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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.

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Key words

(separated by '-')

CRISPR/Cas9 - TRV - *Nicotiana benthamiana* - Genome editing - Targeted modification - Genome engineering - RNA viruses

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## 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.

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

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## 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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Ahmed Mahas and Zahir Ali contributed equally to this work

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.

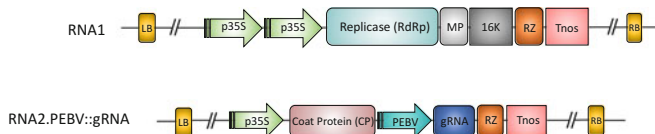
To achieve effective CRISPR/Cas9-mediated targeted genome modifications for crop trait discovery and improvement, Cas9 and its cognate gRNA must be efficiently expressed in the targeted cells, and the genome modification events must be heritable, as they should occur in the germline cells and allow for 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 81  
accomplished by generating transgenic lines that stably express 82  
Cas9 and gRNAs. This goal can be achieved by stably integrating 83  
Cas9 and gRNA cassettes into the plant genome via *Agrobacter-* 84  
*ium*-mediated transformation or using biolistic-based methods, 85  
which should result in the expression of the CRISPR/Cas9 com- 86  
ponents in all cells of the transgenic plant, including germline cells, 87  
thus allowing the targeted modifications to be transmitted to 88  
subsequent generations. While such delivery approaches can result 89  
in the efficient expression of genome engineering reagents and 90  
heritable genome modifications, the production of transgenic 91  
lines is expensive and time consuming, as tissue culture and 92  
repeated transformation are needed to produce every new, targeted 93  
modification. In addition, the use of these genetically modified 94  
plants can raise public concerns, as well as regulatory hurdles. 95

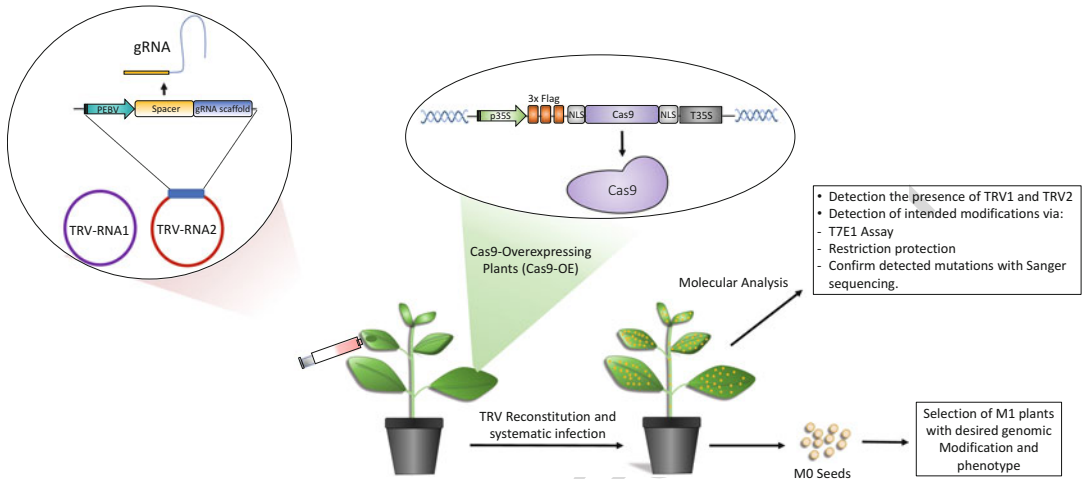
Autonomously replicating DNA and RNA virus-based vectors 96  
offer an alternative means for efficiently delivering genome engi- 97  
neering reagents into plant cells [31]. The advantage of RNA 98  
virus-based vectors versus their DNA viruses-based counterparts 99  
is that they do not integrate into the plant genome, thus avoiding 100  
unintended genome integration. Therefore, plants modified using 101  
RNA viruses are considered to be transgene-free edited plants. 102  
One such virus-based vector is *Tobacco rattle virus* (TRV), which 103  
is widely used for efficient virus-induced gene silencing (VIGS) in 104  
functional genomics studies in diverse plant species [32, 33]. TRV 105  
viruses have bipartite genomes comprising two positive-sense 106  
ssRNAs: TRV1 (RNA1) and TRV2 (RNA2). TRV1 is essential 107  
for virus replication and movement, and TRV2 harbors genes 108  
encoding nonstructural proteins that can be replaced by multiple 109  
cloning sites, into which different exogenous fragments of the 110  
target gene to be silenced can be inserted [34]. When the recom- 111  
binant TRV vector is introduced into plant cells via *Agrobacter-* 112  
*ium*-mediated infiltration, the virus expression system mediates 113  
the synthesis of the recombinant viral RNA inside the infected 114  
plant cells. These initially infected cells presumably serve as a 115  
source for further replication of viral RNAs and their systemic 116  
infection and spread into a variety of tissues and cells, such as 117  
developing and meristematic tissues, including germline cells 118  
[35, 36]. The small size of the TRV genome facilitates cloning, 119  
multiplexing, and agroinfection, pointing to its great potential for 120  
use as a vector for delivering genome engineering reagents. 121

