INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0639/2018/20–1–109–118
DOI: 10.17957/IJAB/15.0456
http://www.fspublishers.org

Full Length Article

TALE-Mediated Inhibition of Replication of Begomoviruses

Zulqurnain Khan[1], Sultan Habibullah Khan[1,2*], Bushra Sadia[1,2], Amer Jamil[3] and Shahid Mansoor[4]
[1]*Centre of Agricultural Biochemistry & Biotechnology (CABB), University of Agriculture Faisalabad 38040, Pakistan*
[2]*U.S.-Pakistan Center for Advanced Studies in Agriculture and Food Security (US-PCAS-AFS), University of Agriculture Faisalabad 38040, Pakistan*
[3]*Department of Chemistry and Biochemistry, University of Agriculture Faisalabad 38040, Pakistan*
[4]*National Institute for Biotechnology and Genetic Engineering (NIBGE), Jhang Road, Faisalabad, Pakistan*
[*]For correspondence: sultan@uaf.edu.pk

Abstract

During the last decade, unprecedented progress in the field of genome modification has been witnessed with various applications in basic and applied biology. Genome editing with specific DNA binding proteins has shown higher specificity and fidelity. Artificially engineered proteins such as zinc fingers (ZFs), transcription activator-like effectors (TALEs) and clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided nucleases (e.g., Cas9) have been used to edit genomes of several plants species such as wheat, rice, soybean, potato, tomato, tobacco, Arabidopsis etc. Engineered proteins with nuclease domain, ZFNs, TALENs, CRISPR/Cas9, can be used to induce double-strand breaks (DSB) in the target genomes. In eukaryotic systems, double strand breaks are repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR) based repair mechanisms resulting in knockdown or malfunction of the targeted gene. Begomoviruses are becoming a serious threat to a number of crops in Pakistan. The present study was initiated with the objective to demonstrate suppression of replication of cotton leaf curl virus (CLCuV) using TALE technology. The most conserved DNA sequence of begomoviruses, nonanucleotide, was targeted to achieve a broad-spectrum resistance against CLCuV prevalent in Pakistan. Activity of TALEs for virus suppression was successfully demonstrated in *Nicotiana benthamiana* by challenging with infectious clones of cotton leaf curl Kokhran virus (CLCuKV). Virus accumulation was determined by qPCR. The plants showed varying degrees of resistance to CLCuKV in three ways; attenuated virus infection, delayed symptoms and lower virus titer. Our results successfully demonstrated the potential of TALE technology for CLCuV suppression and offer a broader genome targeting platform for suppression of other viruses. © 2018 Friends Science Publishers

Keywords: ZFNs; TALENs; CRISPR/Cas9; Begomoviruses; CLCuV

Introduction

For the management of begomovirus diseases, several strategies have been utilized by researchers including conventional crop breeding, transgenic approaches by expressing viral proteins or non-pathogen derived antiviral agents, and RNA silencing (Vanderschuren *et al*., 2007a; Vanderschuren *et al*., 2007b; Shepherd *et al*., 2009; Vanderschuren *et al*., 2009; Nahid *et al*., 2011). Though, after conducting several field trials, commercially-available broad-spectrum virus resistant crops are still very rare. Transgenic plants expressing DNA-binding proteins such as zinc-finger proteins with the ability to bind to begomovirus genomic DNA specifically, can confer resistance to the corresponding begomovirus (Sera, 2005; Mori *et al*., 2013). In a previous study, it was reported that ZFNs, which were designed to specifically bind a conserved DNA sequence motif within begomoviruses genomes, can halt the replication of multiple begomoviruses (Chen *et al*., 2014). These studies suggest that using artificial DNA binding protein technology can be a promising method for developing broad-spectrum resistance against begomoviruses. TALEs, being more specific and less laborious, are emerging as a potential tool for resistance against begomoviruses.

TALEs are a specific class of DNA binding proteins secreted by plant pathogenic bacteria, *Xanthomonas*, which infect more than 350 plant species and cause serious diseases (Chan and Goodwin, 1999). TALEs are secreted into plant to modulate host gene expression favoring disease development. In case of bacterial blight of rice, it was observed that *Xanthomonas oryzae* pv. *oryzae* secretes TALEs which activate susceptibility genes of the plant. Once the TALE function was revealed, the bacterial blight resistant rice was developed by expressing artificial TALEs (Li *et al*., 2012). The inimitable DNA recognition system of

To cite this paper: Khan, Z., S.H. Khan, B. Sadia, A. Jamil and S. Mansoor, 2018. TALE-mediated inhibition of replication of begomoviruses. *Int. J. Agric. Biol*. 20: 109–118

TALEs allows a quick assembly of artificial TALEs with a higher DNA binding specificity to target any DNA sequence. Therefore, TALE technology has been widely and rapidly, exploited in biotechnology to design and engineer customizable transcription factors and sequence-specific nucleases for genome editing (Bogdanove and Voytas, 2011). Moreover, construction of artificial TALEs with high affinity and specificity is more cost effective and is less time consuming than artificial zinc-finger technology (DeFrancesco, 2012).

