Heritable CRISPR-Cas9 editing of plant genomes using RNA virus vectors

Mireia Uranga[1,2], Verónica Aragonés[1], José-Antonio Daròs[1,*], Fabio Pasin[1,*]

[1] Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas – Universitat Politècnica de València, Avenida de los Naranjos s/n, 46022 Valencia, Spain

[2] Present address: VIB-UGent Center for Plant Systems Biology, Technologiepark-Zwijnaarde 71, 9052 Zwijnaarde (Gent), Belgium

*Correspondence: jadaros@ibmcp.upv.es, f.pasin@csic.es

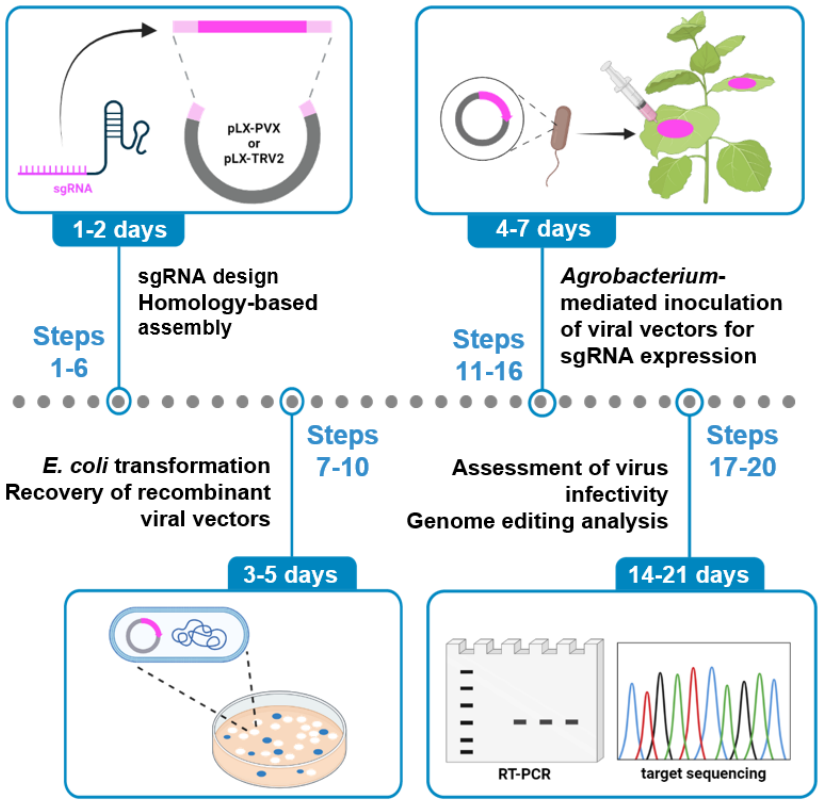
Summary

Viral vectors hold enormous potential for genome editing in plants as transient delivery vehicles of CRISPR-Cas components. Here, we describe a protocol to assemble plant viral vectors for single guide RNA (sgRNA) delivery. The obtained viral constructs are based on compact T-DNA binary vectors of the pLX series and are delivered into Cas9-expressing plants through agroinoculation. This approach allows rapidly assessing sgRNA design for plant genome targeting, as well as the recovery of progeny with heritable mutations at targeted loci.

For complete details on the use and execution of this protocol, please refer to Uranga et al. (2021) **and** Aragonés et al. (2022)**.**

24 ## Graphical abstract

25



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28 ## Highlights

29 - Mini binary vectors for potato virus X (PVX) and tobacco rattle virus (TRV) delivery
30 - Single guide RNA (sgRNA) design and one-step cloning into PVX and TRV vectors
31 - Viral vector expression of sgRNA constructs in Cas9 transgenic plants
32 - Genome editing of plant somatic cells and recovery of mutant progeny

