A Simplified Method to Engineer CRISPR/Cas9-Mediated Geminivirus Resistance in Plants

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Abstract

Throughout the world, geminiviruses cause devastating losses in economically important crops, including tomato, cotton, cassava, potato, chili, and cucumber; however, control mechanisms such as genetic resistance remain expensive and ineffective. CRISPR/Cas9 is an adaptive immunity mechanism used by prokaryotes to defend against invading nucleic acids of phages and plasmids. The CRISPR/Cas9 system has been harnessed for targeted genome editing in a variety of eukaryotic species and in plants, CRISPR/Cas9 has been used to modify or introduce

many traits, including virus resistance. Recently, we demonstrated that the CRISPR/Cas9 system could be used to engineer plant immunity against geminiviruses by directly targeting the viral genome for degradation. In this article, we describe a detailed method for engineering CRISPR/Cas9-mediated resistance against geminiviruses. This method may provide broad, durable viral resistance, as it can target conserved regions of the viral genome and can also be customized to emerging viral variants. Moreover, this method can be used in many crop species, as it requires little or no knowledge of the host plant's genome.

Keywords

CRISPR/Cas9, Virus resistance, Plant virus, Geminivirus, Viral immunity

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CRISPR/Cas9-mediated geminivirus resistance in plants

1 Introduction

Viruses belonging to the Geminiviridae family infect important crops of several plant families throughout the world, including Cucurbitaceae (gourds, squash, watermelon, and melon), Euphorbiaceae (cassava), Solanaceae (tobacco, petunia, pepper, tomato, and potato), Malvaceae (okra and cotton), and Fabaceae (cowpea, mung bean, common bean, lima bean, and soybean) [1-3]. Conventional breeding strategies for improving plant resistance to diseases are expensive, time consuming, labor intensive, and often ineffective, especially in the case of rapidly-mutating viral diseases [4]. Other strategies seek to boost the plant's cellular immunity against viruses [5]. Several novel approaches have been used to attempt to enhance resistance against viruses,

including RNA interference-mediated targeting of viral protein production, expression of mutant proteins that impair viral replication, and targeting of host genes that cause symptoms [6]. The Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/ CRISPR-associated protein-9 (Cas9) nuclease (CRISPR/Cas9) system, derived from an adaptive immunity mechanism that bacteria and Archaea use to defend against invading nucleic acids such as phages and plasmids, has received special interest as a genome-engineering tool because of its ease, simplicity, efficiency, and reproducibility of use. Although established recently, CRISPR/Cas9 has been used in important model and crop plants such as rice, wheat, maize, tomato, potato, tobacco, cotton, soybean, and *Arabidopsis thaliana* [7]. Recent studies have used CRISPR/Cas9 to engineer virus resistance in plants, either by directly targeting and cleaving the viral genome, or by modifying the host plant genome to introduce genetic viral immunity [8-16]. Recently, we demonstrated that CRISPR/Cas9 can efficiently target the geminivirus genome, leading to interference and degradation of the viral genome and resulting in a significant reduction in viral symptoms [12]. To establish immunity against geminiviruses, we first generated *Cas9*-overexpressing (*Cas9*-OE) *Nicotiana benthamiana* transgenic lines and developed a *Tobacco rattle virus* (TRV)-based system for the delivery of the single guide RNA (sgRNA) into the *Cas9*-OE *N. benthamiana* lines [17, 18]. Engineered TRV genomic RNAs RNA1 and RNA2 were transformed into *Agrobacterium* and agro-infiltration was used to systemically infect the optimized TRV for sgRNA delivery into the *Cas9*-OE *N. benthamiana* plants. After the systemic establishment of TRV in the *Cas9*-OE *N. benthamiana* plants, the plants were infected with different geminiviruses. Plants with a complete CRISPR/Cas9 system exhibited robust resistance against *Tomato yellow leaf curl virus* (TYLCV), *Beet curly top virus* (BCTV), and *Merremia mosaic virus* (MeMV) [11, 12]. Other studies in *N. benthamiana* have

demonstrated that the CRISPR/Cas system can interfere with infection by *Bean yellow dwarf virus* (BeYDV) and *Beet severe curly top virus* (BSCTV) or even plant RNA viruses *Turnip mosaic virus* (TuMV) [13-15]. Moreover, we have also demonstrated that this technology can be used to target and cleave *Cotton leaf curl Kokhran virus* (CLCuKoV) and showed that targeting the conserved nonanucleotide sequence can simultaneously target multiple begomoviruses (CLCuKoV, TYLCV, TYLCSV, MeMV, BCTV-Worland, and BCTV-Logan), conferring broad-spectrum geminivirus resistance [11, 19]. These studies showed that *N. benthamiana* plants expressing the CRISPR/Cas9 system displayed reduced viral titers, which abolished or significantly reduced disease symptoms.