TALEs can be fused with other effector domains such as FokI (Cermak *et al*., 2011), KRAB (Kruppel-associated box) (Zhang *et al*., 2014), VP16 and VP64 (Boch, 2011; Miller *et al*., 2011), TET1 (translocation methylcytosine deoxygenase 1) (Maeder *et al*., 2013), LSD (lysine specific demethylase 1A) (Joung *et al*., 2013). TALENs (TALEs fused with FokI nuclease domain) are used to create double strand breaks in the targeted regions in the DNA sequences. For assembly of TALEs and TALENs, a number of methods have been developed. The method of modular assembly has been developed for prompt production of designer TALENs according to a specific DNA sequence (Li *et al*., 2011). This technique can be used to engineer ten TALEN constructs targeting specific sequence in native yeast genome. All TALEN construct tested induced higher rates of targeted gene mutations and disruption with expected results. Consequently, dTALENs (designer TALEN) was developed for insertion of transgene through homologous recombination with higher efficacy. It was shown that TALEN constructs could simply be developed and demonstrated with high activity in primary cells.

Prevention of virus infection is an objective of prime importance in both agricultural and animal sciences. The concept of inhibition of virus replication to suppress viral infection was demonstrated by Sera (2005). Sera (2005) produced an artificial zinc finger protein (AZP) to target the origin of replication of the beet severe curly top virus (BSCTV). It was demonstrated that the AZP efficiently occupied the binding site of the viral replication protein (Rep) at the replication origin in DNA binding assays. The artificial AZP was expressed in all transgenic Arabidopsis plants tested and the replication of the virus was found inhibited. Furthermore, it was observed that all transgenic plants showed varying resistance to virus infection. No symptoms of viral infection were shown by 84% of the transgenic plants. The viral DNA replication mechanism is well understood among animals and plants. This approach of blocking of viral replication could be applied to prevent virus infection in plants as well as animals (Khan *et al*., 2016). Koshino-Kimora *et al.* (2008) used the same AZP technology against tomato yellow leaf curl virus (TYLCV). The AZP was designed to block the binding of TYLV replication protein (Rep) to the replication origin. They found that the AZP had higher affinity to bind replication origin then the rep protein *in vitro*. The AZP gene was introduced using *Agrobacterium*-mediated transformation method to generate transgenic plants. In the present study, we targeted the most conserved nonanucleotide region of the begomoviruses which acts as *ori* (origin of replication) for initiation of viral replication. This region can be targeted for the development of broad-spectrum resistance against begomoviruses.

Materials and Methods

In Silico Analysis of Virus DNAA Sequence to Find Potential Target Sites

DNA sequences of prevailing cotton leaf curl viruses, cotton leaf curl Kokhran virus, cotton leaf curl Burewala virus, cotton leaf curl Multan virus, cotton leaf curl Rajasthan virus and cotton leaf curl Alabad virus, were retrieved from NCBI and aligned using CLUSTALW software to find conserved sequences for TALE-mediated targeting. The selected DNA sequence was further blast to find similarities among DNA sequences of begomoviruses.

Designing and Construction of TALEs

We used TAL Effector Nucleotide Targeter 2.0 to design TALE proteins against target sites. The program is freely available online (Doyle *et al*., 2012). We used HD, NN, NG and NI RVDs to target C, G, T and A DNA bases, respectively. Golden gate cloning protocol (Fig. 1) of Cermak *et al*. (2011) was used to assemble TALE protein using Golden Gate TALEN and TAL Effector Kit 2.0 plasmids provided by Addgene. TALE was amplified using TALE-F1 and TALE-R1 (Table 1) and cloned into *BamHI* site (pJIT163) under 2x35S promoter. TALE was further sub-cloned in pCambia 2300 into *BamHI* and *PstI* sites under 35S promoter and OCS terminator for plant expression.

Expression of TALE-gfp in Protoplast

To check TALE expression in plants, TALE protein fused with *gfp* (green fluorescent protein) was expressed in *N. benthamiana* protoplast. Protoplasts were isolated from *N. benthamiana* seedlings grown in 1/2 MS medium 7–10 days. Chopped leaves of *N. benthamiana* seedlings were transferred immediately to 0.6 *M* mannitol for 10 min and were kept in the dark. Mannitol was discarded and chopped leaves were incubated in an enzyme solution (1.5% Cellulase RS, 0.6 M mannitol, 0.75% Macerozyme R-10, 10 mM $CaCl_2$, 10 mM MES at pH 5.7, and 0.1% BSA) for 4–5 h in the dark with gentle shaking (60–80 rpm). After enzyme digestion, an equal volume of W5 solution (154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$ and 2 mM MES at pH 5.7) was added, following vigorous shaking by hand for 10 sec. Protoplasts were obtained by filtering through 40 μm nylon meshes into round bottom tubes with 3–5 times washing of the strips using W5 solution. The pelleting was