We recently developed TRV as a vehicle for systemic delivery of 122  
gRNAs for targeted genome modification in *N. benthamiana* plants 123  
overexpressing *Cas9* [37, 38]. To construct this TRV-mediated 124  
genome editing system, we generated *Cas9*-overexpressing (*Cas9*- 125  
OE) *N. benthamiana* transgenic lines. We then generated a TRV 126  
RNA2 genome-derived vector for systemic gRNA delivery. In the 127  
TRV RNA2 vector, the gRNA was expressed under the control of 128

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), 129  
 permitting the expression of the gRNA from the virus expression 130  
 system. We then reconstituted the TRV virus in *N. benthamiana* 131  
 leaves via agroinfiltration of mixed *Agrobacterium* cultures harbor- 132  
 ing the RNA1 genome in combination with different RNA2 vec- 133  
 tors, in which gRNAs with binding specificity for a single target (the 134  
 phytoene desaturase [*PDS*] gene) or multiplex targets (*PCNA* and 135  
*PDS*) were driven by the PEBV promoter (pRNA2.PEBV::PDS/ 136  
*PCNA.gRNA*) (Fig. 1a). This TRV-mediated genome engineering 137  
 resulted in highly efficient targeted genome modification in both 138

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.

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## 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**

	<b>Primer name</b>	<b>Sequence (5'----3')</b>	<b>Use</b>
t.1	Cas9-gw-Fw	CACCAITGGACTATAAAGGACCACG	To clone <i>Cas9</i>
t.2	Cas9-R	TT ACTTTTTTCTTTTTTTTGCCTGGC	To clone <i>Cas9</i> and as a reverse primer for <i>Cas9</i> detection via RT-PCR
t.3	CAS9-SEQ-F6	GCCCTCCAAATATGTGACTTCC	Forward primer for <i>Cas9</i> detection via RT-PCR
t.4	Cas9-R-No-Stop	CTTTTTCTTTTTTTTGCCTGGC	To construct <i>Cas9-GFP</i>
t.5	NB-ACTINI-RT-F	TGAAGA TCCTCACAGAGCGTGG	RT-PCR normalization control
t.6	NB-ACTINI QRT-LIU-R	TTGT A TGTGGTCTCGTGA TTC	
t.7	TRV1-Replicase-RT-F	CT ACTGGGAGAGCAGCAACC	To detect systemic movement of TRV-RNA1
t.8	TRV1-Replicase-RT-R	CTGAGCGCAAAAAGT ACACCA	
t.9	TRV2-CP-RT-F	TTGGGTGGAATCAGTTTCGT	To detect systemic movement of TRV-RNA2
t.10	TRV2-CP-RT-R	TCTTCCAAAAGTCGAGCCAGT	
t.11	NB-PDS3-TR1,2-gDNA-F2	GAAACACATCACCTAGGCGG	For PCR of the region flanking the <i>PDS</i> target
t.12	NB-PDS3-TR1,2-gDNA-R	GGCGTGAGGAAAGT ACGAAA	
t.13	NBPDS3-gDNA-404 bp-F	GTAAAATGCCCCAAAATTGGACTTGT	To amplify the 404-bp target-flanking region around <i>PDS</i>
t.14	NBPDS3-gDNA-404 bp-R	CGTGAGGAAAGTACGAAAATGATGATGA	
t.15	NB_PCNA gDNA F1	CCTAACCCCTAATTTCCCCCAG	For PCR of the region flanking the <i>PCNA</i> target
t.16	NB_PCNA gDNA R1	TCACTGTCAATGTCCATCAG	



