



Virus-Mediated Genome Editing in Plants Using the CRISPR/Cas9 System

Ahmed Mahas, Zahir Ali, Manal Tashkandi, and Magdy M. Mahfouz

Abstract

Targeted modification of plant genomes is a powerful strategy for investigating and engineering cellular systems, paving the way for the discovery and development of important, novel agricultural traits. Cas9, an RNA-guided DNA endonuclease from the type II adaptive immune CRISPR system of the prokaryote *Streptococcus pyogenes*, has gained widespread popularity as a genome-editing tool for use in a wide array of cells and organisms, including model and crop plants. Effective genome engineering requires the delivery of the Cas9 protein and guide RNAs into target cells. However, *in planta* genome modification faces many hurdles, including the difficulty in efficiently delivering genome engineering reagents to the desired tissues. We recently developed a *Tobacco rattle virus* (TRV)-mediated genome engineering system for *Nicotiana benthamiana*. Using this platform, genome engineering reagents can be delivered into all plant parts in a simple, efficient manner, facilitating the recovery of progeny plants with the desired genomic modifications, thus bypassing the need for transformation and tissue culture. This platform expands the utility of the CRISPR/Cas9 system for *in planta*, targeted genome modification. Here, we provide a detailed protocol explaining the methodologies used to develop and implement TRV-mediated genome engineering in *N. benthamiana*. The protocol described here can be extended to any other plant species susceptible to systemic infection by TRV. However, this approach is not limited to vectors derived from TRV, as other RNA viruses could be used to develop similar delivery platforms.

Key words CRISPR/Cas9, TRV, *Nicotiana benthamiana*, Genome editing, Targeted modification, Genome engineering, RNA viruses

1 Introduction

Precise genetic manipulation of living cells via the introduction of controlled, targeted alterations in specific genomic sequences is an important goal with implications for fields ranging from functional biology to biotechnology and medicine [1, 2]. In plants, targeted genome editing has enormous potential for facilitating the analysis of gene function and uncovering and developing novel traits for crop improvement and sustainable agriculture [3]. Precise genome

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modification relies on the induction of DNA double-strand breaks (DSBs) at a targeted genomic locus and the subsequent repair mechanism, which is mediated by the two major repair pathways, the error-prone non-homologous end joining (NHEJ) mechanism and the precise homology-directed repair (HDR) mechanism, both of which can be harnessed to achieve the desired genome engineering outcome [4]. The use of various programmable, site-specific nucleases capable of precisely generating DNA DSBs has accelerated targeted genome engineering in a wide range of cell types and organisms, including plants [5].

The recent development of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats [CRISPR]/CRISPR-associated endonuclease 9 [Cas9]) system as a genome-editing platform has dramatically simplified the field of genome engineering [1, 6]. The CRISPR/Cas9 system, which is based on the adaptive immune system of the prokaryote *Streptococcus pyogenes*, is composed of two components: the RNA-guided DNA endonuclease Cas9 and an engineered single guide RNA (gRNA) capable of guiding the Cas9 endonuclease to the genomic sequence of interest, which is complementary to the user-defined 20-nucleotide targeting or “spacer” sequence within the gRNA. In addition to the spacer sequence, which confers specificity to Cas9, target recognition and cleavage require the presence of a DNA protospacer-adjacent motif (PAM) immediately adjacent to the 3' end of the 20-nt target sequence [7]. The simplicity and robustness of this system have led to its extensive use for genome engineering and efficient genetic manipulation across a wide array of plants, including model plants such as *Arabidopsis* [8–10] and *Nicotiana benthamiana* [9, 11] and crop plants such as rice [12, 13], wheat [14, 15], maize [16, 17], tomato [18], and sweet orange [19]. Furthermore, other CRISPR/Cas variants have been developed by engineering of catalytically inactivated Cas variants (nuclease-deficient or nuclease-deactivated (dCas) in combination with various nucleases, transcriptional repressors, activators, or epigenetic modifiers, resulting in enhanced targeting efficiency and specificity and enabling sequence-specific regulation of gene expression and chromatin state [20–22]. In addition, the CRISPR/Cas9 system has been successfully harnessed to engineer plants with increased resistance to a wide range of plant pathogenic viruses [23–30], highlighting its potential to fundamentally transform agriculture.

