al.*, 2009).

Our results show development of partial resistance against viral infection (Fig. 8). Partial resistance has also been achieved by Cheng *et al*. (2015) against begomoviruses in *N. benthamiana* using TALE proteins targeting conserved motifs in the viral genome. Cheng *et al*. (2015) used TALE to target DNA A of the begomovirus and found promising results. Our present results are also in line with Cheng *et al.* (2015) and our previous results (Khan *et al.*, 2018) though comparatively less efficient. Low virus titer, attenuated symptoms and viral infection and delay in symptoms development were observed similar to the above studies (Table 1).

CRISPR/Cas9 is a unique RNA-guided DNA binding protein system which has been reported for targeting DNA sequences with more comparative efficiency than ZFNs and TALENs (Kim and Kim, 2014; Mubarik *et al.*, 2016; Demirci *et al.*, 2018). Cost-effectiveness, designing comfort and easy to use has made this approach a method of choice. Although, some researchers have reported comparatively higher off-target activity of CRISPR/Cas9 (Fu *et al.*, 2013). CRISPR/Cas9 has been strongly proposed for engineering resistance against plant viruses (Ali *et al.*, 2015). In particular, it has been successfully used for suppression of plant DNA viruses (Ji *et al.*, 2015; Ali *et al.*, 2016). However, it has been reported that plant viruses can withstand small deletions or insertions produced by Cas9 and can escape after repair of double strand break (Ji *et al*., 2015; Ali *et al*., 2016).

Similarly, other ssDNA proteins such as VirE2 of *Agrobacterium tumefeciens* have also been used to suppress cotton and tomato leaf curl viruses (Yousaf *et al.*, 2015). Yousaf *et al.* (2015) observed 68% resistance against CLCuV compared to 60-80% resistance (Fig. 9) in our study. Yousaf *et al.* (2015) also found 56% resistance in case of using VirE2 against ToLCNDV. DNA binding/targeting efficiency of TALEs and CRISPR/Cas9 is well established and has been widely applied. Lower efficiency of dCas9 in our study (less than 80% compared to more than 80%) may be due to the fact that TALEs are natural transcription factors and DNA binding proteins whose function is well adapted in plant cells as compared to dCas9 which are functionally evolved in bacteria only (Gao *et al*., 2014). But the comfort of designing and cost-effectiveness of Cas9 has made it common technique for researchers.

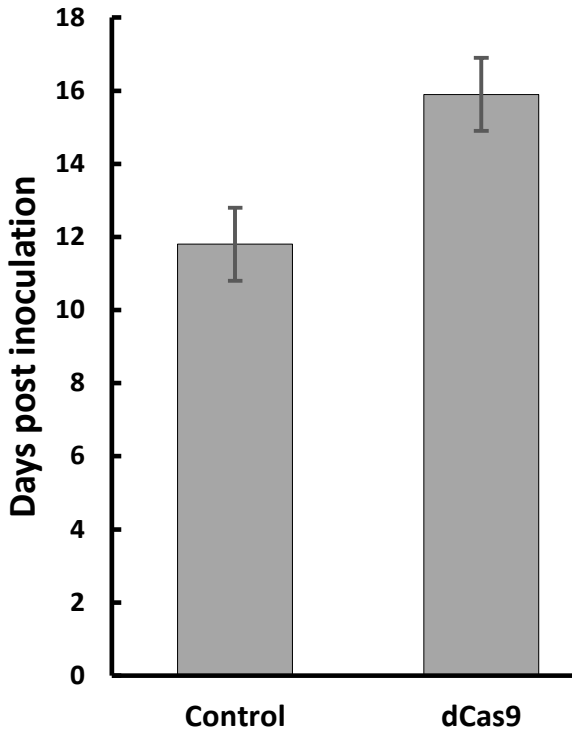

Fig. 10: Delay in symptoms development. Control plants were infiltrated with CLCuKV/CLCuMB only. dCas9 plants were co-infiltrated with CLCuKV/CLCuMB and dCas9

Moreover, multiplexing is easy in case of Cas9 (Xing *et al.*, 2014) and different multiplexing platforms have been developed for genome editing in monocots and dicots plants (Ma *et al.*, 2015). Therefore, multiplexing approach could be used to get more efficient and effective suppression of viruses.

Conclusion

On the basis of our previous and current study, it is concluded that the DNA binding proteins such as TALEs and CRISPR/dCas9 can be used to suppress replication of begomoviruses. Occupying nonanucleotide region in the viral DNA was found very helpful in this regard. Moreover, binding of the engineered proteins at the regulatory regions in the viral DNA could also be resulted in viral interference. For blocking/occupying multiple sites in the viral DNA simultaneously, multiplexed CRISPR/dCas9 can be used efficiently.

Acknowledgments

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