Further studies have refined the system and defined key parameters for successful induction of virus resistance. For example, *Cas9* expression has been correlated with the levels of virus suppression, indicating the need to use a single background transgenic line with optimum expression of *Cas9* and the sgRNA for practical applications [14]. Furthermore, catalytically inactive *Cas9* (d*Cas9*) can be used to mediate virus interference, thereby eliminating concerns about off-target activities in the plant genome [13, 20]. Our work, therefore, returns CRISPR/Cas9 to its origins as an immunity mechanism and our findings highlight the potential for using *Cas9* overexpression to induce viral immunity without time-intensive tissue culturing practices. Here, we provide a stepwise method for the development of plant lines with CRISPR/Cas9-mediated immunity against Geminiviruses.

2 Materials

2.1 Designing and Cloning the sgRNA

1. TYLCV sequence data (see 3.1 and Note 1)

2. CRISPR-P: <http://crispr.hzau.edu.cn/CRISPR2/> [21, 22] or CCTop: <https://crispr.cos.uni-heidelberg.de/> [21, 22] (*see* Subheading 3.1 and **Note 2**)
3. Phusion polymerase: Phusion high-fidelity DNA polymerase (New England Biolabs)
4. dNTP mixture: Mixture of an equal concentration (e.g., 2.5 mM) of dATP, dCTP, dGTP and dTTP.
5. TYLCV-IR-T1 and SPDK-gRNA-R (*see* Subheading 3.1 for primers designing and Table 1 for the sequences.)
6. Plasmid with sgRNA scaffold sequence (*see* **Note 3**)
7. Thermal cycler
8. TRV (*Tobacco rattle virus*) vector RNA2 construct: A modified pYL156 having Pea early browning virus (PEBV) subgenomic promoter in the upstream of insertion site. Provided by Savithramma Dinesh-Kumar, University of California at Davis.
9. Restriction enzymes: *Xba*I and *Xma*I or other restriction enzymes as per design of experiment, preferably high-fidelity (New England Biolabs).
10. T4 DNA ligase
11. *E. coli* competent cells: Any types of competent cells of *Escherichia coli* strains such as DH5 α .
12. LB (Luria Bertani) medium: 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl. For agar plate medium, add 1.5 % (w/v) agar. Sterilize by autoclave.
13. LB agar plates and LB liquid medium containing 50 mg/L kanamycin
14. Mini-prep kit for plasmids
15. Sequencing facility

2.2 Cloning Cas9

1. Phusion polymerase
2. dNTP mixture
3. Cas9-gw-F and Cas9-R (Table 1)
4. pX330 plasmid [20]
5. Thermal cycler
6. pENTR/D-TOPO (Life Technologies)
7. pK2GW7 binary vector Gateway compatible [21]
8. LR clonase (Life Technologies, Invitrogen)
9. *E. coli* competent cells: Any types of competent cells of *Escherichia coli* ccdB-sensitive strains such as DH5 α .
10. LB agar plates and LB liquid medium containing 50 mg/L kanamycin
11. LB agar plates and LB liquid medium containing 50 mg/L spectinomycin
12. Mini-prep kit
13. Sequencing facility

2.3 Generation of *N. benthamiana* Plants Overexpressing Cas9 (Cas9-OE)

1. Electro-competent cells of *Agrobacterium tumefaciens* GV3101 strain
2. LB agar plates and LB liquid medium containing 100 mg/L spectinomycin, 30 mg/L gentamycin, and 25 mg/L rifampicin.
3. MS medium: 4.4 g/L Murashige and Skoog (MS) basal salt mixture (Sigma-Aldrich or others), 1 ml/L Murashige and Skoog Vitamin Solution (1000 $\times$; Sigma-Aldrich or others), 3 % (w/v) sucrose, pH 5.8. For agar plates, add 0.8 % (w/v) agar (plant tissue culture grade). Sterilize by autoclave.

4. Sterile plastic Petri dishes
5. Autoclavable plant boxes such as Magenta™ vessel GA-7 (Sigma-Aldrich)
6. *Nicotiana benthamiana*: 2-week-old plants grown aseptically on MS agar medium in plant boxes.
7. Transformation medium: MS liquid medium
8. Sterile filter paper
9. MS co-cultivation medium: MS agar medium containing 1 mg/L 6-benzylaminopurine and 0.1 mg/L 1-naphthaleneacetic acid
10. MS regeneration and selection medium: MS co-cultivation medium supplemented with 50 mg/L kanamycin and 200 mg/L timentin.
11. Root-inducing medium: ½ MS media [2.2 g/L Murashige and Skoog (MS) basal salt mixture, 0.5 ml/L Murashige and Skoog Vitamin Solution (1000 ×), 1.5 % (w/v) sucrose, pH 5.8] added with 0.8 % (w/v) agar (plant tissue culture grade), sterilized by autoclave, added with 50 mg/L kanamycin, and poured into plant boxes.
12. Soil, pots and plastic domes for plant growth