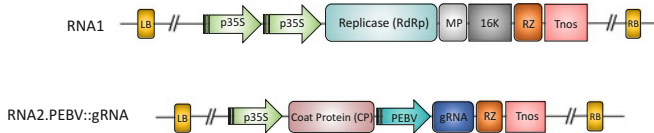
To achieve effective CRISPR/Cas9-mediated targeted genome modifications for crop trait discovery and improvement, Cas9 and its cognate gRNA must be efficiently expressed in the targeted cells, and the genome modification events must be heritable, as they should occur in the germline cells and allow for subsequent recovery of progeny with the desired DNA modification. Therefore, the efficient delivery of genome engineering reagents into plant cells is crucial for the effective use of this technology. *In planta* delivery of

these editing components for heritable modification is often accomplished by generating transgenic lines that stably express Cas9 and gRNAs. This goal can be achieved by stably integrating Cas9 and gRNA cassettes into the plant genome via *Agrobacterium*-mediated transformation or using biolistic-based methods, which should result in the expression of the CRISPR/Cas9 components in all cells of the transgenic plant, including germline cells, thus allowing the targeted modifications to be transmitted to subsequent generations. While such delivery approaches can result in the efficient expression of genome engineering reagents and heritable genome modifications, the production of transgenic lines is expensive and time consuming, as tissue culture and repeated transformation are needed to produce every new, targeted modification. In addition, the use of these genetically modified plants can raise public concerns, as well as regulatory hurdles.

Autonomously replicating DNA and RNA virus-based vectors offer an alternative means for efficiently delivering genome engineering reagents into plant cells [31]. The advantage of RNA virus-based vectors versus their DNA viruses-based counterparts is that they do not integrate into the plant genome, thus avoiding unintended genome integration. Therefore, plants modified using RNA viruses are considered to be transgene-free edited plants. One such virus-based vector is *Tobacco rattle virus* (TRV), which is widely used for efficient virus-induced gene silencing (VIGS) in functional genomics studies in diverse plant species [32, 33]. TRV viruses have bipartite genomes comprising two positive-sense ssRNAs: TRV1 (RNA1) and TRV2 (RNA2). TRV1 is essential for virus replication and movement, and TRV2 harbors genes encoding nonstructural proteins that can be replaced by multiple cloning sites, into which different exogenous fragments of the target gene to be silenced can be inserted [34]. When the recombinant TRV vector is introduced into plant cells via *Agrobacterium*-mediated infiltration, the virus expression system mediates the synthesis of the recombinant viral RNA inside the infected plant cells. These initially infected cells presumably serve as a source for further replication of viral RNAs and their systemic infection and spread into a variety of tissues and cells, such as developing and meristematic tissues, including germline cells [35, 36]. The small size of the TRV genome facilitates cloning, multiplexing, and agroinfection, pointing to its great potential for use as a vector for delivering genome engineering reagents.

We recently developed TRV as a vehicle for systemic delivery of gRNAs for targeted genome modification in *N. benthamiana* plants overexpressing *Cas9* [37, 38]. To construct this TRV-mediated genome editing system, we generated *Cas9*-overexpressing (*Cas9*-OE) *N. benthamiana* transgenic lines. We then generated a TRV RNA2 genome-derived vector for systemic gRNA delivery. In the TRV RNA2 vector, the gRNA was expressed under the control of