Table 1: List of primers

Primer	Sequence	Application
pCR8_F1	ttgatgcctggcagttccct	Colony PCR
pCR8_R1	cgaaccgaacaggcttatgt	Colony PCR
TAL_F1	ttggcgtcggcaaacagtgg	Colony PCR
TAL_R2	ggcgacgaggtggtcgttgg	Colony PCR
TAL_Seq_5-1	catcgcgcaatgcactgac	Sequencing
TAL_R3	ggctcagctgggccacaatg	Sequencing
CR-F1	GGAGACCAAGTCGAAGAATC	DNAA amplification
CR-R1	TCGACATAATTCCTAGCCCT	DNAA amplification
CLCuKV-F1	CTGTTAAATGAGTTCCCCGA	qPCR
CLCuKV-R1	TTATATCTGCTGGTCGCTTC	qPCR
PPR-F	ATGAGGGRCCATTTGAGTGAC	qPCR
PPR-R	AGGCTGATGTTGGAATCTGG	qPCR
TALE-F1	GGAAGATCTAACTCGAGAAAGATA	TALE amplification
TALE-R1	GAAGATCTTCACTAGCTGGGATCTAGATAT	TALE amplification

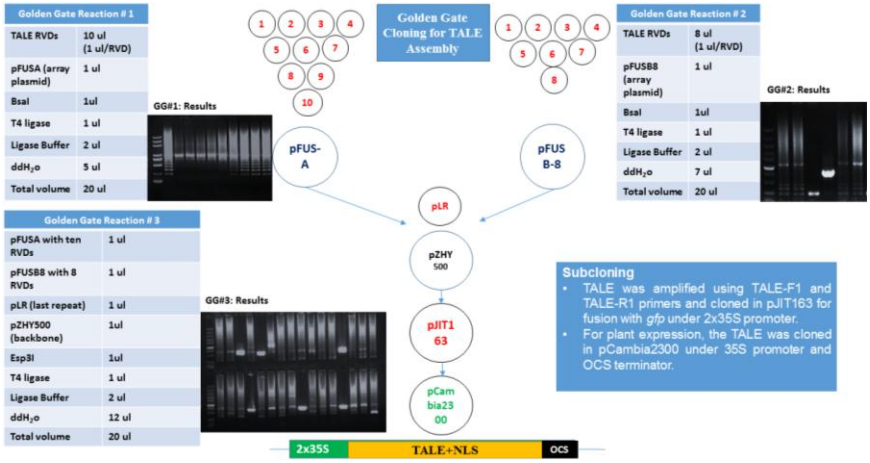

Fig. 1: Golden gate cloning strategy for TALE construction and further sub-cloning of TALE for plant expression. Complete TALE RVDs were assembled in three steps. Ten RVDs assembled in GG#1, eight RVDs assembled in GG#2 while remaining half repeat, GG#1 and GG#2 RVDs were assembled in GG#3. Colony PCR results shown in GG#1 (a brighter band of 1.2 kb with laddering and smear), GG#2 (a brighter band of around 1kb with laddering and smear) and GG#3 (a smear around 2-3 kb with laddering) are similar as given by Cermak *et al*. (2011)

done by centrifugation at 1,500 rpm for 3 min with a swinging bucket. After washing with W5 solution, the pellet was re-suspended in MMG solution (15 mM $MgCl_2$, 0.4 M mannitol and 4 mM MES at pH 5.7) at a concentration of 2 × 106 cells/mL. Finally, PEG-mediated transfections were carried out as described by Yoo *et al*. (2007).

Plasmid DNA (5–10 µg) was mixed for each sample with 100 µL protoplasts. The total plasmid DNA was between 10 µg and 15 µg for co-expression assays. A volume of 110 µL of freshly prepared PEG solution [40% (w/v) PEG 4000; Fluka, 0.1 M $CaCl_2$ and 0.2 M mannitol] was added into protoplast and plasmid mixture and the mixture was incubated at 25ºC for 15 min in the dark. Afterwards, W5 solution (440 µL) was added to the mixture slowly. The tubes were inverted gently to mix the solution well. Protoplasts were pelleted by centrifugation at 1500 rpm for 3 min. About 1 mL of WI solution (0.5 M mannitol, 20 mM KCl and 4 mM MES at pH 5.7) was used to re-suspended the protoplasts. Finally, the protoplasts were transferred and cultured into multi-well plates under light or dark at room temperature for 6–16 h. Expression of *gfp* in transfected cells was observed under OLYMPUS SZX18 fluorescent microscope.

Evaluation of TALE for Virus Suppression

Plant growth conditions: Seeds of *N. benthamiana* were grown in plastic pots containing standard germination soil at 25°C with 16 h light and 8 h dark photoperiod with appropriate light intensity. Seedlings were transfered to another plastic pot after 8 days of sowing containing potting soil (one seedling per pot) and kept at 25°C with 16 h light and 8 h dark photoperiod (about 150 µmol m^{-2} s^{-1}). Three weeks after transplantation, the plants attaining optimal developmental stage were used for agroinfiltration. At that stage, the plant had 4–5 fully developed true leaves without visible flower buds.