2.4 Cas9 Confirmation and Expression Analysis

1. MS medium seedling selection plates: the same as root-inducing medium (*see* Subheading 2.3, Item 11)
2. Soil and pots
3. RNA extraction kit such as RNeasy mini kit
4. SuperScript 3 reverse transcriptase (Life Technologies, Invitrogen)
5. Oligo-dT primer
6. Phusion polymerase

7. dNTP mixture
8. Cas9-seqF6 and Cas9-R primers (Table 1).
9. Thermal cycler
10. Agarose gel electrophoresis
11. Protein extraction buffer: 100 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 3 mM DTT and protease inhibitors (1 mM PMSF, 5 $\mu\text{g ml}^{-1}$ leupeptin, 1 $\mu\text{g ml}^{-1}$ aprotinin, 1 $\mu\text{g ml}^{-1}$ pepstatin, 5 $\mu\text{g ml}^{-1}$ antipain, 5 $\mu\text{g ml}^{-1}$ chymostatin, 2 mM Na_2VO_3 , 2 mM NaF and 50 μM MG132).
12. SDS-PAGE system
13. PVDF membrane
14. Electric blotter
15. TBS-T: 50 mM Tris-HCl, 0.138 M NaCl, 0.0027 M KCl, 0.05 % Tween 20, pH 8.0. Dissolve Tris Buffered Saline, with Tween[®] 20, pH 8.0 powder (Sigma-Aldrich) in 1 L distilled water.
16. 5 % skim milk: 5 % (w/v) skim milk powder in TBS-T
17. 1 % skim milk: 1 % (w/v) skim milk powder in TBS-T
18. Anti-FLAG antibody (Sigma-Aldrich)
19. Goat anti-mouse IgG-HRP secondary antibody (Santa Cruz)
20. Enhanced Chemiluminescence (ECL)-detection reagent (Thermo Scientific)
21. Chemiluminescence imaging system

2.5 TRV-mediated sgRNA Delivery for Cas9 against TYLCV

1. *N. benthamiana* Cas9-OE plants: The transgenic plants should be grown according to the relevant law and regulations in each nation/state/province. Preferably grown in a greenhouse on the soil.
2. TRV vectors: pYL192 for TRV-RNA1 (Provided by Savithramma Dinesh-Kumar, University of California at Davis), and TRV-RNA2:TYLCV (constructed in 3.1)
3. Infectious clone of TYLCV: Infectious clones should be constructed by each of the research groups or obtained from other groups in the same nation/state/province because of the phytosanitary regulations.
4. Electro-competent cells of *Agrobacterium tumefaciens* GV3101 strain
5. LB agar plates and LB liquid medium containing 100 mg/L spectinomycin, 30 mg/L gentamycin, and 25 mg/L rifampicin.
6. Infiltration medium: 10 mM MES (2-(N-morpholino)ethanesulfonic acid), 10 mM CaCl₂. Adjust pH to 5.7 with KOH and sterilize by autoclave. Then add 200 μ M acetosyringone (add 1/500 volume of 100 mM stock solution in dimethyl sulfoxide).
7. Spectrophotometer
8. 1 ml needleless syringes

2.6 Mutation Detection by T7EI Assay, Restriction Site Loss Assay, and Sanger Sequencing

1. DNA extraction buffer: 100 mM Tris-Cl, 1 mM EDTA, 100 mM NaCl, 100 mM LiCl, 100 mM β -mercaptoethanol, and 0.4 % RNase.
2. PCI: Mixture of phenol: chloroform: isoamyl alcohol at a ratio of 25:24:1 saturated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
3. Phusion polymerase
4. dNTP mixture

- 200 5. TYLCV2.3-IR-T-F and TYLCV2.3-IR-T-R primers (Table 1).
- 201 6. Thermal cycler
- 202 7. PCR purification kit
- 203 8. Nanodrop spectrophotometer
- 204 9. T7EI: T7 endonuclease I supplied with NEBuffer 2(New England Biolabs)
- 205 10. SspI restriction enzyme
- 206 11. Agarose gel electrophoresis
- 207 12. pJET2.1 (Thermo Scientific) or other PCR cloning vector for blunt-ended PCR products
- 208 13. T4 DNA ligase
- 209 14. *E. coli* competent cells
- 210 15. LB agar plates and LB liquid medium containing 50 mg/L Ampicillin
- 211 16. Mini-prep kit
- 212 17. Sequencing facility

213 ***2.7 Viral Quantification with Semi-Quantitative PCR, Rolling Circle Amplification, and***
214 ***Southern Blot Analysis***