A



B

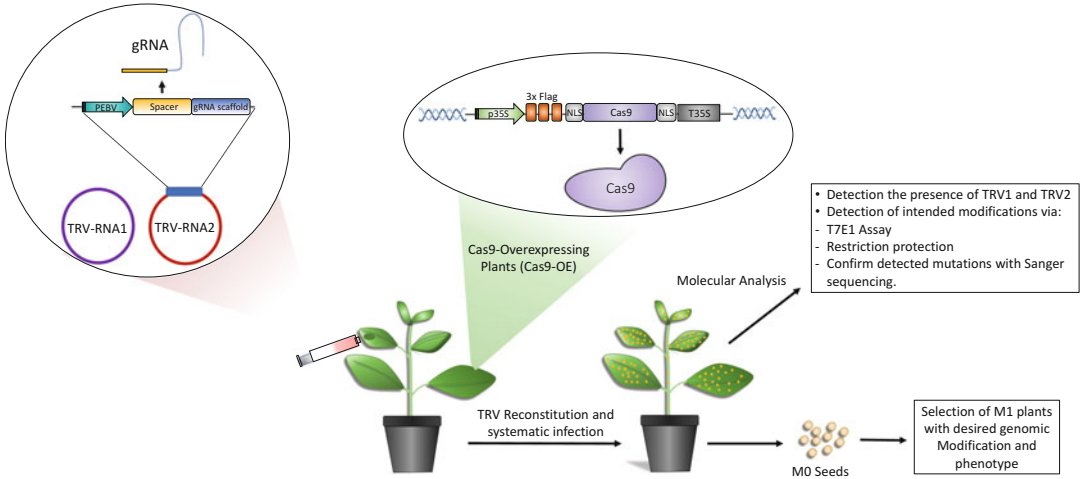


Fig. 1 TRV-mediated genome editing in *N. benthamiana*. **(a)** Schematic representation of the genome organization of TRV RNA1 and RNA2. RNA1 in the *Agrobacterium* binary vector system: LB (left border), 2Xp35S (2X CaMV 35S promoter), RdRNAP (134/194 kDa RNA-dependent RNA polymerase, RdRp), MP (movement protein), 16k (cysteine rich protein), Rz (self-cleaving ribozyme), Tnos (nopaline synthase terminator), RB (right border). RNA2 in the *Agrobacterium* binary vector system: LB, p35S, CP (coat protein), Rz, Tnos, and RB. In RNA2, the gRNA is under the control of the *Pea early browning virus* (PEBV) promoter (pPEBV::gRNA). **(b)** Experimental scheme for TRV-mediated genome editing. A 20-nucleotide target sequence (shown in yellow) preceding the PAM sequence is cloned into the gRNA backbone (shown in blue) under the control of the PEBV promoter in the RNA2 genome. *Agrobacterium* cultures carrying the engineered TRV RNA2 genome (conferring user-selected sequence specificity) and the RNA1 genome are co-infiltrated into the leaves of *N. benthamiana* overexpressing *Cas9* (*Cas9*-OE) via agroinfection. After agroinfection, the plants are analyzed for the presence of the targeted modification. Leaf disks carrying modified genomes can be regenerated to recover mutant plants, or the seed progeny can be screened for the presence of the modification, thereby bypassing the need for tissue culture

the *Pea early browning virus* (PEBV) promoter (PEBV::gRNA), permitting the expression of the gRNA from the virus expression system. We then reconstituted the TRV virus in *N. benthamiana* leaves via agroinfiltration of mixed *Agrobacterium* cultures harboring the RNA1 genome in combination with different RNA2 vectors, in which gRNAs with binding specificity for a single target (the phytoene desaturase [*PDS*] gene) or multiplex targets (*PCNA* and *PDS*) were driven by the PEBV promoter (pRNA2.PEBV::PDS/*PCNA*.gRNA) (Fig. 1a). This TRV-mediated genome engineering resulted in highly efficient targeted genome modification in both

the inoculated and systemic leaves of *Cas9*-OE *N. benthamiana*. In addition, the ability of the TRV to infect germline cells resulted in the detection of targeted genome modifications in the seeds of the agro-infiltrated plants, indicating that we successfully recovered the desired modification in the progeny [38]. Moreover, the TRV-mediated CRISPR/Cas9 activity persisted for up to 30 days post-agroinfiltration, and the genetic modification was specific, as no off-target activity was detected [37].

TRV-mediated CRISPR/Cas9 is a simple, versatile genome-editing platform for *in planta* targeted genome modification that eliminates the need for transformation and tissue culture to produce targeted modifications. This system meets several important requirements for highly efficient, multiplexed editing. For example, TRV can be used to systemically infect many different plant species, both naturally and under laboratory conditions. In addition, the virus is easily introduced into plants via *Agrobacterium* and systemic delivery into growing points of the plant. Moreover, the small genome size of TRV facilitates cloning, multiplexing, library construction, and agroinfection. Finally, the viral RNA genome does not integrate into the plant genome, overcoming the regulatory hurdles that might impede the commercialization of engineered plants. Thus, our work expands the utility of the CRISPR/Cas9 system for functional genomic studies in plants and for agricultural biotechnological applications. Here, we present our stepwise method for TRV-mediated genome engineering in plants.

2 Materials

2.1 Reagents

1. Enzymes: Phusion high-fidelity DNA polymerase, T7 endonuclease 1 (T7E1), *Nco*I, *Xba*I, and *Xma*I (or other restriction enzymes as required, preferably high-fidelity) (New England Biolabs), SuperScript 3 reverse transcriptase (Invitrogen), T4 DNA ligase (Promega).
2. Growth media: Luria Bertani (LB) medium for bacterial culture and Murashige and Skoog (MS) basal salt mixture for plant tissue culture (Sigma-Aldrich).
3. Antibiotics: Spectinomycin, gentamicin, rifampicin, kanamycin, and Timentin (Sigma-Aldrich).
4. Primers, as detailed in Table 1.
5. MES buffer (Sigma-Aldrich).
6. Growth hormones: 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BA) (Sigma-Aldrich).
7. Acetosyringone (Sigma-Aldrich).
8. Anti-FLAG antibody (Sigma-Aldrich).