	9. Goat anti-mouse IgG-HRP secondary antibody (Santa Cruz).	181
	10. Enhanced Chemiluminescence (ECL)-detection reagent (Thermo Scientific).	182 183
	11. Skim milk powder, Tween 20, Sodium dodecyl sulfate (SDS), HEPES, Tris buffer, Glycerol, Agarose powder, Ethidium bromide, Lithium chloride, Bleach (Sodium hypochlorite).	184 185 186
	12. Polyacrylamide gels (Precise Tris-HEPES Gels, Thermo Scientific).	187 188
	13. TBS-T: Tris-buffered saline with Tween 20, pH 8.0.	189
	14. Infiltration medium: 10 mM MES, 10 mM CaCl <sub>2</sub> , 200 μM acetosyringone, pH 5.7.	190 191
	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.	192 193 194
	16. Root-inducing medium: 2.2 g/L MS salts with vitamins, 50 mg/L kanamycin.	195 196
	17. DNA extraction buffer: 100 mM Tris-Cl, 1 mM EDTA, 100 mM NaCl, 100 mM LiCl, 100 mM β-mercaptoethanol, 0.4% RNase.	197 198 199
	18. PCI solution: Phenol:chloroform:isoamyl alcohol.	200
	19. CDP-Star Chemiluminescent Substrate (Roche).	201
	20. PVDF membrane.	202 203
<b>2.2 Plasmids and Vectors</b>	1. <i>Tobacco rattle virus</i> (TRV) RNA1 (pYL192) and RNA2 (pYL156 modified with the PEBV promoter), provided by Savithramma Dinesh-Kumar, University of California at Davis.	204 205 206
	2. pK2GW7 binary vector, which is Gateway compatible [39].	207
	3. pX330 plasmid [40].	208
	4. pEarleyGate 103.	209
	5. pENTR/D-TOPO (Life Technologies).	210
	6. LR Clonase (Life Technologies, Invitrogen).	211
	7. TOPO TA cloning vector (Thermo Scientific).	212 213
<b>2.3 Kits</b>	1. RNeasy Mini kit.	214
	2. PCR purification.	215
	3. Plasmid Miniprep kit.	216 217
<b>2.4 Organisms</b>	1. <i>Nicotiana benthamiana</i> .	218
	2. <i>Agrobacterium tumefaciens</i> strain GV3101.	219 220
<b>2.5 Software and Programs</b>	1. CRISPR-P/CCTop [41, 42].	221
	2. ImageJ ( <a href="http://rsb.info.nih.gov/ij">http://rsb.info.nih.gov/ij</a> ).	222