Before you begin

Viral vectors are a promising alternative for the transient delivery of the CRISPR-Cas system in many biological systems including plants, following the so-called virus-induced genome editing (VIGE) approach (Uranga and Daròs, 2022). The systemic spread of the viral infection promotes an accumulation of the editing components within plant tissues. This leads to efficient and fast genome editing, thus providing an ideal screening tool to assess the effectivity and specificity of single guide RNA (sgRNA) design. Several plant RNA virus-based replicons have been successfully used for the delivery of sgRNAs in transgenic plants constitutively expressing Cas9 nuclease (Ali et al., 2015, 2018; Cody et al., 2017; Ellison et al., 2020; Hu et al., 2019; Jiang et al., 2019; Li et al., 2021; Luo et al., 2021; Mei et al., 2019). However, each viral vector has its own molecular biology properties and is restricted to a specific host range. Here, we describe the engineering of two novel viral vectors derived from potato virus X (PVX; genus *Potexvirus*) and tobacco rattle virus (TRV; genus *Tobravirus*) for the delivery of unspaced sgRNAs in the model species *Nicotiana benthamiana*. The obtained viral constructs are based on compact T-DNA binary vectors of the pLX series (Pasin et al., 2017), which have been successfully used for starting RNA and DNA virus infections by *Agrobacterium*-mediated inoculation (agroinoculation) (Pasin et al., 2018; Pasin, 2021). Recombinant viral replicons with sgRNA constructs are first delivered into Cas9-expressing plants via agroinoculation and following systemic spread they produce a high rate of heritable modifications in the progeny of infected plants.

Biosafety

Bacteria containing recombinant nucleic acid molecules need to be handled and disposed according to institutional regulations. Plant viruses pose potential agronomical and environmental risks; therefore, researchers must comply with governmental and institutional regulations for virus propagation in plants and working with plant infectious agents. Biosafety considerations and working practices for handling plant viruses and derived infectious clones were revised elsewhere (Brewer et al., 2018). Additional institutional regulations must be considered when using transgenic plant lines such as the Cas9-expressing *N. benthamiana* described here.

Cas9-expressing plants and growth conditions

Timing: 4-5 weeks

Start preparing the plant material four to five weeks in advance as follows:

1. Sow *N. benthamiana* Cas9 seeds in a well-watered soil mixture [1 part of inorganic substrate (e.g. perlite) and 2 parts of peat (OPM)] and let them germinate in a growth chamber set at 25°C and long-day conditions (16 h-light/8 h-dark).

CRITICAL: A variety of Cas nuclease effectors have been reported (Uranga and Daròs, 2022). This protocol has been validated using a *N. benthamiana* transgenic line that constitutively expresses a human codon-optimized version of *Streptococcus pyogenes* Cas9 fused to a nuclear localization signal (NLS) at the carboxyl terminus. The Cas9 gene is under the control of the cauliflower mosaic virus 35S promoter and *A. tumefaciens nopaline synthase* terminator (Bernabé-Orts et al., 2019).

2. Two weeks after sowing, transfer three seedlings per plastic pot filled with the same soil mixture and water them every 2 days to ensure that the soil is humid.
   CRITICAL: Avoiding water excess in the tray.
3. Four- to five-week-old plants (i.e. five-leaf stage) are ideal to be used for *Agrobacterium*-mediated inoculation of viral vectors.

CRITICAL: The quality and age of the plants are essential factors for observing homogeneous symptoms of systemic viral infection and achieving successful genome editing. Healthy plants are obtained only following good practices in a well-conditioned growth chamber, including a disease-free environment and careful watering.

Note: Plants can grow at different pace in different places, therefore following a leaf stage can be more appropriate than an age stage. For optimal agroinoculation conditions and time saving, ensure to coordinate the preparation of plants and *Agrobacterium* strains hosting the viral vectors.