Table 1
Primers used in this protocol

Primer name	Sequence (5'----3')	Use
Cas9-gw-Fw	CACCAATGGACTATAAGGACCACG	To clone <i>Cas9</i>
Cas9-R	TT ACTTTTTCTTTTTTTTGCCTGGC	To clone <i>Cas9</i> and as a reverse primer for <i>Cas9</i> detection via RT-PCR
CAS9-SEQ-F6	GCCCTCCAAATATGTGACTTCC	Forward primer for <i>Cas9</i> detection via RT-PCR
Cas9-R-No-Stop	CTTTTTCTTTTTTTTGCCTGGC	To construct <i>Cas9-GFP</i>
NB-ACTIN1-RT-F	TGAAAGA TCCTCACAGAGCGTGG	RT-PCR normalization control
NB-ACTIN1QRT-LIU-R	TTGT A TGTGGTCTCGTGGG TTC	
TRV1-Replicase-RT-F	CT ACTGGGAGAGCAGCAACC	To detect systemic movement of TRV-RNA1
TRV1-Replicase-RT-R	CTGAGCGCAAAAAGT ACACCA	
TRV2-CP-RT-F	TTGGGTGGAATCAGTTTCGT	To detect systemic movement of TRV-RNA2
TRV2-CP-RT-R	TCTTCCAAAAGTCGAGCCAGT	
NB-PDS3-TR1,2-gDNA-F2	GAAACACATCACCTAGGCGG	For PCR of the region flanking the <i>PDS</i> target
NB-PDS3-TR1,2-gDNA-R	GGCGTGAGGAAAGT ACGAAA	
NBPDS3-gDNA-404 bp-F	GTAATAATGCCCCAAAATTGGACTTTGT	To amplify the 404-bp target-flanking region around <i>PDS</i>
NBPDS3-gDNA-404 bp-R	CGTGAGGAAAGTACGAAAATGATGATGA	
NB_PCNA gDNA F1	CCTAACCCCTAATTTCCCCCAG	For PCR of the region flanking the <i>PCNA</i> target
NB_PCNA gDNA R1	TCACTGTCAATGTCCATCAG	

9. Goat anti-mouse IgG-HRP secondary antibody (Santa Cruz).
10. Enhanced Chemiluminescence (ECL)-detection reagent (Thermo Scientific).
11. Skim milk powder, Tween 20, Sodium dodecyl sulfate (SDS), HEPES, Tris buffer, Glycerol, Agarose powder, Ethidium bromide, Lithium chloride, Bleach (Sodium hypochlorite).
12. Polyacrylamide gels (Precise Tris-HEPES Gels, Thermo Scientific).
13. TBS-T: Tris-buffered saline with Tween 20, pH 8.0.
14. Infiltration medium: 10 mM MES, 10 mM CaCl₂, 200 μM acetosyringone, pH 5.7.
15. Regeneration and selection medium: 4.4 g/L MS salts with vitamins, 1 mg/L BA, 0.1 mg/L NAA, 30 g sucrose, 50 mg/L kanamycin, 200 mg/L Timentin, pH 5.8.
16. Root-inducing medium: 2.2 g/L MS salts with vitamins, 50 mg/L kanamycin.
17. DNA extraction buffer: 100 mM Tris-Cl, 1 mM EDTA, 100 mM NaCl, 100 mM LiCl, 100 mM β-mercaptoethanol, 0.4% RNase.
18. PCI solution: Phenol:chloroform:isoamyl alcohol.
19. CDP-Star Chemiluminescent Substrate (Roche).
20. PVDF membrane.

2.2 Plasmids and Vectors

1. *Tobacco rattle virus* (TRV) RNA1 (pYL192) and RNA2 (pYL156 modified with the PEBV promoter), provided by Savithramma Dinesh-Kumar, University of California at Davis.
2. pK2GW7 binary vector, which is Gateway compatible [39].
3. pX330 plasmid [40].
4. pEarleyGate 103.
5. pENTR/D-TOPO (Life Technologies).
6. LR Clonase (Life Technologies, Invitrogen).
7. TOPO TA cloning vector (Thermo Scientific).

2.3 Kits

1. RNeasy Mini kit.
2. PCR purification.
3. Plasmid Miniprep kit.

2.4 Organisms

1. *Nicotiana benthamiana*.
2. *Agrobacterium tumefaciens* strain GV3101.

2.5 Software and Programs

1. CRISPR-P/CCTop [41, 42].
2. ImageJ (<http://rsb.info.nih.gov/ij/>).

3 Methods

3.1 Designing and Cloning of sgRNA

1. Select the genomic region of the plant genome to be targeted. In this example, we selected the phytoene desaturase (*PDS*) gene for single targeting and the proliferating cell nuclear antigen gene (*PCNA*) for multiplex targeting (*PCNA* and *PDS*, simultaneously), as mutations at these genes can result in obvious visual phenotypes. Target site selection can be performed manually by looking for an “NGG” as the protospacer-adjacent motif (PAM); use the 20 bp upstream sequence, avoiding five or more thymines (T) in a row. Software-assisted selection can be performed using CRISPR-P or CCTop [41, 42] (see **Note 1**). Manual selection is preferable for targeting a specific structure, motif, or domain.
2. Use a PCR-based restriction ligation procedure to clone sgRNAs targeting the *PDS* and/or *PCNA* genes into the TRV RNA2 vector under the control of the *Pea early browning virus* (PEBV) promoter (see **Note 2**).
3. Use a forward primer containing an *Xba*I recognition site, a 20-nucleotide target sequence, and an 84-nucleotide Cas9-binding sgRNA scaffold with a reverse primer containing complementary sequence to the sgRNA end, a 7-T repeat (terminator), and an *Xma*I recognition site to amplify a 116-bp PCR fragment.
4. PCR amplify the fragment containing the 20-nucleotide target sequence, the 84-bp Cas9 binding loop for sgRNA, and a 7-T repeat (as a terminator), using a backbone containing the 84 bp sgRNA Cas9 binding scaffold as template.
5. Digest both the PCR product and the RNA2 vector with *Xba*I and *Xma*I and gel purify.
6. Using T4 ligase, clone the 116-bp PCR fragment of the sgRNA for each target into the TRV RNA2 vector under the control of the PEBV promoter (Fig. 1a).
7. Perform Sanger sequencing to confirm all cloned sequences.

