Review Article

Gemini Virus: an emerging threat and engineering plants resistance with CRISPER/CAS9 system for it

Abstract

Background: Gemini viruses account for destructive crop losses that endanger food security [1]. The genome of geminivirus comprises of a circular, single-stranded DNA molecule that becomes a double-stranded DNA replication center in the plant cell nucleus and translate four to seven multifunctional proteins.**Objectives:**Plant trait engineering needs effectual targeted genome-editing technologies. Clustered regularly interspaced palindromic repeats (CRISPRs)/ CRISPR associated (CAS) type II system is used for targeted genome editing applications beyond eukaryotic species containing plants that cause resistance of these devastating geminvirus.**Method:** we reported the evolution of *Tobacco rattle virus* (TRV)-mediated genome editing in *Nicotiana benthamiana recently*. TRV affectsthe growing points and holds small genome size; which aid cloning, multiplexing, and agro infections. Here, we address on the constant action and specificity of the TRV-mediated CRISPR/Cas9 system for targeted modification of the *Nicotiana benthamiana* genome. **Result** Our data report the perseverance of the TRV- mediated Cas9 activity for up to 30 d post-agroinefection. In addition, our data demonstrate that TRV-mediated genome editing illustrated no off-target activities at potential off-targets arguing the exactness of the system for plant genome engineering. **Conclusion:** Taken together, our data authorize the feasibility and arousing chances of using virus mediated CRISPER/Cas9 for targeted engineering of plant genomes.

Keywords: Palindromic, Multiplexing, CRISPR/Cas9, Post-agroinefection, DNA replication

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Introduction:

Gemini viruses, the twinned icosahedral particles, affect food and fibre crops, ornamental plants and weeds and substantial crop losses are caused by them around the world(Zaidi, Mansoor et al. 2016). The occurrence and severity of geminivirus diseases has greatly

enhancedin the past 20 years1, 2. Geminivirus disease greatly impact in agriculture, maize streak disease, cassava mosaic disease and cotton leaf curl disease have caused complete losses in infected fields in Africa and Asia3–5(Ali, Ali et al. 2016). One of the major viral diseases of tomato worldwide6 is tomato yellow leaf curl disease. These Gemini viruseshaving small DNA genomes with limited coding capacities and rely strongly on host cellular machineries and during infection interact with a wide range of plant proteins and processes(Raja, Sanville et al. 2008).To induce the replication of both viral and plant chromosomal DNA,Gemini virusesreprogram the cell cycle of infected cells. They alter host gene expression patterns, arrest cell death pathways, change macromolecular trafficking and interfere with cell signaling and protein turnover to redirect or block host defaces and hormone signaling(Sahu and Prasad 2015). Gemini viruses encode multiple silencing suppressors that interfere with plant small interfering RNA (siRNA) production and change plant DNA methylation and microRNA (miRNA) pathways, usually causing developmental abnormalities(Morilla, Castillo et al. 2006).

Gemini viruses mostly exist in disease complexes, andmultiple viruses7can infect individual plants.To increase viral diversity,Gemini virus genomes can undergo high levels of mutation, recombination and reassortment8-11. In particular whiteflies, the development of insecticide resistance and the evolution of new vector biotypes have allowed geminiviruses to infect new regions and to bring together new combinations of viruses in disease complexes2(Nagar, Pedersen et al. 1995). Geminivirusesadapt rapidly to new hosts and environments due to these properties. This and the global spread of geminivirus complexes due to human activity and adverse weather now pose great threats to food security12–14.

FamilyGeminiviridae virusesare insect-transmitted pathogens of plants and their genomes are small, circular, single-stranded (ss) DNA. Gemini viruses have either one or two circular ssDNA components of, 2.6–2.8 kb particles(Nash, Dallas et al. 2011). Mostlyof begomoviruses have two components, named as DNA A and DNA B, both are necessary for virus proliferation. The common region shared by components contains motifs that are required for the control of gene expression and replication, notably conserved reiterated motifs and a reported stem–loop structure containing the highly conserved mononucleotide TAATATTAC that play role in the initiation of rolling circle replication(Nawaz-ul-Rehman and Fauquet 2009). These viruses often

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spontaneously produce abouthalf sized defective DNA B components also, that function as defective interfering (DI) DNA. The DI DNA might serve a biological role during infection to reduce the severity of the disease by competing with the genomic components for cellular resources(Morilla, Castillo et al. 2006).

Following are recent efforts to engineer Gemini virus resistance in crops which include the strategies are given as(i)viral protein- mediated resistance,(ii)non-viral protein-mediated resistance,(iii)viralRNA- mediated resistance(iv)host-derived resistance(Mansoor, Zafar et al. 2006).However, the drawback of these strategies is that geminiviruses evolve fast byrecombination, component capture, andmutation, enabling theseviruses torapidlyevade thesestrategies [3]. A substantial resistance strategy will require overcoming these challenges(Mansoor, Briddon et al. 2003).