Alternatives: The protocol uses viral vectors based on viruses naturally infecting species of the Solanaceae family, including *N. benthamiana*. Alternative plant species may be used depending on the known host range of the viral vectors and availability of transgenic lines expressing a Cas nuclease.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Viral vectors		
pLX-PVX	(Uranga et al., 2021)	n/a
pLX-TRV1	Addgene	180515
pLX-TRV2	Addgene	180516
Bacterial strains		
Electrocompetent *E. coli*	DH5α or any other laboratory common strain	n/a
A. tumefaciens C58C1 with disarmed pTi	(Aragonés et al., 2022)	n/a
Chemicals		
Kanamycin	Sigma-Aldrich	K1377
Gentamycin	Sigma-Aldrich	G1264
Rifampicin	Sigma-Aldrich	R3501
5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (X-Gal)	Thermo Fisher Scientific	B1690
2-(N-Morpholino)ethanesulfonic acid hydrate (MES)	Sigma-Aldrich	M8250
Magnesium chloride ($MgCl_2$)	PanReac-AppliChem	131396.1210

3´,5´-Dimethoxy-4-hydroxyacetophenon (Acetosyringone)	Sigma-Aldrich	D134406
Guanidinium thiocyanate	PanReac-AppliChem	A1499,0250
Sodium acetate (NaAc)	PanReac-AppliChem	A131632.1211
Tris(hydroxymethyl)aminomethane (Tris base)	PanReac-AppliChem	A1086,1000
Acetic acid	PanReac-AppliChem	131008.1214
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma-Aldrich	E5134
β-Mercaptoethanol	PanReac-AppliChem	A4338,0100
Sodium chloride (NaCl)	PanReac-AppliChem	131659.1211
Tryptone	Condalab	1612
Yeast Extract	Condalab	1702
Bacteriological Agar	Condalab	1800
Agarose D1 Medium EEO	Condalab	8019
Liquid nitrogen	n/a	n/a
Critical commercial assays		
NEBuilder® HiFi DNA Assembly Master Mix	New England Biolabs	E2621S
Phusion™ High-fidelity DNA polymerase	Thermo Fisher Scientific	F530S
MluI (10 U/µL)	Thermo Fisher Scientific	ER0561
BsaIHF®v2 (20 U/µL)	New England Biolabs	R3733S
DNA Clean & Concentrator™-5	Zymo Research	D4004
NucleoSpin® Plasmid DNA purification	Macherey-Nagel	740588.250
RiboLock RNase Inhibitor (40 U/µL)	Thermo Fisher Scientific	EO0381
RevertAid™ Reverse Transcriptase	Thermo Fisher Scientific	EP0441
Thermus thermophilus (Tth) DNA polymerase	Biotools	10.003
Experimental models: Organisms/strains		
Nicotiana benthamiana Cas9	(Bernabé-Orts et al., 2019)	n/a
Oligonucleotides		
D2409 ATTTATATTATTCATACAATCAAACC	(Uranga et al., 2021)	n/a
D2410 TGGTGGTGGTAGAGTGACAAC	(Uranga et al., 2021)	n/a
D3436 ATGTCAGGCCTGTTCACTATCC	(Uranga et al., 2021)	n/a
D4337 GAATATGGTATCACCCACCCTC	(Aragonés et al., 2022)	n/a
D4338 ATGGGAGATATGTACGATGAAT	(Aragonés et al., 2022)	n/a
D4339 GGGATTAGGACGTATCGGACC	(Aragonés et al., 2022)	n/a
D4444 GAACCAGAATATTGAAAAAAC	(Aragonés et al., 2022)	n/a
D4445 CTCCTAATCTAATCAGTTGGG	(Aragonés et al., 2022)	n/a
Software and algorithms		
Nicotiana benthamiana draft genome sequence v1.0.1	SolGenomics Network	https://solgenomics.net/organism/Nicotiana_benthamiana/genome