3.2 Cloning of Cas9

1. PCR amplify the complete 3XFlag–NLS–Cas9–NLS cassette with Cas9-GW-F and Cas9-R primers using the pX330 plasmid as template [40].
2. Clone the gel-purified PCR product into pENTR/D-TOPO.
3. Transfer *Cas9* into the pK2GW7 binary vector via the LR Clonase reaction to generate the 35S::*Cas9*-*T35S* construct. Use this construct to produce the *N. benthamiana* *Cas9*-OE line (see **Note 3**).

4. Subject the clones to restriction digestion and Sanger sequencing to confirm the authenticity of the clones and the in-frame translational fusions.

3.3 Cloning the Cas9: GFP Reporter

1. PCR amplify the complete 3XFlag-NLS-Cas9-NLS cassette with Cas9-GW-F and Cas9-R-no-stop primers (to remove the stop codon) using the pX330 plasmid as template [40].
2. Clone the gel-purified PCR product into pENTR/D-TOPO.
3. Transfer *Cas9* into the pEarleyGate 103 binary vector via the LR Clonase reaction to generate the *35S::Cas9:GFP-T35S* construct. Use this construct to transiently express the GFP-fusion variant in *N. benthamiana* leaves for subcellular localization analysis of Cas9 (see **Note 4**).
4. Subject the clones to restriction digestion and Sanger sequencing to confirm the authenticity of the clones and the in-frame translational fusions.

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

1. Introduce the pK2GW7.Cas9 binary vector obtained in Subheading 3.2 into *Agrobacterium tumefaciens* strain GV3101 by electroporation.
2. Grow single colonies carrying the T-DNA vector overnight at 28 °C in 5 mL *Agrobacterium* culturing medium.
3. Inoculate the overnight culture in 50 mL of LB containing the selective antibiotics in 5 mM MES buffer.
4. Grow the cells to OD₆₀₀ of 1.0, resuspend them in transformation medium to 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 disks. Make small incisions on the leaf surface with the scalpel to maximize contact of the *Agrobacteria* with the plant cells.
6. Incubate leaf disks in *Agrobacterium* culture for 30 min with occasional shaking.
7. Remove excess *Agrobacterium* cells with sterile filter paper and cocultivate the leaf disks in *Agrobacterium* on MS medium in a growth chamber at ~25 °C for 2 days.
8. Transfer the leaf disks onto the regeneration and selection medium.
9. Excise shoot tissues after 3–4 weeks and place them onto root-inducing medium.
10. After proper roots have formed (2–3 weeks), acclimate the plantlets in soil under plastic domes.

3.5 Confirmation of Cas9 Expression at RNA and Protein Levels

3.5.1 RNA Confirmation by Semiquantitative RT-PCR

1. Extract total RNA from the leaves using an RNeasy Mini kit.
2. Synthesize first-strand cDNA using SuperScript 3 reverse transcriptase.
3. PCR amplify a fragment corresponding to the C-terminus of Cas9 with Phusion Polymerase.
4. Perform RT-PCR under the following conditions: 98 °C for 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.
5. Confirm the presence of the Cas9 by electrophoresis.

3.5.2 Protein Confirmation by Western Blot

1. Extract total proteins from 100 µg leaf tissue and separate the proteins by SDS polyacrylamide gel electrophoresis.
2. Perform immunoblot analysis using primary mouse anti-FLAG antibody (1:1000) in 5% skim milk in Tris-buffered saline with Tween 20 (TBS-T) and secondary goat anti-mouse (1:1000; Santa Cruz) in 1% skim milk in TBS-T.
3. Detect reacting proteins based on chemiluminescence using ECL detection reagent.

3.6 TRV-Mediated Delivery of Cas9/sgRNA

The complete strategy for TRV-mediated genome editing is depicted in Fig. 1b and is described as follows.