- 215 1. DNA extracted in 3.6.1
- 216 2. Phusion polymerase
- 217 3. dNTP mixture
- 218 4. TYLCV2.3-IR-T-F and TYLCV2.3-IR-T-R primers (Table 1).
- 219 5. NbActin-F and NbActin-R primers (Table 1).
- 220 6. Thermal cycler
- 221 7. Agarose gel electrophoresis

8. Rolling circle amplification kit: TempliPhi 100 Amplification kit (Sigma-Aldrich or GE Healthcare)
9. NcoI restriction enzyme
10. DIG DNA labeling and detection kit (Roche Diagnostics GmbH)
11. DNA Molecular Weight Marker II, DIG-labeled (Roche Diagnostics GmbH)
12. Nylon membrane
13. CDP-Star chemiluminescent substrate (Roche Diagnostics GmbH)
14. Chemiluminescence imaging system such as Alpha Innotech digital imaging system
15. ImageJ: <http://rsb.info.nih.gov/ij>

3 Methods

3.1 Designing and Cloning the sgRNA

1. Select the genomic region of the geminivirus to be targeted. In this example, we selected the intergenic region (IR) of TYLCV (**Figure 1A**). (*see Note 1*). Target site selection can be done manually by looking for an “NGG” as the protospacer-adjacent motif (PAM); use the 20 bp upstream, avoiding five or more consecutive thymines (T) in a row. Software-assisted selection is possible with CRISPR-P or CCTop [20, 21] (*see Note 2*). Manual selection is preferable to target a specific structure, motif, or domain. (Fig. 1A).
2. Design a forward primer to contain an XbaI recognition site with a 4-nucleotide 5' extension, a 20-nucleotide target sequence, and a 84-nucleotide Cas9-binding sgRNA scaffold, and a reverse primer to contain complementary sequence to the sgRNA 3'end, a 7-T repeat (terminator), and an XmaI recognition site with a 4-nucleotide 5' extension. Perform PCR with these primers to amplify a 125-bp PCR fragment using Phusion polymerase with 5x Phusion HF buffer, and a backbone containing the 84 bp sgRNA

Cas9 binding scaffold as a template. The fragment should contain a 4-nucleotide extension, 6-nucleotide XbaI recognition site, the 20-nucleotide target sequence, the 84-bp Cas9 binding loop for sgRNA including a 7-T repeat, 6-nucleotide XmaI recognition site, and a 5-nucleotide extension. (*see Note 3*)

Clone the amplified fragment using a restriction ligation procedure to have an sgRNA targeting the TYLCV IR region in the downstream of PEBV promoter of the TRV RNA2 vector. Cut both the PCR product and the RNA2 vector with XbaI and XmaI and gel purify.

- Using T4 ligase, insert the gel-purified 116-bp restriction fragment of the sgRNA for TYLCV target into the TRV RNA2 vector.
- Transform *E. coli* and plate onto LB agar plate containing 50 mg/L kanamycin.
- Pick up a few single colonies, grow them in LB medium containing 50 mg/L kanamycin and extract plasmid DNA using a mini-prep kit.
- Perform Sanger sequencing to confirm all cloned sequences.

3.2 Cloning of Cas9

- PCR amplify the complete 3XFlag–NLS-Cas9-NLS cassette with Cas9-GW-F and Cas9-R primers using Phusion polymerase with Phusion 5x HF buffer, and the pX330 plasmid as a template [20].
- Clone the gel-purified PCR product into pENTR/D-TOPO according to manufacturer's protocol. Transform *E. coli* and plate onto LB agar plate containing 50 mg/L kanamycin. Extract plasmid DNA from a few clones as mentioned above.

3. Confirm clones with restriction digestion and Sanger sequencing using Cas9-seq series primers (Table 1) for authenticity of clones and the in-frame translational fusions.
4. By LR Clonase reaction under manufacturer's protocol, move *Cas9* coding sequence into the pK2GW7 binary vector to generate a *35S::Cas9-T35S* construct. Transform *E. coli* and plate onto LB agar plate containing 100 mg/L spectinomycin. Pick up a few single colonies, grow them in LB medium containing 100 mg/L spectinomycin and extract plasmid DNA using a mini-prep kit. Confirm clones with restriction digestion. Use this construct for the production of the *N. benthamiana* *Cas9*-OE line (see **Note 4**).