### 3 Methods

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#### 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.	266 267 268 269
<b>3.3 Cloning the Cas9: GFP Reporter</b>	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].	270 271 272
	2. Clone the gel-purified PCR product into pENTR/D-TOPO.	273
	3. Transfer <i>Cas9</i> into the pEarleyGate 103 binary vector via the LR Clonase reaction to generate the <i>35S::Cas9:GFP-T35S</i> construct. Use this construct to transiently express the GFP-fusion variant in <i>N. benthamiana</i> leaves for subcellular localization analysis of Cas9 (see <b>Note 4</b> ).	274 275 276 277 278
	4. Subject the clones to restriction digestion and Sanger sequencing to confirm the authenticity of the clones and the in-frame translational fusions.	279 280 281 282
<b>3.4 Generation of <i>N. benthamiana</i> Plants Overexpressing Cas9 (Cas9-OE)</b>	1. Introduce the pK2GW7.Cas9 binary vector obtained in Subheading 3.2 into <i>Agrobacterium tumefaciens</i> strain GV3101 by electroporation.	283 284 285
	2. Grow single colonies carrying the T-DNA vector overnight at 28 °C in 5 mL <i>Agrobacterium</i> culturing medium.	286 287
	3. Inoculate the overnight culture in 50 mL of LB containing the selective antibiotics in 5 mM MES buffer.	288 289
	4. Grow the cells to OD <sub>600</sub> of 1.0, resuspend them in transformation medium to OD <sub>600</sub> of 0.4, and pour onto a Petri dish.	290 291
	5. Use a surgical blade to cut leaf tissues from aseptically grown 2-week-old <i>N. benthamiana</i> plants into small (approximately 1 cm) leaf disks. Make small incisions on the leaf surface with the scalpel to maximize contact of the <i>Agrobacteria</i> with the plant cells.	292 293 294 295 296
	6. Incubate leaf disks in <i>Agrobacterium</i> culture for 30 min with occasional shaking.	297 298
	7. Remove excess <i>Agrobacterium</i> cells with sterile filter paper and cocultivate the leaf disks in <i>Agrobacterium</i> on MS medium in a growth chamber at ~25 °C for 2 days.	299 300 301
	8. Transfer the leaf disks onto the regeneration and selection medium.	302 303
	9. Excise shoot tissues after 3–4 weeks and place them onto root-inducing medium.	304 305
	10. After proper roots have formed (2–3 weeks), acclimate the plantlets in soil under plastic domes.	306 307

**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. 308-310
2. Synthesize first-strand cDNA using SuperScript 3 reverse transcriptase. 311
3. PCR amplify a fragment corresponding to the C-terminus of Cas9 with Phusion Polymerase. 312-313
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. 314-316
5. Confirm the presence of the Cas9 by electrophoresis. 317-318

**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. 319-320
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. 321-324
3. Detect reacting proteins based on chemiluminescence using ECL detection reagent. 325-327

**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. 328-329

1. Grow *N. benthamiana* Cas9-OE plants in soil in a greenhouse to the 6–8 leaf stage (2–3 weeks). 330-331
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). 332-336
3. Grow the transformed single colonies overnight in selective medium to OD<sub>600</sub> of 1.2. 337-338
4. Collect *Agrobacterium* cells by centrifugation and resuspend in the infiltration medium to an OD<sub>600</sub> of 0.3. 339-340
5. Incubate the cultures at ambient temperature in the dark for 2–4 h. 341-342
6. Prior to infiltration, combine bacterial cultures (at OD<sub>600</sub> 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). 343-346
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. 347-349
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) 350-352

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.

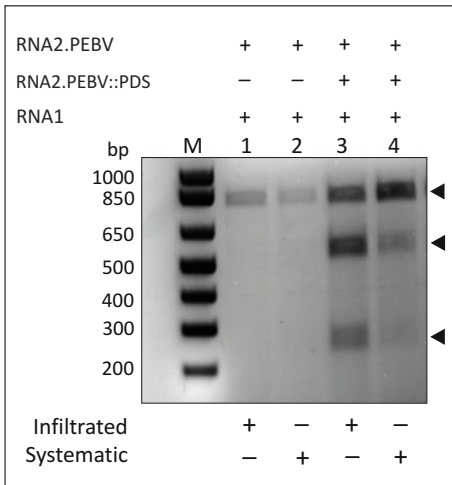
**3.8 Mutation Detection by the T7EI Assay, Loss of Restriction Enzyme Recognition Site Assay, and Sanger Sequencing**

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).