1. Grow *N. benthamiana* Cas9-OE plants in soil in a greenhouse to the 6–8 leaf stage (2–3 weeks).
2. Introduce vectors containing the TRV RNA1 and recombinant TRV RNA2 genomes separately into *A. tumefaciens* strain GV3101 and spread the cells on LB agar plates containing kanamycin (50 mg/L), gentamicin (30 mg/L), and rifampicin (25 mg/L).
3. Grow the transformed single colonies overnight in selective medium to OD₆₀₀ of 1.2.
4. Collect *Agrobacterium* cells by centrifugation and resuspend in the infiltration medium to an OD₆₀₀ of 0.3.
5. Incubate the cultures at ambient temperature in the dark for 2–4 h.
6. Prior to infiltration, combine bacterial cultures (at OD₆₀₀ 0.1) harboring TRV-RNA1 and TRV-RNA2::PDS at a 1:1 ratio (for single targeting) or TRV-RNA1, TRV-RNA2::PDS, PCNA at a 1:1:1 ratio (for multiplexed targeting; see Note 5).
7. Infiltrate the bacterial culture mix into the abaxial sides of 3–4-week-old fully extended leaves of Cas9-OE *N. benthamiana* plants using a needleless 1 mL syringe.
8. Collect leaf disk samples from inoculated and systemic leaves at 5, 10, 15, and 30 days post-infiltration and subject them to targeted genome modification analysis (see Subheading 3.8)

to determine and quantify mutations induced by Cas9 (*see Note 6*).

3.7 Confirmation of TRV RNA1 and TRV RNA2 in Systemic Leaves by Semiquantitative RT-PCR

1. Extract total RNA from systemic leaves of interest using an RNeasy Mini kit.
2. Synthesize first-strand cDNA using SuperScript 3 reverse transcriptase.
3. To detect the presence of TRV RNA1, perform PCR with primers TRV1-Replicase-RT-F and R (Table 1) to amplify a fragment corresponding to part of the Replicase (RNA-dependent RNA polymerase) gene in TRV RNA1.
4. To detect the presence of recombinant TRV RNA2, perform PCR with primers TRV2-CP-RT-F and R (Table 1) to amplify a fragment corresponding to part of the Coat protein (CP) gene in TRV RNA2.
5. Perform RT-PCR using the following conditions: 98 °C for 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.
6. Confirm the presence of the TRV RNA1 and RNA2 by electrophoresis.

3.8 Mutation Detection by the T7EI Assay, Loss of Restriction Enzyme Recognition Site Assay, and Sanger Sequencing

3.8.1 Mutation Detection by the T7EI Assay and Sanger Sequencing

1. Extract genomic DNA from samples collected at 5, 10, and 30 days post-infiltration using DNA extraction buffer and PCI solution, followed by ethanol precipitation.
2. PCR amplify the fragment encompassing the target sequence using the appropriate primers (*see Table 1* for the PDS and PCNA primers used in this study) and High-Fidelity Phusion Polymerase (*see Note 7*).
1. In a total reaction volume of 20 μ L, denature and reanneal 200 ng PCR products in New England Biolabs Buffer 2 (1.8 μ L) in a thermal cycler to allow for hetero-duplex formation using the 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 on hold.
2. Treat the hybrid PCR products with T7EI by adding 0.5 μ L of T7EI and 0.2 μ L Buffer 2 and incubate at 37 °C (*see example results in Fig. 2a*).
3. Calculate the mutation rates using ImageJ software.
4. To validate the mutations detected by the T7EI assay, clone the PCR products into the TOPO TA cloning vector and subject the clones to Sanger sequencing.

3.8.2 Mutation Detection by Restriction Fragment Length Polymorphism (RFLP) Assay and Sanger Sequencing

The target sequence in the *PDS* gene contains an *NcoI* restriction enzyme recognition site that overlaps with the Cas9 cleavage site (about 3 bp ahead of the NGG PAM). Cas9 induced mutations are likely to destroy this *NcoI* recognition site, allowing for mutation detection based on an RFLP assay.

1. In a 20- μ L reaction volume, add *NcoI* to 300 ng of PCR product containing the region flanking the *PDS* target sequence.
2. To ensure complete digestion, the reaction should be performed for 8 h at 37 °C.
3. Confirm mutations based on uncut bands (*see* Fig. 2b).
4. Gel purify and clone the undigested PCR product into the TOPO TA cloning vector and confirm that mutations via Sanger sequencing (*see* Fig. 2c).

3.9 Screening Progeny Plants for Heritable Genome Modifications

1. Collect seed capsules (a total of 100–200 seeds) from plants successfully infected with recombinant TRV-RNA2 carrying the desired gRNA at various stages of plant growth (Fig. 1b).
2. Grow seeds in soil, 10 seeds in per pot, for 10–15 days.
3. Collect cotyledonary leaves from all 10 seedlings (per pot) as one pool.
4. From each seedling pool, extract genomic DNA as one pool. Follow the procedure in Subheading 3.8 for genomic DNA extraction.
5. PCR amplify the fragment encompassing the target sequence.
6. Detect mutations using the T7E1 assay (as described in Subheading 3.8.1) or RFLP assay (as described in Subheading 3.8.2).
7. Clone positive PCR products into the TOPO TA cloning vector and subject the clones to Sanger sequencing.
8. If mutations are detected, screen each plant from the positive pool separately to identify plants carrying the intended genomic modifications.
9. Remove any negative plant from the pot and allow the selected mutants to set seed (*see* Note 8).