CRISPOR	(Concordet and Haeussler, 2018)	http://crispor.tefor.net
NEBuilder Assembly Tool	New England Biolabs	https://nebuilder.neb.com
NEBiocalculator Tool	New England Biolabs	https://nebiocalculator.neb.com
Inference of CRISPR Edits (ICE)	Synthego	https://ice.synthego.com
Other		
Materials and equipment for molecular biology (tabletop centrifuges, dry block heaters, horizontal electrophoresis system, nucleic acid staining dyes, blue light transilluminator, water purification system, etc.)	n/a	n/a
Materials and equipment for bacterial handling and culturing (petri dishes, tubes, autoclave, microbiological cabinet, static and shaking incubators, etc.)	n/a	n/a
Materials and equipment for plant maintenance (soil substrate, pots, greenhouse or plant growth chamber, etc.)	n/a	n/a

n/a, not applicable

Materials

Preparation of stock solutions and media

Timing: 1-2 days

Unless otherwise indicated, standard molecular cloning methods and reagents are used (Sambrook and Russel, 2001).

- Kanamycin (50 mg/mL): Dissolve 0.5 g kanamycin powder in 10 mL MilliQ water, sterilize through a 0.2 µm filter, and store 1-mL aliquots at -20°C, for up to 1 year.
- Gentamycin (20 mg/mL): Dissolve 0.2 g gentamycin powder in 10 mL MilliQ water, sterilize through a 0.2 µm filter, and store 1-mL aliquots at -20°C, for up to 1 year.
- Rifampicin (50 mg/mL): Dissolve 0.5 g rifampicin powder in 10 mL DMSO, and store 1-mL aliquots at -20°C in the dark, for up to 6 months.
- X-Gal (40 mg/mL): Dissolve 0.4 g X-Gal powder in 10 mL N, N-dimethylformamide, and store 1-mL aliquots at -20°C in the dark, for up to 6 months.
- MES pH 5.5 (1 M): Dissolve 9.762 g MES powder in 40 mL MilliQ water, adjust pH to 5.5 with 1 N KOH, take the volume up to 50 mL with MilliQ water, and sterilize through a 0.2 µm filter.
- $MgCl_2$ (2 M): Dissolve 9.52 g MgCl2 in MilliQ water to a final volume of 50 mL, and sterilize through a 0.2 µm filter.

- Acetosyringone (0.1 M): Dissolve 98 mg acetosyringone powder in 5 mL DMSO, and store in single-use 150-µL aliquots at -20°C in the dark, for up to 6 months.
- EDTA (0.5 M): Mix 93.05 g EDTA and 0.4 L distilled water on a magnetic stirrer, adjust pH to 8.0 with ~10 g NaOH pellets (EDTA will not go into solution until the solution reach pH~8.0), take the volume up to 0.5 L with distilled water and autoclave
- Oligonucleotides: Custom DNA oligonucleotides are purchased desalted after synthesis as 100 µM stock solutions. Stocks are diluted in autoclaved MilliQ water to have 10 µM aliquots for PCR, and 2 µM aliquots for cDNA synthesis.
- Media:

Lysogenic broth (LB)

Reagent	Final concentration	Amount
Tryptone	1.0%	10 g
Yeast Extract	0.5%	5 g
NaCl	1.0%	10 g
MilliQ water		Up to 1 L

Note: Sterilize by autoclaving, and store at room temperature. Aliquot and supplement antibiotics as needed before use; handle in a microbiological cabinet to prevent contaminations.

LB agar

Reagent	Final concentration	Amount
Tryptone	1.0%	10 g
Yeast Extract	0.5%	5 g
NaCl	1.0%	10 g
Bacteriological agar	1.5%	15 g
MilliQ water		Up to 1 L

Note: Sterilize by autoclaving, cool the medium down to ~50–60°C and, in a microbiological cabinet, supplement antibiotics and mix vigorously. Aliquot into petri dishes, and once solidified store at 4°C in the dark.

Note: X-Gal solution can be spread onto LB agar plates before use.