Agroinfiltration-based infectivity assay: Agrobacterium

Table 2: Evaluation of TALE protein 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
TALE+ CLCuKV/CLCuMB	1	10	2/10	20	Mild	3-5	Yes	+7/10	0.22
	2	10	4/10	40	Mild	3-5	Yes	+8/10	0.34
	3	10	3/10	30	Mild	3-5	Yes	+7/10	0.42

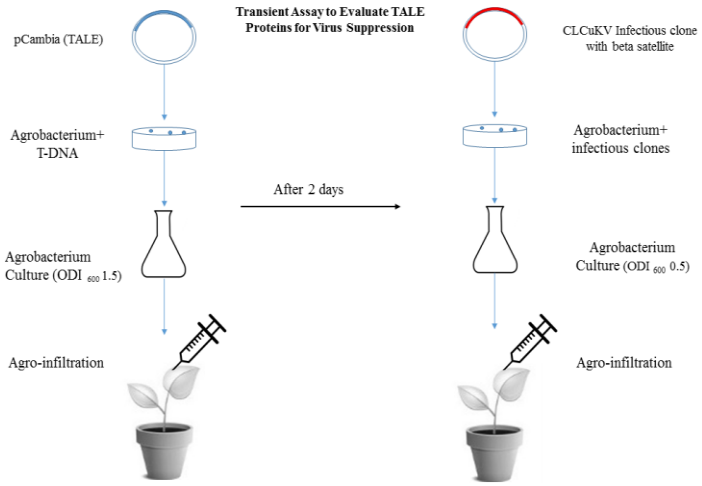

Fig. 2: TALE-CLCuKV/CLCuMB co-infiltration strategy for evaluation of virus suppression

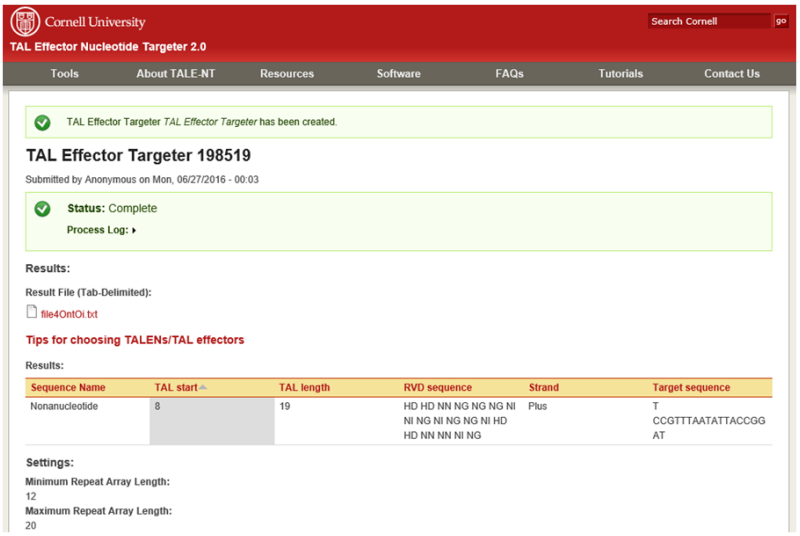

Fig. 3: TALE target site selection by TAL Effector Nucleotide Targeter 2.0. Every target is preceded by T. 19 RVDs were given to target 19 bp of viral DNA sequence

strains GV3101 with infectious clones of CLCuKV/CLCuMB were grown at 28°C. Culture was harvested and pellet was dissolved in infiltration solution (10 mM $MgCl_2$, MES 10 mM pH 5.6,100 uL Acetosyringone). Infiltration solution was left on bench for four hours. Three weeks old plant leaves were infiltrated using needless syringe. The symptoms were recorded at 14 dpi (days post inoculation). Plant DNA was extracted from symptomatic leaves and CLCuKV DNAA was amplified with specific primers.

Disease resistance assay: To check out TALE efficiency for virus suppression, TALE and CLCuKV/CLCuMB were co-infiltrated in *N. benthamiana* leaves (Fig. 2). Briefly, GV3101 with pCambia (TALE) was grown at 28°C. The culture was harvested and pellet was dissolved in infiltration solution. Three weeks old young *N. benthamiana* leaves were infiltrated with infiltration solution. The infiltrated leaves were further infected with CLCuKV/CLCuMB at 2 dpi. Delay in symptoms development was recorded from 9–21 dpi. Virus accumulation was determined using CFX96 Real-Time System (Biorad) with specific primers. PPR (*Pentatricopeptide repeat protein*) primers were used as internal control.

Results

Designing and Construction of TALE to Target Nonanucleotide

To find an appropriate and potential target site in the conserved region of the viral DNAA, the DNA sequence was subjected to TAL Effector Nucleotide Targeter 2.0 with some limitations i.e., target site must be preceded with T (thymine). The design and detail of RVDs used in TALE construction is given in Fig. 3. To construct TALE, first ten RVDs were assembled in pFUSA and remaining (n-1) were assembled in pFUSB8 vector. Each clone was sequenced before next cloning step. Final TALE array along with half repeat was assembled in pZHY500. The clone was sequenced and the assembly and orientation of the RVDs was confirmed by sequencing and BLASTx analysis (Fig. 4 and Fig. 5). The amino acid sequences were found 99% similar with the expected sequence. All of the RVDs were assembled in the desired orientation.