4 Notes

1. We highly recommend checking the off-targeting activity of the designed sgRNA using an online tool such as CRISPR-P or CCTop [41, 42].
2. Alternatively, the sgRNA clones can be custom synthesized in the pUC19 (-MCS) plasmid using the 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 and an 84 bp Cas9 binding loop, followed by a tandem repeat sequence of seven thymines (T) as the transcriptional terminator. The 116 bp sgRNA fragment of each target can be subcloned into the TRV RNA2 vector under the control of the PEBV viral promoter by restriction ligation cloning using the restriction enzymes *Xba*I and *Xma*I.

3. In this protocol, we describe TRV-mediated genome editing in *N. benthamiana*, but any plant species where CRISPR/Cas9 and TRV has been experimentally demonstrated can be used.
4. This experiment is performed to investigate and confirm the subcellular localization of Cas9 in plants.
5. The OD₆₀₀ of the combined TRV1 and TRV2(n) cultures should not exceed 0.5, as higher concentrations might be toxic to the infiltrated leaves. For multiplexed editing on more than four targets, an OD₆₀₀ as low as 0.05 can be used efficiently.
6. Photograph the plants at each stage and compare the molecular data with phenotypic data.
7. Perform PCR using undigested genomic DNA or genomic DNA predigested with restriction enzymes to enrich for the modification of interest.
8. Seeds collected from late flowers typically show lower mutation frequencies than seeds from early flowers.

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References

1. Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157(6):1262–1278. <https://doi.org/10.1016/j.cell.2014.05.010>
2. Gaj T, Gersbach CA, Barbas CF 3rd (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 31(7):397–405. <https://doi.org/10.1016/j.tibtech.2013.04.004>
3. Pennisi E (2010) Sowing the seeds for the ideal crop. *Science* 327(5967):802–803. <https://doi.org/10.1126/science.327.5967.802>
4. Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* 32(4):347–355. <https://doi.org/10.1038/nbt.2842>
5. Voytas DF (2013) Plant genome engineering with sequence-specific nucleases. *Annu Rev Plant Biol* 64:327–350. <https://doi.org/10.1146/annurev-arplant-042811-105552>
6. Doudna JA, Charpentier E (2014) Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346(6213):1258096. <https://doi.org/10.1126/science.1258096>

7. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096):816–821. <https://doi.org/10.1126/science.1225829>
8. Jiang W, Yang B, Weeks DP (2014) Efficient CRISPR/Cas9-mediated gene editing in *Arabidopsis thaliana* and inheritance of modified genes in the T2 and T3 generations. *PLoS One* 9(6):e99225. <https://doi.org/10.1371/journal.pone.0099225>
9. Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat Biotechnol* 31(8):688–691. <https://doi.org/10.1038/nbt.2654>
10. Eid A, Ali Z, Mahfouz MM (2016) High efficiency of targeted mutagenesis in *Arabidopsis* via meiotic promoter-driven expression of Cas9 endonuclease. *Plant Cell Rep* 35(7):1555–1558. <https://doi.org/10.1007/s00299-016-2000-4>
11. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31(8):691–693. <https://doi.org/10.1038/nbt.2655>
12. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Res* 42(17):10903–10914. <https://doi.org/10.1093/nar/gku806>
13. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol* 31(8):686–688. <https://doi.org/10.1038/nbt.2650>
14. Upadhyay SK, Kumar J, Alok A, Tuli R (2013) RNA-guided genome editing for target gene mutations in wheat. *G3* 3(12):2233–2238. <https://doi.org/10.1534/g3.113.008847>
15. Shan Q, Wang Y, Li J, Gao C (2014) Genome editing in rice and wheat using the CRISPR/Cas system. *Nat Protoc* 9(10):2395–2410. <https://doi.org/10.1038/nprot.2014.157>
16. Svitashv S, Young JK, Schwartz C, Gao H, Falco SC, Cigan AM (2015) Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. *Plant Physiol* 169(2):931–945. <https://doi.org/10.1104/pp.15.00793>
17. Feng C, Yuan J, Wang R, Liu Y, Birchler JA, Han F (2016) Efficient targeted genome modification in maize using CRISPR/Cas9 system. *J Genet Genomics* 43(1):37–43. <https://doi.org/10.1016/j.jgg.2015.10.002>
18. Brooks C, Nekrasov V, Lippman ZB, Van Eck J (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiol* 166(3):1292–1297. <https://doi.org/10.1104/pp.114.247577>
19. Jia H, Wang N (2014) Targeted genome editing of sweet orange using Cas9/sgRNA. *PLoS One* 9(4):e93806. <https://doi.org/10.1371/journal.pone.0093806>
20. Aouida M, Eid A, Ali Z, Cradick T, Lee C, Deshmukh H, Atef A, AbuSamra D, Gadhoum SZ, Merzaban J, Bao G, Mahfouz M (2015) Efficient fdCas9 synthetic endonuclease with improved specificity for precise genome engineering. *PLoS One* 10(7):e0133373. <https://doi.org/10.1371/journal.pone.0133373>
21. Wang H, La Russa M, Qi LS (2016) CRISPR/Cas9 in genome editing and beyond. *Annu Rev Biochem* 85:227–264. <https://doi.org/10.1146/annurev-biochem-060815-014607>
22. Komor AC, Badran AH, Liu DR (2017) CRISPR-based technologies for the manipulation of eukaryotic genomes. *Cell* 168(1–2):20–36. <https://doi.org/10.1016/j.cell.2016.10.044>
23. Ali Z, Ali S, Tashkandi M, Zaidi SS, Mahfouz MM (2016) CRISPR/Cas9-mediated immunity to geminiviruses: differential interference and evasion. *Sci Rep* 6:26912. <https://doi.org/10.1038/srep26912>
24. Ali Z, Abulfaraj A, Idris A, Ali S, Tashkandi M, Mahfouz MM (2015) CRISPR/Cas9-mediated viral interference in plants. *Genome Biol* 16:238. <https://doi.org/10.1186/s13059-015-0799-6>
25. Baltés NJ (2015) Conferring resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. *Nat Plants* 1(10):15145
26. Ji X, Zhang H, Zhang Y, Wang Y, Gao C (2015) Establishing a CRISPR-Cas-like immune system conferring DNA virus resistance in plants. *Nat Plants* 1:15144. <https://doi.org/10.1038/nplants.2015.144>
27. Pyott DE, Sheehan E, Molnar A (2016) Engineering of CRISPR/Cas9-mediated potyvirus resistance in transgene-free *Arabidopsis* plants. *Mol Plant Pathol* 17(8):1276–1288. <https://doi.org/10.1111/mpp.12417>
28. Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M,