3.3 Generation of *N. benthamiana* Plants Overexpressing *Cas9* (*Cas9*-OE)

1. Introduce the pK2GW7.Cas9 binary vector from the previous step into the *Agrobacterium tumefaciens* GV3101 strain by electroporation. Plate the bacteria onto LB agar plate containing 100 mg/L spectinomycin, 30 mg/L gentamycin, and 25 mg/L rifampicin.
2. Pick up and grow single colonies carrying the T-DNA vector overnight at 28 °C in 5 mL LB medium containing 100 mg/L spectinomycin, 30 mg/L gentamycin, and 25 mg/L rifampicin.
3. Use the overnight culture to inoculate 50 mL of the same medium.
4. Collect the cells at an OD₆₀₀ of 1.0, re-suspend in transformation medium to an OD₆₀₀ of 0.4 and pour onto a Petri dish.
5. Use a surgical blade to cut leaf tissues from aseptically grown 2-week-old *N. benthamiana* plants into small (approximately 1 cm) leaf discs. Make small incisions on the leaf surface with the scalpel to maximize contact of *Agrobacteria* with the cells.

6. Incubate leaf discs with an *Agrobacterium* culture for 30 min with occasional shaking.
7. Remove extra *Agrobacterium* cells with sterile filter paper and keep the leaf discs for 2 days for co-cultivation with *Agrobacterium* in the growth chamber on MS co-cultivation media.
8. After 2 days, transfer leaf discs onto MS regeneration and selection medium.
9. Excise shoot tissues appearing after 3–4 weeks and place on the root-inducing medium.
10. After proper roots have formed (2–3 weeks), carefully remove the agar medium from the roots, transfer the plantlets to soil and acclimate them to ambient atmosphere under plastic domes.
11. Grow the plants until seed setting and collect T1 seeds.

3.4 Cas9 Confirmation and Expression Analysis

3.4.1 Plant Growth

1. Grow the T1 generation of *N. benthamiana* Cas9-OE plants on MS medium seedling selection plates.
2. Transfer some kanamycin resistant plants to soil and grow them further for the analysis.

3.4.2 Confirmation of the Expression of Cas9 in the T1 Plants by Semi-quantitative RT-PCR

1. Extract total RNA from the leaves of *N. benthamiana* Cas9-OE plants using the RNAeasy mini kit or equivalent.
2. Synthesize first-strand cDNA using SuperScript 3 reverse transcriptase with oligo-dT primer.
3. Amplify a fragment corresponding to the C-terminal end of Cas9 using a primer pair (Cas9-seqF6 and Cas9-R) with Phusion polymerase with 5x Phusion HF buffer. Input PCR condition as follows: 98 °C for 1 min 30 s, 30 cycles of 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s, followed by 72 °C for 5 min.

4. Analyze PCR products on an agarose gel. The expected size of the PCR product should be 471 bp.

3.4.3 Confirmation of the Presence of the Cas9 Protein by Western Blotting Using Anti-Flag Antibody

1. Extract total proteins from 100 µg leaf tissue of *N. benthamiana* Cas9-OE plants with 100 µL of extraction buffer, clear the extract by centrifugation at 10,000 x g for 5 min at 4 °C.
2. Denature the proteins in a sample buffer and separate them on a 6 % SDS polyacrylamide gel electrophoresis by a standard protocol.
3. Transfer the proteins onto PVDF membrane using electric blotter by a standard protocol.
4. Perform immunoblot analysis by a standard protocol. Use primary mouse anti-FLAG antibody (1:1,000) in 5 % skim milk and secondary goat anti-mouse (1:1,000) in 1 % skim milk.
5. Detect reacting proteins by chemiluminescence using ECL detection reagent and a chemiluminescence imaging system according to manufacturers' protocol.
6. Select transgenic lines with good Cas9 protein expression and grow them to collect T2 seeds.

3.5 TRV-Mediated sgRNA Delivery for Cas9 against TYLCV

The complete strategy is provided in (Fig. 1B) and described as follows.

1. Grow *N. benthamiana* Cas9-OE plants on the soil in a greenhouse to the 6–8 leaf stage (2–3 weeks).

2. Introduce vectors containing the TRV RNA1 and TRV RNA2 genomes separately into the *A. tumefaciens* GV3101 strain and spread on LB agar plates containing 50 mg/L kanamycin, 30 mg/L gentamycin, and 25 mg/L rifampicin.
3. Introduce vectors containing the infectious clone of TYLCV (or any geminivirus under experiment) into the *A. tumefaciens* GV3101 strain and spread on the appropriate selection plates.
4. Grow the transformed single colonies for 36–48 hours in selective medium to OD₆₀₀ of 1.2, spin down, re-suspend and dilute to an OD₆₀₀ of 0.3 with infiltration medium.
5. Incubate cultures at ambient temperature in the dark for 2–4 h.
6. Prior to infiltration, mix bacterial cultures at OD₆₀₀ at a 1:1:0.05 ratio for TRV-RNA1:TRV-RNA2:TYLCV.
7. Infiltrate the bacterial culture mix into the lower (abaxial) side of two lower leaves of 3- to 4-week-old *Cas9*-OE *N. benthamiana* plants using a needleless 1 mL syringe.
8. Alternatively, first infiltrate the mixture of TRV-RNA 1:TRV-RNA2 at a ratio of 1:1 to pre-establish the CRISPR/Cas9 system for 5 days and then superimpose TYLCV by agroinfiltration to 1 lower leaf at an OD₆₀₀ of 0.05.
9. Collect leaf disc samples from infiltrated and upper uninfiltrated leaves at 5, 10, 15, and 30 days post-infiltration and subject to targeted genome modification analysis to determine and quantify the activity of the Cas9 endonuclease mutations resulting from double-strand break repair through the non-homologous end joining pathway (*see Note 5*).