Cloning and Expression of TALE

After successful TALE assembly, TALE was fused with *gfp* (green fluorescent protein) to express in *N. benthamiana* protoplast (Fig. 6). The TALE was finally cloned in pCambia 2300 for plant expression (Fig. 7). The expression of TALE-gfp was comparable to *gfp* control expression (Fig. 8).

Evaluation of Infectious Clones for Infectivity in *N. benthamiana*

Plants infiltrated with CLCuKV/CLCuMB were observed for symptoms between 9–14 days' post inoculation (dpi). All the infected plants showed symptoms on 10–13 dpi and the control plant, the plant without virus infiltration, did not show any symptoms. The symptoms of the virus were severe at 21 dpi. The pattern of the symptoms was irregular, from upward leaf curling to downward leaf curling, vein thickening, plant yellowing, stunted growth etc. PCR amplification of viral DNA and qPCR results to check virus accumulation are shown in Fig. 9. Viral DNA was amplified from all symptomatic leaves while the control did not show any amplification. The symptoms are shown in Fig. 10.

Evaluation of TALE for Virus Suppression

Three weeks old plants were infiltrated with pCambia (TALE). After two days, the plants were infiltrated with infectious clones of CLCuKV/CLCuMB. Symptoms were appeared on control plants, infiltrated with virus only, at 10–13 dpi. While the delay in symptoms development (5–6 days) was observed in the plants co-infiltrated with TALE and CLCuKV/CLCuMB. TALE showed suppression of virus in terms of less and mild symptoms, delay in symptoms and low virus accumulation (Table 2). The experiment was conducted in triplicate.

Determination of Virus Accumulation by qPCR

TALE was evaluated for virus suppression in *N. benthamiana*. It was clear by symptom scoring that TALE was promising in decreasing virus infection in plant (Fig. 11). Virus accumulation in the co-infiltrated plants was checked by qPCR analysis. It was shown that virus titer was decreased up to 60–80% in case of TALE (Fig. 12).

Delay in Symptom Development

Development of symptoms of leaf curl disease after infiltration with infectious clones was observed in all control plants with equal frequency. Virus infected plants started to show disease symptoms at 10–13 dpi. It was found that plants co-infiltrated with TALE and CLCuKV/CLCuMB showed symptoms in 15–18 dpi (Fig. 13). The decrease in symptom development further indicated the lower level of infection of virus. Moreover, along with delay in symptoms, it was also observed that the plants co-infiltrated with TALE were recovered completely at 30 dpi.

Discussion

Begomoviruses are whitefly transmitted ssDNA viruses which infect and damage many economically important crops and vegetables such as cotton, tomato, bean, cassava and squash (Kenyon *et al*., 2014).

Their replication depends on replication initiator protein (Rep) which is encoded by *rep* gene (C1) of virus (Hanley-Bowdoin *et al*., 1999). The rep gene suppression may lead to viral replication inhibition. This idea has been utilized by different researchers. Sera (2005) selected binding site of rep protein in the promoter region of the *rep* gene to occupy it with AZPs and found promising results with respect to inhibition of viral replication. Other researchers have also selected this site for inhibition of viral replication (Mino *et al*., 2006; Koshino-kimora *et al*., 2008; Takenaka *et al*.,

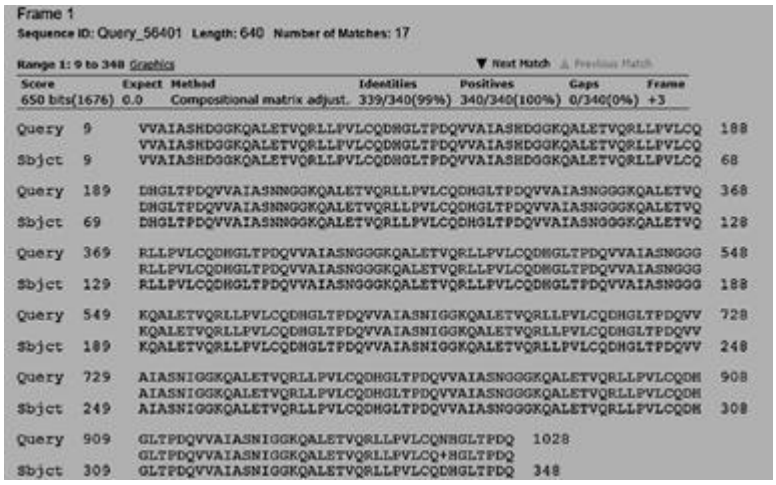

Fig. 4: BLASTx alignment results of TALE in pZHY500 sequenced with forward primer. Translated nucleotide were found to have 99% similarity with 0.0 E value

Fig. 5: BLASTx alignment results of TALE in pZHY500 sequenced with reverse primer. Sequence data have similarity of 99% with expected sequence with 0.0 E value

2008; Mori *et al*., 2013). Nonanucleotide is the most conserved sequence of the viral genome and is the origin of replication (*ori*) of the virus in which rep protein creates a nick to initiate viral replication (Heyraud *et al*., 1993). Targeting this region for suppression of viral replication could produce broad spectrum resistance against a number of viruses. We found delay in symptoms and attenuated virus infection by targeting nonanucleotide region with TALEs.