- Sherman A, Arazi T, Gal-On A (2016) Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Mol Plant Pathol* 17(7):1140–1153. <https://doi.org/10.1111/mpp.12375>
29. Zaidi SS, Mansoor S, Ali Z, Tashkandi M, Mahfouz MM (2016) Engineering plants for geminivirus resistance with CRISPR/Cas9 system. *Trends Plant Sci* 21(4):279–281. <https://doi.org/10.1016/j.tplants.2016.01.023>
 30. Zaidi SS, Tashkandi M, Mansoor S, Mahfouz MM (2016) Engineering plant immunity: using CRISPR/Cas9 to generate virus resistance. *Front Plant Sci* 7:1673. <https://doi.org/10.3389/fpls.2016.01673>
 31. Zaidi SS, Mansoor S (2017) Viral vectors for plant genome engineering. *Front Plant Sci* 8:539. <https://doi.org/10.3389/fpls.2017.00539>
 32. Macfarlane SA (2010) Tobravirus—plant pathogens and tools for biotechnology. *Mol Plant Pathol* 11(4):577–583. <https://doi.org/10.1111/j.1364-3703.2010.00617.x>
 33. Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. *Plant J* 31(6):777–786
 34. Senthil-Kumar M, Mysore KS (2014) Tobacco rattle virus-based virus-induced gene silencing in *Nicotiana benthamiana*. *Nat Protoc* 9(7):1549–1562. <https://doi.org/10.1038/nprot.2014.092>
 35. Senthil-Kumar M, Mysore KS (2011) Virus-induced gene silencing can persist for more than 2 years and also be transmitted to progeny seedlings in *Nicotiana benthamiana* and tomato. *Plant Biotechnol J* 9(7):797–806. <https://doi.org/10.1111/j.1467-7652.2011.00589.x>
 36. Lu R, Martin-Hernandez AM, Peart JR, Malcuit I, Baulcombe DC (2003) Virus-induced gene silencing in plants. *Methods* 30(4):296–303
 37. Ali Z, Abul-Faraj A, Piatek M, Mahfouz MM (2015) Activity and specificity of TRV-mediated gene editing in plants. *Plant Signal Behav* 10(10):e1044191. <https://doi.org/10.1080/15592324.2015.1044191>
 38. Ali Z, Abul-faraj A, Li L, Ghosh N, Piatek M, Mahjoub A, Aouida M, Piatek A, Baltes NJ, Voytas DF, Dinesh-Kumar S, Mahfouz MM (2015) Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. *Mol Plant* 8(8):1288–1291. <https://doi.org/10.1016/j.molp.2015.02.011>
 39. Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci* 7(5):193–195. [https://doi.org/10.1016/S1360-1385\(02\)02251-3](https://doi.org/10.1016/S1360-1385(02)02251-3)
 40. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339(6121):819–823. <https://doi.org/10.1126/science.1231143>
 41. Lei Y, Lu L, Liu HY, Li S, Xing F, Chen LL (2014) CRISPR-P: a web tool for synthetic single-guide RNA design of CRISPR-system in plants. *Mol Plant* 7:1494. <https://doi.org/10.1093/mp/ssu044>
 42. Stemmer M, Thumberger T, del Sol Keyer M, Wittbrodt J, Mateo JL (2015) CCTop: an intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. *PLoS One* 10(4):e0124633. <https://doi.org/10.1371/journal.pone.0124633>