3.6 Mutation Detection by T7EI Assay, Loss of Restriction Site Assay, and Sanger Sequencing

3.6.1 PCR Amplification of DNA Fragments Containing the Target Site

1. Extract genomic DNA from the samples collected as mentioned above by a standard protocol using DNA extraction buffer, PCI extraction, chloroform extraction, and ethanol precipitation. Quantify the DNA using Nanodrop spectrophotometer.
2. PCR amplify the fragment encompassing the target sequence (*see Note 6*). In this example, a 560 bp fragment of the IR flanking region was amplified using TYLCV2.3-IR-T-F and TYLCV2.3-IR-T-R primers with Phusion Polymerase with 5x Phusion HF buffer. PCR condition was as follows: 98 °C for 1 min 30 s, 30 cycles of 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s, followed by 72 °C for 5 min.
3. Confirm the amplification of the fragments on an agarose gel, purify the DNA fragments using a PCR purification kit and quantify using Nanodrop. Mutation can be detected by T7EI assay in most cases. A loss of restriction site assay can be used to detect mutations if a restriction enzyme recognition site was included in the target DNA. In both cases, mutations detected should be validated by Sanger sequencing.

3.6.2 Mutation Detection by a T7EI Assay

1. Prepare hetero-duplex formation reaction by mixing 200 ng purified PCR products, 1.8 µL NEBuffer 2, and sterile water to 18 µL total volume. Denature and re-anneal the PCR products in a thermal cycler to allow hetero-duplex formation using a following cycling program: 95 °C for 10 min, 85 °C for 2 min, 75 °C for 3 min, 65 °C for 3 min, 55 °C for 3 min, 45 °C for 3 min, 35 °C for 3 min, 25 °C for 3 min, and 4 °C.
2. Treat the re-annealed PCR products with T7EI by adding 0.5 µl of T7EI and 0.2 µL Buffer 2 and 1.3 ul of sterile water, incubate at 37 °C for 1h. If the thermal cycler is to be used for the incubation reaction, the led temperature should not be more than 37 °C.

- 382 3. Analyze the reaction product on an agarose gel, capture the gel image and quantify the
383 cut and uncut bands using ImageJ. Calculate the molar ratio of cut fragments to the total
384 fragments. The mutation rates is calculated as follows:

$$385 \text{ Mutation rate (\%)} = 100 \times (1 - (1 - \text{ratio of cut fragment})^{1/2}).$$

386 **3.6.3 Mutation Detection by a Loss of Restriction Site Assay**

- 387 1. For TYLCV, the IR contains an *SspI* restriction enzyme recognition site, and can be used
388 in restriction enzyme protection analysis. In a 20- μ l reaction, 300 ng of the IR flanking
389 PCR products were subjected to *SspI* restriction and the results are shown in (Fig. 1C).
390 To ensure complete digestion, the reaction should be performed for 8 h at 37 °C. If the
391 thermal cycler is to be used for the incubation reaction, the set temperature should not be
392 more than 37 °C.
- 393 2. Analyze the reaction product on an agarose gel, capture the gel image and quantify the
394 cut and uncut bands using ImageJ. Uncut bands represent the modified target sequence,
395 and therefore, the molar ratio of the uncut fragments is the mutation rate.

396 **3.6.4 Validation of Mutations Detected by Sanger Sequencing**

- 397 1. Ligate the PCR products to pJET2.1 using T4 DNA ligase. To validate the mutations
398 detected by T7EI assay, the original PCR products from 3.6.1 are used. It is preferable to
399 gel purify the undigested DNA fragments in the loss of restriction site assay and clone
400 them.
- 401 2. Transform *E. coli* and spread the bacteria onto the LB agar plate containing 50 mg/L
402 Ampicillin.
- 403 3. Pick up 8 or more colonies per sample, grow the bacteria in LB medium containing 50
404 mg/L Ampicillin and extract plasmid DNA using a mini-prep kit.

4. Sequence the flanking regions of the target site to identify the mutation occurred. The representative results are shown in Fig. 1D.