Sera (2005) selected Rep protein binding site which is in the promoter region of the *rep* gene to block viral replication and to decrease virus infection.

They observed 84% decrease in viral symptoms using AZFs. The mechanism of replication of viral DNA is well conserved among animals and plants this approach is applicable with equal efficiency, not only to plant protection but also to the prevention of viral diseases in humans (Sera, 2005). Previously, an artificial zinc-finger protein (AZP) was designed to block replication associated protein (Rep) of beet severe curly top virus (BSCTV) from binding to its origin of replication and demonstrated that AZP expressing transgenic *Arabidopsis* plants were completely resistant to the virus infection. After wards, AZP technology was applied against tomato yellow leaf curl virus (TYLCV) which infect tomato an important agricultural crop. The binding site of the rep protein was blocked and the replication of the virus was halted (Takenaka *et al*., 2008).

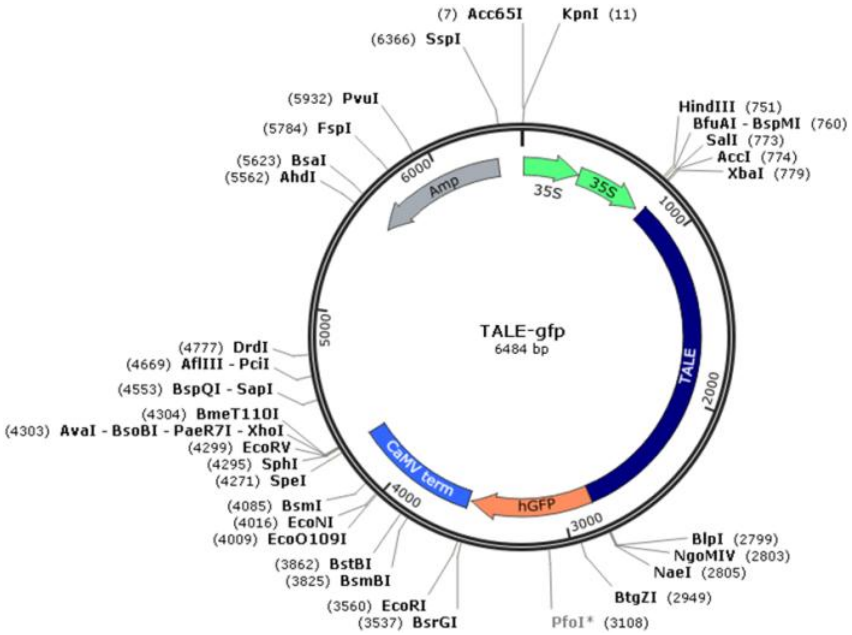

Fig. 6: Map of TALE-gfp. TALE was fused with *gfp* under 2x35S promoter to check its expression in protoplast

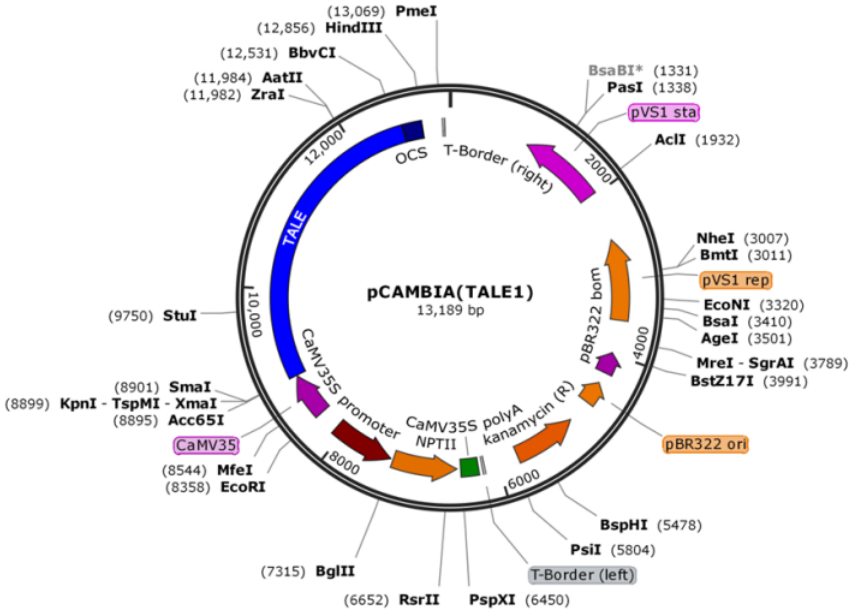

Fig. 7: Map of pCambia(TALEI). TALE was finally cloned in pCambia, a binary vector, to express in *N. benthamiana* plants

TALEs and TALENs have been proved as successful DNA targeting tools during the last few years. Due to their single nucleotide binding affinity, TALENs were used to target viral genome. In case of ZFNs, three DNA bases can be targeted with one monomer of protein (Pavletich and Pabo, 1991) making it difficult to find out the target sites frequently and in selected regions. In case of TALENs, any DNA sequence can be targeted. Repeat variable di-residues (RVDs) consist of two amino acids are responsible to target single DNA base. So, for four DNA bases (A, T, G and C) four RVDs (NI, NG, NN and HD) can be used respectively (Boch *et al*., 2009; Moscou and Bogdanove, 2009). Golden gate assembly of Cermak *et al*. (2011) is a well-recognized approach for efficient, robust and cost-effective construction of TALEs/TALENs.