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Aprokaryotic molecular immunitysystemagainst invading virusesis Clustered regularlyinterspacedshortpalindromerepeats (CRISPRs)/CRISPR- associated 9(Cas9) andhasbeenappliedasa powerful toolfortargetedgenomicediting. Bacterial andarchaeal incorporate short piecesofDNA, calledspacers, from invading viruses, withintheirgenome, wherethey work as a molecularmemory(Legg and Fauquet 2004). During subsequentinfections, spacers are transcribedascrisper RNA and aftertranscriptionandmaturation,gRNAguidestheCas9 endonuclease toscaninvadingDNAandcleave the targetsequence [4] at asiteleading the protospacer-associatedmotif (PAM), a trinucleotidesequencethatis recognized byCas9andnecessaryforits binding totargetDNA.The PAM helps theCRISPR/Cas9machinery todifferentiatebetweenselfandnon- self DNA(Kong and Hanley-Bowdoin 2002). Fortargetedmodification of genomic sites, transgenicproductionofa single guideRNA (sgRNA)provides specificity totheCas9endonuclease, allowing targeted cleavageofspecific DNA sequences ineukaryoticcells(Kaliappan, Choudhury et al. 2012).

One major concern for CRISPR/Cas9 approaches is the potential for off-target affects on the plant genome and this review highlights either our crisperCAS 9 system exhibit these off target activities or not(Hanley-Bowdoin, Bejarano et al. 2013).Recentstudydemonstratedthat the CRISPR/Cas9systemcouldbe harnessed toconferresistanceagainst geminiviruses inplantsbyusingsgRNAs designed totargetviralgenomicDNAs. Ali et al. showed that CRISPR/Cas9 technologycouldimpart molecular immunityagainst gemini- viruses [i.e., Tobacco curl virus, in N. benthamiana plants , andrevealedthata sgRNA designedtotargetaconserved sequence (TAATATTAC) inthe viral intergenic regioncouldbeusedtotarget multiple geminivirusessimultaneously(Gutierrez 2000).

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TRV developed as vector to systemically to carry gRNA and determined high capacity of targeted modification in *Nicotiana benthamiana recently(Zaidi, Tashkandi et al. 2016)*.

Methodolgy

for virus-induced gene silencing (VIGS) for functional genomics studies in various plant species,*Tobacco rattle virus* (TRV) is used as a potent vector bipartite RNA1 and RNA2 genomes of the TRV, and the RNA2 genome can be modified to convey exonic gene fragments for post-transcriptional VIGS(Fondong 2013). The cargo capacity of TRV is restricted to 2–3 kb and insufficient to deliver Cas9 endonuclease into plants but it can be used to deliver one or more gRNAs.. To determine whether the TRV was capable of producing and delivering the gRNA molecules systemically and the persistence of genomic modification in growing tissues, the TRV virus was delivered by agroinfiltration and reconstituted in the leaves of *N.benthamiana* expressing Cas9(Briddon, Pinner et al. 1990).

Mixed *Agrobacterium* cultures harboring the RNA1 genome (pYL192) in combination with an RNA2 vector was used, in which a gRNA with binding specificity for the *PDS* gene was driven by the PEBV promoter (pRNA2.PEBV::PDS.gRNA) (**Fig. 1A**). A clone of gRNA empty vector was used as the negative control. Samples were obtained at 7, 15 and 30 d post-infiltration (dpi), and in 3 independent plants by PCR amplifying a 797 bp fragment flanking the target site, the targeted editing of the PDS target sequence was assessed. Then, PCR products were instantly directed to the T7EI assay(Baltes, Hummel et al. 2015).

Results and analysis

High levels of genome editing at 7, 15 and 30 d are showed by our results (**Fig. 1B**). By usingImage software as described previously (http://imageJ.nih.gov/ij/)the modification efficiency was analyzed(Navas-Castillo, Fiallo-Olivé et al. 2011). The high ability of TRV-mediated CRISPR/Cas9 editing indicated a constant activity of the system, which is essential for the modification of germline cells and recovery of mutant seed progeny(Gonzales-Salazar, Cecere et al. 2017).