3.7 Viral Quantification with Semi-Quantitative PCR, Rolling Circle Amplification, and Southern Blot Analysis

3.7.1 Semi-Quantitative PCR for TYLCV

1. Amplify the DNA fragment encompassing the IR of TYLCV, using the TYLCV2.3-IR-T-F and -R primers (Table 1) with Phusion polymerase with 5x Phusion HF buffer and the 50 ng genomic DNA extracted in 3.6.1 from all samples including the controls as templates.
2. As the experimental control, amplify a partial fragment of *N. benthamiana* Actin gene using the NbActin -F and -R primers (Table 1) with Phusion polymerase with 5x Phusion HF buffer and the genomic DNA extracted from all samples including the controls as templates.
3. Run a 20–23 cycle PCR with conditions as follows: 98 °C for 1 min 30 s, 20–23 cycles of 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s, followed by 72 °C for 5 min.
4. Run the PCR product on a 1 % agarose gel. Representative results from the uninoculated upper leaves are shown in Fig. 1E.

3.7.2 Rolling Circle Amplification for TYLCV

Amplify the TYLCV genomic DNA following the rolling circle amplification kit protocol. The amplified DNA can be detected on an agarose gel electrophoresis after cutting into unit size molecules by restriction digestion.

1. Incubate the 50 ng genomic DNA for 3 minutes at 95 °C in sample buffer and place on ice for 5 min.
2. Add enzyme mix and reaction buffer to the samples and incubate at 30 °C for 12–18 h for amplification.
3. Inactivate the enzyme at 65 °C for 15 min.
4. Add *Nco*I to the samples and incubate for 1 h at 37 °C to linearize the amplified DNA.
5. Resolve DNA samples on a 1 % agarose gel. Representative results from the uninoculated upper leaves are shown in Fig. 1F.

3.7.3 DIG-Based Southern Blot Analysis for the TYLCV Genome

1. Using DNA DIG labeling and detection kit, PCR amplify a fragment of the IR sequence of the TYLCV genome (an approximately 560 bp fragment) using the TYLCV2.3-IR-T-F and -R primers (Table 1).
2. Resolve 2 µg total genomic DNA from 3.6.1 on a 1 % agarose gel alongside the DNA DIG-labeled size marker-II.
3. Blot the DNA fragments onto a nylon membrane by a standard capillary transfer procedure overnight followed by UV-crosslinking. Block the membrane and allow hybridization with the respective IR-DIG labeled probe.
4. Visualize DNA bands using alkaline phosphatase-conjugated anti-DIG (1:10,000) and CDP-star chemiluminescent substrate in a chemiluminescence imaging system. Representative results from the uninoculated upper leaves are shown in Fig. 1G.
5. Quantify virus titer using ImageJ.

4 Notes

1. In this method, we used TYLCV as an example geminivirus. Any geminivirus or DNA virus can be targeted using the described method. A comprehensive list of all geminiviruses can be found at www.talk.ictvonline.org with respective accession numbers that can be used to download viral genome sequences from www.ncbi.nlm.nih.gov.
2. We highly recommend checking the off-targeting activity of the designed sgRNA using online tools like CRISPR-P or CCTop [20, 21].
3. Alternatively, the sgRNA clones can be custom synthesized in a plasmid vector using an oligonucleotide synthesis service company such as BlueHeronBio gene synthesis service (BlueHeronBio, Bothell, WA USA). Each sgRNA, flanked by *Xba*I and *Xma*I restriction sites, should consist of a 116-bp fragment containing the 20-nucleotide target sequence, 77 bp functioning as a Cas9 binding loop followed by a repeat of 7 thymine residues as the transcriptional terminator and 6-nucleotide each of restriction sites. The 116 bp sgRNA fragment of each target can be subcloned into the TRV RNA2 vector under the PEBV viral promoter by restriction ligation cloning using *Xba*I and *Xma*I restriction enzymes.
4. In this example, we described *N. benthamiana*, but any plant species where the efficiency of CRISPR/Cas9 and TRV has been experimentally demonstrated can be used.
5. Take photographs of plants at each stage for comparison of molecular data with the phenotypic data.
6. Perform PCR reactions using undigested genomic DNA. For the cloning and Sanger sequencing purpose, restriction enzyme predigested genomic DNA can be used to enrich

for the modification when the target sequence includes the restriction enzyme recognition site. In this case, design PCR primers such that the amplified regions do not have the recognition sites except for that in the target sequence.

Acknowledgment

We would like to thank members of the laboratory for genome engineering for continuous discussions. This study is supported by King Abdullah University of Science and Technology (KAUST) Office of Sponsored Research (OSR) under Award No. OSR-2015-CRG4-2647.