Preparation of buffers

Timing: 1-2 days

Tris-acetate-EDTA (TAE) Buffer (50X)

Reagent	Final concentration	Amount
Tris-base	2 M	242 g

EDTA pH 8 (0.5 M)	50 mM	100 mL
Acetic acid (100%)	1 M	57.1 mL
MilliQ water		Up to 1 L

144

145 **Infiltration Buffer**

Reagent	Final concentration	Amount
MES-NaOH pH 5.5 (1 M)	10 mM	2.5 mL
$MgCl_2$ (2 M)	10 mM	1.25 mL
Acetosyringone (100 mM)	150 µM	0.375 mL
MilliQ water		Up to 250 mL

146 ***Note:*** Adjust Infiltration Buffer to pH 5.6.

147 ***Note:*** Always add acetosyringone to Infiltration Buffer before use.

148

149 **Extraction Buffer**

Reagent	Final concentration	Amount
Guanidinium thiocyanate	4 M	47.3 g
NaAcO pH 5.5 (3 M)	0.1 M	3.33 mL
EDTA pH 8.0 (0.5 M)	10 mM	2 mL
β-Mercaptoethanol	0.1 M	0.7 mL
MilliQ water		Up to 100 mL

150 ***Note:*** Freshly add β-Mercaptoethanol to Extraction Buffer before use.

151

152 **Wash Buffer**

Reagent	Final concentration	Amount
Ethanol (96%)	70%	73 mL
NaAc pH 5.5 (3 M)	10 mM	0.33 mL
MilliQ water		Up to 100 mL

153

154 **Elution Buffer**

Reagent	Final concentration	Amount
Tris-HCl pH 8.5 (0.1 M)	20 mM	0.2 mL
MilliQ water		Up to 1 mL

155

Step-by-step method details

Homology-based assembly of recombinant viral vectors

Timing: 3-5 days

This section described the use of homology-based assembly to generate full-length clones of potato virus X (PVX; genus *Potexvirus*) and tobacco rattle virus (TRV; genus *Tobravirus*) expressing target-specific sgRNAs. Plasmids for recombinant viral vectors are built by standard molecular biology techniques, as described in Uranga et al. (2021) and Aragonés et al. (2022).

Note: Simultaneous disruption of *Phytoene desaturase 3* (*PDS*) homeologs present in the genome of the allotetraploid *N. benthamiana* results in cells with a photobleaching phenotype typical of carotenoid biosynthetic defects (Ellison et al., 2020; Komatsu et al., 2020; Ma et al., 2020; Liu et al., 2022). *PDS* is used in the described procedures as the target gene to facilitate visual inspection of CRISPR-Cas9-mediated genome editing in somatic cells and progeny.

Note: NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, cat. number E2611S) is used in this protocol for homology-based cloning. Gibson assembly can also be used (Gibson et al., 2009); the required enzymatic mix can be in-house prepared as detailed (Zhao et al., 2020), or purchased from commercial providers (e.g. Gibson Assembly® Master Mix, New England Biolabs, cat. number E2611S). If using Gibson assembly, no protocol changes are required and steps 1–10 should be followed as detailed herein.

1. sgRNA design:
    a. Download the cDNA sequence of the target *N. benthamiana* gene from SolGenomics Network (https://solgenomics.net/organism/Nicotiana_benthamiana/genome/).
    Note: Download Niben101Scf01283g02002.1 and Niben101Scf14708g00023.1 sequences for targeting of *PDS* homeologs
    b. Paste the cDNA sequence into CRISPOR bioinformatics software (http://crispor.tefor.net/)(Concordet and Haeussler, 2018) and set the following parameters:
        i. *N. benthamiana* – SolGenomics.net V1.01 as the genome
        ii. 20 bp-NGG – SpCas9, SpCas9-HF1, eSpCas9 1.1 as the Protospacer Adjacent Motif (PAM)
    c. Select one or two protospacers showing a predicted efficiency of ≥ 45 (i.e. Doench '16 and Mor.-Mateos scores).
    CRITICAL: Avoid protospacers that target splicing junctions and overlap with two different exons.

CRITICAL: *N. benthamiana* genome is allotetraploid and loss-of-function mutations of two homeologs may be required to obtain a mutant phenotype.