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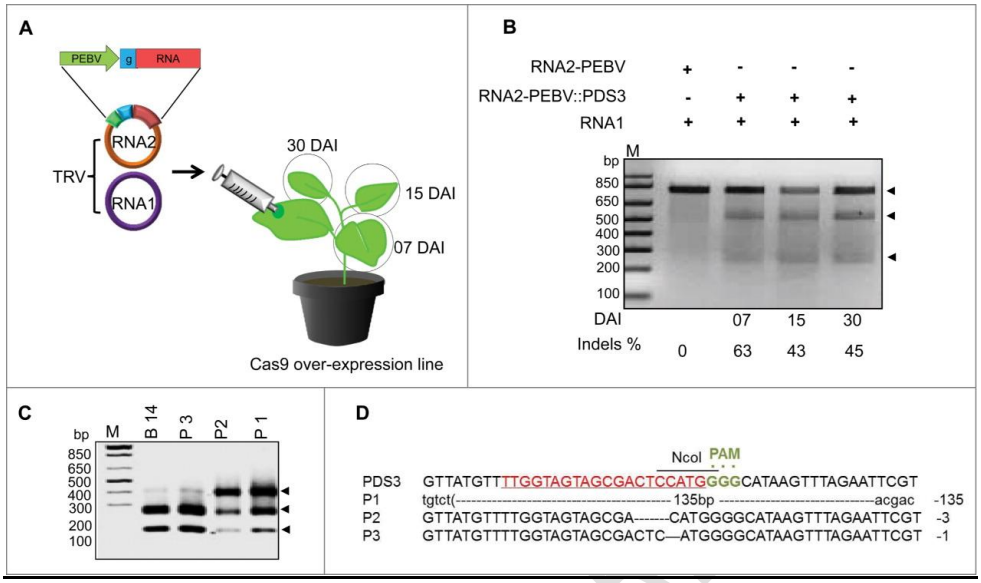

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Figure 1. Persistence of TRV-mediated CRISPR/Cas9 targeted mutagenesis of the *PDS3* gene. (**A**) Establishment of TRV infection in B14 plants, *Nicotiana benthamiana* Cas9 over expression line. *Agrobacterium* cultures containing RNA1 and engineered RNA2 harboring gRNA for targeting *PDS* gene were mixed 1: 1 (OD_{600} 0.1 each) and co-infiltrated to 2 fully expanded true leaves. Systemic leaves were collected 7, 15 and 30 d post-infiltration (dpi). (**B**) T7EI assay for indels detection. Genomic DNA was extracted from the systemic leaves and purified PCR product (200ng) of *PDS* fragment flanking the targeted locus was subjected to T7EI analysis. All three leaves collected at different time points showed high efficiency of targeted mutagenesis (43– 61 %) compared to vector control systemic leaves collected at 30 dpi. (**C**) Analysis of progeny plants for the presence of targeted modification using NcoI recognition site loss assay. DNA was extracted from progeny plants (50 seedlings pooled in one tube) and PCR was performed with a primer set to amplify 404 bp fragment encompassing the target site. Purified PCR product (300 ng) was treated with NcoI and separated on 2% agarose gel. Progeny pool 1 and 2 clearly showed a resistant DNA fragment of 404 bp indicating the targeted mutagenesis. (**D**) Alignment of Sanger sequencing reads showing the presence of indels at the *PDS* target

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sequence. Numbers to the right of sequence alignment indicates the number of nucleotides deleted by targeting the PDS genomic target.

All plant parts including meristematic tissues are infected by TRV, we tested whether the infected seed progeny of plants with pRNA2.PEBV: PDS.gRNAs would carry genomic modifications in the *PDS* gene target(Wright, Nuñez et al. 2016). Next to the infiltrated leavesthree early-matured seed capsules were grouped as one pool. From 50 progeny plants of each pool,Small leaf discs were collected in one tube as one pool for a total of 4 pools containing a WT control(Schaeffer and Nakata 2015). The complementary 404 bp DNA PCR fragments were delivered to NcoI restriction digestion to assess for targeted sequence modification in these pools. An NcoI enzyme-resistant band (404 bp) developed only in pools derived from plants infected with the pRNA2: pEBV:: gRNA.PDS assemble compared to the WT plants, demonstrating the presence of genomic modification in seed progeny (**Fig. 1C**)(Ji, Zhang et al. 2015). To attest these data, the PCR product was cloned into a topo cloning vector and the resultant clones were delivered to Sanger sequencing, which confirmed the existence of modification at the intended target site (**Fig. 1D**)(Ali, Abul-Faraj et al. 2015). These data provide the evidence about the feasibility of recovering plants bringing the targeted modification, even at very low efficiency, and omitting the need for transformation and tissue culture (**Fig. 1D**)(Merchante, Brumos et al. 2015). The detection of germinal transmission only in early flowers indicates that TRV infection and persistence in meristematic cells need to be optimized to improve the recovery of mutated plants from the seed progeny(Slaymaker, Gao et al. 2016). We did not recover plants carrying the targeted mutagenesis from capsules produced later in development(O'Connell, Oakes et al. 2014). Further improvements might increase the frequency of germinal transmission and recovery of mutant plants from the seed progeny(Abouzid, Hiebert et al. 1992).