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543 Tables

544 **Table 1 Primers used in the method.**

Primer name	sequence (5' ---- 3')	Use
TYLCV2.3-IR-T-F	AATTGGGAAAGTGCTTCCTCT	Simi-q PCR, to amplify TYLCV IR flanking region, to make probe for Southern, detection of TYLCV by PCR
TYLCV2.3-IR-T-R	ATAGTCACGGGCCCTTACAACA	
TYLCV-IR-T1	CGAGTCTAGAGGCCATCCGTATA ATATTACGTTTTAGAGCTAGAAA TAGCAAG	To clone TYLCV IR- sgRNA
SPDK-gRNA-R	acatGCCCCGgAAAAAAGCACCG ACTCGG	To clone all sgRNAs
Cas9-gw-F	CACCATGGACTATAAGGACCACG	To clone <i>Cas9</i>
Cas9-R	TTACTTTTTCTTTTTTGCCTGGC	To clone <i>Cas9</i> and as reverse primer in <i>Cas9</i> detection RT-PCR
Cas9-seqF6	GCCCTCCAAATATGTGAACTTCC	Forward primer in <i>Cas9</i> detection by RT-PCR and Cas9 sequencing
NB-Actin-RT-R	GATGAAGATACTCACAGAAAGA	NB. Actin RT control
NB-Actin-Q-RT-F	TAGCTCTTCTCCACGGATGAGC	NB. Actin RT control

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CAS9-SEQ-1860-F	aaatcgagtgcttcgactccgtggaat	Cas9 sequencing
CAS9-SEQ-2442-F	acagaagaacagccgcgagagaatgaa	Cas9 sequencing
CAS9-SEQ-2960-F	acactaagtacgacgagaatgacaagc	Cas9 sequencing
Cas9-SEQ-3726-F	gatcatcaagctgcctaagtactccctg	Cas9 sequencing
CAS9SEQ F1	CCCATCAACGCCAGCGGCGTG	Cas9 sequencing
CAS9-SEQ-1064-F	GACATCCTGAGAGTGAACACCGAGATCA	Cas9 sequencing
CAS9SEQ F2	GCTGCGGAAGCAGCGGACCTTC	Cas9 sequencing
CAS9SEQ F3	CGAGGACATTCTGGAAGATATC	Cas9 sequencing
CAS9SEQ F5	GCGAGCAGGAAATCGGCAAGGC	Cas9 sequencing
Cas9-887-SeqR.	CAGGTCGAAGTTGCTCTTGAAGTT	Cas9 sequencing
Cas9-397-seq	CCATCTCGTTGCTGAAGATCTCTT	Cas9 sequencing

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546

Figures

Fig. 1 CRISPR/Cas9-mediated interference of *Tomato yellow leaf curl virus* (TYLCV) in *N. benthamiana*.

(A) CRISPR/Cas9 machinery (Cas9, sgRNA) designed for the intergenic region (IR) sequence of TYLCV is shown. Black arrows represent the partially overlapping open reading frames (ORFs), and the IR is represented by double arrowheads in the TYLCV genome. (B) Schematic representation of the experimental design. The sgRNA specific for the TYLCV IR sequence engineered to TRV-RNA2 was infiltrated into *Cas9*-OE plants. After establishing CRISPR/Cas9 system targeting the TYLCV IR sequence in plants, TYLCV was agro-infiltrated into the lower leaves. Samples were collected at 5, 10, 15, and 30 days post-infiltration (dpi). PEBV, *Pea early browning virus* promoter. (C) Mutation analysis using a restriction site loss assay. The IR (560 bp) sequence containing an *SspI* recognition site at the target region was analyzed for loss of recognition. The arrow indicates the 269-bp *SspI*-resistant DNA fragment in samples harboring IR-sgRNA, but not in samples harboring the TRV empty vector used as a control. Note that the 560 bp fragment has two additional *SspI* sites outside the target sequence (for detail, see figure 3C in Ref. 11). (D) Alignment of Sanger sequencing reads of PCR amplicons cloned from the IR targeted region. PAM and *SspI* recognition sequences are represented. (E) Semi-quantitative PCR of TYLCV genomic DNA. TYLCV infiltration into CRISPR/Cas9 established plants. IR-sgRNA containing plants accumulated low levels of TYLCV compared to the control plants. Actin of *N. benthamiana* (NB-Actin) was used for normalization. (F) Rolling-circle amplification (RCA) assay of the TYLCV genome. Plants harboring IR-sgRNA accumulated less TYLCV genomic DNA than those with the TRV empty vector controls. (G) Southern blot analysis of TYLCV genomic DNA accumulation. Genomic DNA of TYLCV was detected with

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570 DIG-labeled 560-bp IR probe. IR-sgRNA-harboring plants showed lower accumulation of the
571 TYLCV genome than plants inoculated with the TRV empty vector and TYLCV. Arrowhead
572 indicates the expected size of the TYLCV genome.

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