Previously, TALEs have been used to target begomoviruses. Cheng *et al*. (2015) designed an artificial TALE protein for developing broad-spectrum resistance platform to begomoviruses. They showed that artificial TALEs were promising in conferring partial resistance

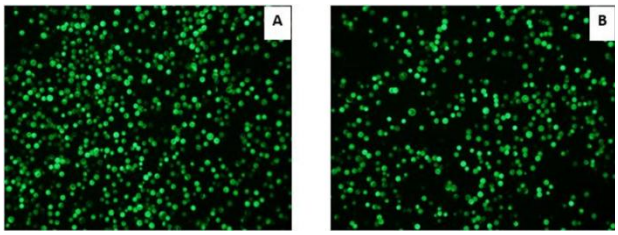

Fig. 8: Expression of TALE-gfp in protoplast of *N. benthamiana*. A, The expression of gfp in protoplast as control. B, The expression of TALE-gfp in protoplast. Image was taken using OLYMPUS SZX16

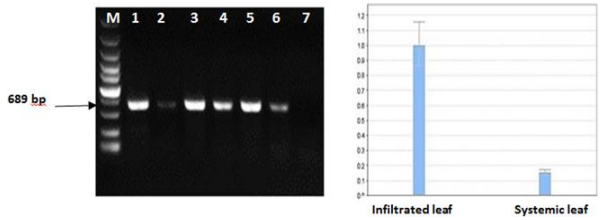

Fig. 9: Confirmation of viral DNA in infected plants. PCR amplified viral DNA fragment of 689 bp on agarose gel. CR-F1 and CR-F2 specific primers were used for PCR amplification of viral DNA. Viral DNA was amplified from all infected plants (Lane 1-6). M is 1kb ladder and Lane 7 is negative control (plant without viral inoculation). qPCR graph results are confirming presence and accumulation of CLCuKV in systemic leaves. CLCuKV-F1 and CLCuKV-R1 specific primers were used for qPCR

against begomoviruses in transgenic *Nicotiana benthamiana* to all three begomoviruses tested. Furthermore, the resistance was also maintained in the presence of betasatellite of the virus. Similar results have been obtained in the present study. The plants co-infiltrated with TALE and CLCuKV/CLCuMB showed delay in symptoms development and decreased virus titer in systemic leaves (Fig. 13). It is observed that nonanucleotide could be a better target for broad-spectrum resistance against begomoviruses. Cheng *et al*. (2015) also observed attenuated virus infection, delayed viral symptoms and decreased virus accumulation by using TALE against begomoviruses.

Plant DNA viruses have also been targeted with CRISPR/Cas9 for targeted DSBs (Ali *et al*., 2015; Ji *et al*., 2015). It was found that DNA viruses can be targeted with engineered nuclease for creation of DSBs to mutate the target site.9

Replication of the virus can be inhibited by disruption of the Rep binding site and/or occupation of nonanucleotide. Rolling circle replication mechanism is initiated by rep protein which involves DNA double strand intermediate form of the virus genome (Saunders *et al*., 1991; Ilyina and Koonin, 1992; Koonin and Ilyina, 1992).

Fig. 10: Infectivity assay in *N. benthamiana* using infectious clones of CLCuKV/CLCuMB. Agrobacterium culture of infectious clones were infiltrated in young *N. benthamiana* leaves of 2 weeks old plants. The symptoms appeared at 12 dpi are shown in the figure

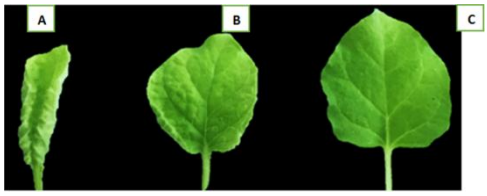

Fig. 11: Inhibition of CLCuV infection using TALE. A, control plant infiltrated with CLCuKV/CLCuMB. B, plant co-infiltrated with TALEs and CLCuKV/CLCuMB. C, Control plant infiltrated with mock vector. The picture was taken at 21 dpi

TALENs can only bind double stranded DNA, while begomoviruses are ssDNA viruses. But it was considered that TALEN proteins bound DNA in its double stranded replicative form and created double strand break in the targeted region. The DSB leads to mutation of the DNA sequence by creating Indels (insertion/deletions) which ultimately result in mutation in the target site. Begomoviruses have been targeted by artificial TALEs which resulted in site specific binding of TALEs and suppression of virus (Cheng *et al*., 2015).