CRISPR/Cas9 genome editing is off-target activities.

In applications of CRISPR/Cas9 genome editing is off-target activities is one of primary concern(Murant and Mayo 1982).however, this issue is more important in human applications than plants, we attempted to demonstrate whether our system showed off-target activities(Fauquet, Bisaro et al. 2003).

Methodology

The draft genome of *N. benthamiana* was screened for imperfect matches (i.e., allowing many mismatches) to the 20-nucleotide gRNA sequence (**Fig. 2A**)to identify candidate unintended targets of genome editing, and 13 candidates were then delivered to T7EI and restriction-protection assays.

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Consistent with former reports, no genomic modifications were detected at any of the predicted unintended targets (Fig. 2B). Therefore, we concluded that nooff-target activities are detected by our system, or these activities occurred at very low levels so not to detected by the modification-detection assays used(Thresh, Otim-Nape et al. 1998).

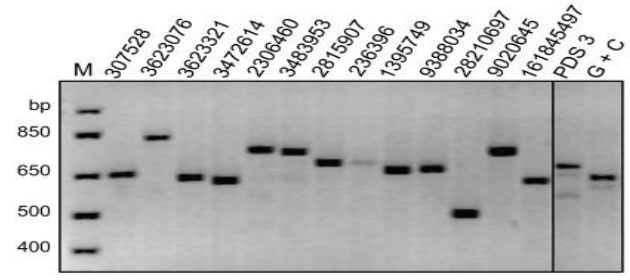
Results:

Figure 2. TRV-based CRISPR/Cas9 system exhibited no apparent off-target effects in *N. benthamiana* genome. (**A**) Table showing combinatorics approach to identify potential off-target binding of *PDS3* gRNA in *N. benthamiana* genome. The putative off-target binding sites were subjected to further annotation, where sequences were split into 2 groups of conserved and not conserved NcoI restriction site directly preceding the PAM sequence and sites containing mutation in seed and non-seed sequence. Based on dissimilarities of 1 to 7 nucleotides with *PDS3* gRNA, a total of 4265 potential off targets were detected, out of which 375 have NcoI site proximal to PAM sequence. (**B**) T7EI assay for the presence of indels at potential off target sites. DNA fragment flanking potential 13 targets were amplified by PCR with their respective primers. TRV-mediated CRISPR/Cas9 system exhibited no detectable activities at all tested targets using the T7EI assays. The respective contig number of each of 13 potential off target site are represented on the top of gel

A

Number of mismatches	Total combinations of gRNAs	Total hits	Total hits with NcoI sequence
1	60	0	0
2	1,710	0	0
3	30,780	0	0
4	392,444	1	0
5	3,767,412	60	4
6	28,255,525	515	46
7	169,532,551	3,689	275

B



Discussion

However, off-target Cas9 activities in the plant genome have been disclosed hardly(Czosnek and Laterrot 1997). These activities can be further diminished by engineering short lived Cas9 expression that is activated by viral infection or chemical induction(Rojas, Noueiry et al. 1998). Also, Ali et al. showed that one sgRNA targeting the TRV genome could advise plant resistance without inducing cleavage; this advises that catalytically immobile Cas9 (dCas9) can be used to interpose virus interference and thereby discard interests of off-target activities in the plant genome(Briddon, Mansoor et al. 2001). Moreover, fresh work has identified Cas9 enzymes that give few off-target effects, thus further weaken this interest in plants(Briddon, Bull et al. 2003). However,further research is required to test whether the platform can be adapted to target RNA viruses using other Cas9 variants(Eagle, Orozco et al. 1994).

Conclusion

In conclusion, our work drives the endurance of TRV-mediated CRISPR/Cas9 editing and the possibilityof optimizing this method to convert progeny plants carrying the targeted modifications thereby depart from the need for tissue culture or repeated transformation.

Future Applications

This method will elaborate the efficacy of the CRISPR/Cas9 system for plant functional genomics and targeted improvements of crop traits. In addition, the use of heterozygous Cas9 overexpressing plants with this apparent and facile genome-editing platform permits the engineering and production of plants free of foreign DNA. This might beat the regulatory hinders that disorder the commercialization of engineered plants. Applying the CRISPR/Cas9 machinery to engineer plant combat to viral pathogens also opens the chances of addressing main questions in virus infection and plant host resistance. For instance, the CRISPR/Cas9 platform could be used to check out the evolution of the viral genome to resist plant immunity, by assaying the genomes of viruses that rescue detection by the CRISPR/ Cas9 system. To identify host factors,The CRISPR/Cas9 platform could also be used for targeted mutagenesis that control plant resistance and vulnerability to viral infection. Thus, CRISPR/Cas9 technology offers an encouraging access for understanding and engineering resistance to single and multiple viral infections in plants.

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