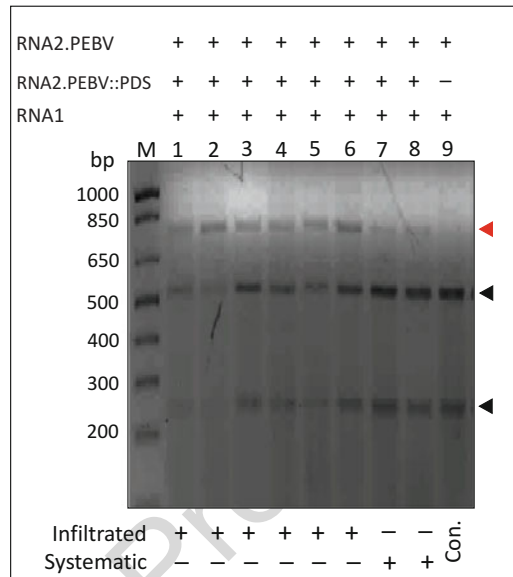
**3.8.1 Mutation Detection by the T7EI Assay and Sanger Sequencing**

1. In a total reaction volume of 20 µL, denature and reanneal 200 ng PCR products in New England Biolabs Buffer 2 (1.8 µL) 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 µL of T7EI and 0.2 µL 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.

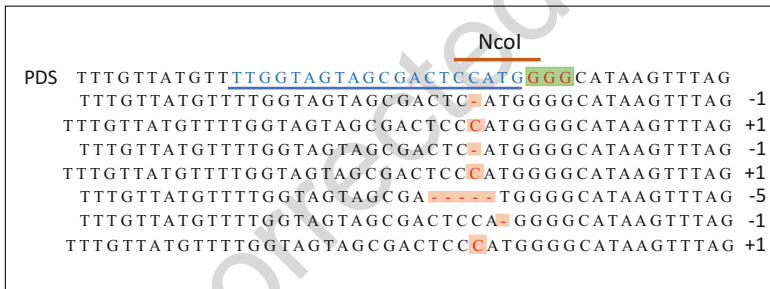
**A**



**B**



**C**



**Fig. 2** Analysis of TRV-mediated CRISPR/Cas9 targeted mutagenesis of *PDS3*. **(a)** T7EI-based mutation detection in systemic leaves. Mutagenesis was detected in inoculated (lane 3) and systemic (lane 4) leaves co-infiltrated with RNA1 and RNA2 carrying pPEBV::PDS.gRNA compared to the vector control (lanes 1 and 2). Arrowheads indicate the restriction digestion products. **(b)** RFLP-based mutation detection in systemic leaves. DNA was extracted from *Cas9*-OE plants and PCR was performed with a primer set to amplify a fragment encompassing the target site. Purified PCR product (300 ng) was treated with *NcoI* and separated on a 2% agarose gel. Inoculated plants clearly showed an *NcoI*-resistant (uncut) DNA fragment of 802 bp (red arrowhead), indicating the occurrence of targeted mutagenesis. Mutations were detected in inoculated leaves (lanes 1, 2, 3, 4, 5, and 6) and systemic leaves (lanes 7 and 8) in plants co-infiltrated with RNA1 and RNA2. PEBV::PDS-gRNA compared to the control (lane 9). Arrowheads indicate the restriction products. **(c)** Sanger sequencing of indels at the *PDS* target site from inoculated plants. **(b)** The wild-type (WT) sequence is shown at the top (the target sequence is shown in blue and underlined, the *NcoI* site is indicated by a line, and the protospacer-associated motif (PAM) is shown in red and highlighted in green). Different indel mutations are shown (“-” indicates deletion and “+” indicates insertion)

**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- $\mu$ 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).

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**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 OD<sub>600</sub> 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 OD<sub>600</sub> 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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# Author Queries

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Query Refs.	Details Required	Author's response
AU1	References [40] and [43] were identical and Reference [43] has been deleted. The subsequent references have been renumbered. Please check and confirm if appropriate.	

Uncorrected Proof