Cotton leaf curl disease is a complex of viruses which cause infection. So, to make TALE strategy broad spectrum targeting maximum viruses, sequences of prominent cotton leaf curl viruses were aligned to find out consensus sequence in the conserved region of viruses. Moreover, there is a problem of recombination and pseudo-recombination in virus which increase the evasion of virus from targeting proteins. In the past, scientists targeted different viral genes like V1 and V2 to suppress viruses. But unfortunately, no complete resistance has been achieved hitherto. Targeting conserved sequences in the virus genome can be helpful in creating broad spectrum resistance against begomoviruses (Cheng *et al*., 2015). Cheng *et al*. (2015) achieved partial resistance in *Nicotiana benthamiana* against begomoviruses using TALE proteins targeting conserved motifs in the viral genome. Chen *et al*.

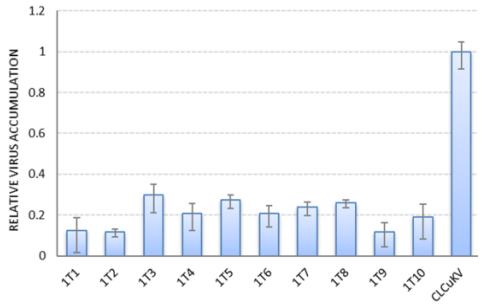

Fig. 12: qPCR results of virus accumulation. CLCuKV was used as reference. CLCuKV graph bar is showing relative virus accumulation as 1. T1 to T10 is showing low virus accumulation relative to reference in plants targeted with TALE

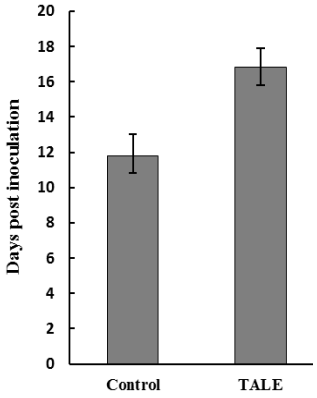

Fig. 13: Delay in symptoms development. Control plants were infiltrated with CLCuKV/CLCuMB only. TALE plants were co-infiltrated with CLCuKV/CLCuMB and TALE

(2014) used artificial zinc finger nuclease (AZFNs) to target *rep* gene conserved motifs in begomoviruses. It was found that engineered nuclease cleaved the target site and disrupted the *rep* gene of the virus resulting in inhibition of replication and resistance against virus. TALEs and TALENs are more specific and highly efficient comparing to AZFNs and have almost no off targets. In previous studies, it was also observed that begomoviruses have the ability to tolerate small mutations/deletions in the coding region (Ali *et al*., 2015). Moreover, it was also observed that the virus is able to repair after DSBs without any mutation in the DNA sequence. Ali *et al*. (2016) also found similar results by targeting CLCuKV with CRISPR/Cas9. It was found that target sites repaired after DSBs without any mutation in the sequence.

A novel DNA binding proteins, CRISPR/Cas9, has been developed to target DNA more efficiently (Deltcheva *et al*., 2011; Jinek *et al*., 2012; Cho *et al*., 2013; Kim and Kim, 2014; Hendel *et al*., 2015; Ledford, 2015; Bolukbasi *et al*., 2015; Mubarik *et al*., 2016). The ease of designing and less cost has made CRISPR/Cas9 more fascinating approach to target DNA sequence. Although there are more off-targets in case of CRISPR/Cas9 (Fu *et al*., 2013) than that of TALENs. But multiplexing (targeting multiple sequences with multiple gRNA simultaneously) has made it very distinctive technology (Cong *et al*., 2013; Xing *et al*., 2014). In a recent study, it was found that begomoviruses can escape Cas9 after mutation in the target site. Mutated target sites will not be available further for the binding of Cas9 (Ali *et al*., 2016). Moreover, it is reported that begomoviruses can tolerate some of the small deletions or insertions at the target site in the coding region of the virus (Ji *et al*., 2015). Ali *et al*. (2016) also found similar results when CP and RCRII were targeted with CRISPR/Cas9.

Decrease in the titer of the virus by using engineered nucleases have been achieved by many researchers (Sera, 2005; Ji *et al*., 2015; Ali *et al*., 2016). We found that titer of the virus was decreased more than 80% when the non-coding regions of the virus, nonanucleotide was targeted with TALEs and TAENs respectively. Cotton leaf curl viruses have also been targeted by other DNA binding proteins like VirE2 (Yousaf *et al*., 2015). VirE2 are *Agrobacterium tumefeciens* proteins which have the ability to bind ssDNA. Yousaf *et al*. (2015) observed 68% resistance against CLCuKoV/CLCuMuB and 56% against ToLCNDV. The binding ability of TALEs for dsDNA is well established. Moreover, TALEs are highly specific and efficient in binding specific sequence in a DNA. Inhibition of virus replication is an attractive approach to virus suppression. Owing to the well-established and highly conserved mechanism of viral DNA replication, this approach can be helpful in developing broad-spectrum resistance against viruses in plants as well as animals and humans.

Acknowledgments

This work was supported by Higher Education Commission (HEC) of Pakistan under Indigenous Fellowship Program. We are thankful to Professor Caixia Goa, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, for her help and support in accomplishment of this study.

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(Received 10 June 2017; Accepted 08 August 2017)