Note: Select the protospacer TTGGTAGTAGCGACTCCATG conserved in Niben101Scf01283g02002.1 and Niben101Scf14708g00023.1 for simultaneous targeting and disruption of two *PDS* homeologs.

d. To obtain the full sgRNA, append downstream the 20-bp protospacer the following sequence corresponding to the conserved *S. pyogenes* Cas9 binding scaffold: TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

e. To create the fused sgRNA-tFT sequence, append downstream the sgRNA the first 102-bp of the *Arabidopsis thaliana Flowering locus T* (i.e. truncated FT, tFT; TAIR ID: AT1G65480) coding region.

Note: For details on full and truncated *A. thaliana* FT sequences, please refer to Ellison et al. (2020).

Note: The designed sgRNA-tFT sequence for *PDS* targeting consists of the gene-specific protospacer (in bold), Cas9 scaffold (underlined) and tFT (italicized) as follows:

TTGGTAGTAGCGACTCCATG<u>TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC</u>*ATGTCTATAAATATAAGAGACCCTCTTATAGTAAGCAGAGTTGTTGGAGACGTTCTTGATCCGTTTAATAGATCAATCACTCTAAAGGTTACTTATGGCCAA*

f. Flank the obtained sgRNA-tFT sequence with cloning adapters suitable for homology-based assembly.

Note: To assemble sgRNA-tFT into pLX-PVX, append the sequence GAGGTCAGCACCAGCTAGCA to the 5' terminus, and TAGGGTTTGTTAAGTTTCCC to the 3' terminus of the sequence from the previous step.

Note: To assemble sgRNA-tFT into pLX-TRV2, append the sequence CACTTACCCGAGTTAACGCC to the 5' terminus, and TGTCCCGAAGACATTAAAC to the 3' terminus of the sequence from the previous step.

Note: Sequences of the fragments sgPDS3-PVX and sgPDS3-TRV2 for homology-based cloning of a sgRNA-tFT that targets *PDS* into pLX-PVX or pLX-TRV2, respectively, are as follows:

Fragment	**Sequence***
sgPDS3-PVX	gaggtcagcaccagctagca**TTGGTAGTAGCGACTCCATG**TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC*ATGTCTATAAATATAAGAGACCCTCTTATAGTAAGCAGAGTTGTTGGAGACGTTCTTGATCCGTTTAATAGATCAATCACTCTAAAGGTTACTTATGGCCAA*tagggtttgttaagtttccc
sgPDS3-TRV2	cacttacccgagttaacgcc**TTGGTAGTAGCGACTCCATG**TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC*ATGTCTATAA*

	*ATATAAGAGACCCTCTTATAGTAAGCAGAGTTGTTGGAGACGTTCTTGATCCGTTTAATAG ATCAATCACTCTAAAGGTTACTTATGGCCAA*tgtcccgaagacattaaac

* Cloning adapters for homology-based assembly are shown in lowercase, sgRNA-tFT sequence for PDS targeting in uppercase

225
226 2. *In silico* assembly of recombinant virus clones.
227 ***Note***: NEBuilder Assembly Tool (https://nebuilder.neb.com/) and Benchling bioinformatics tool
228 (https://benchling.com/) can be used for the assembly simulation.
229 a. For sgRNA-tFT assembly into pLX-PVX
230 i. Retrieve the pLX-PVX sequence (see Supporting Information)
231 ii. Perform an *in silico* restriction digest with MluI to linearize pLX-PVX
232 ***Note:*** Digestion results in a 9.9-kb linearized fragment without the *lacZ* region.
233 iii. Simulate the homology-based assembly reaction including the linearized
234 vector and the linear sgRNA-tFT fragment designed in step 1f.
235 ***Note:*** sgPDS3-PVX is used for *PDS* targeting
236 b. For sgRNA-tFT assembly into pLX-TRV2
237 i. Retrieve the pLX-TRV2 sequence (GenBank: OM372496)
238 ii. Perform an *in silico* restriction digest with BsaI-HF to linearize pLX-TRV2
239 ***Note:*** Digestion results in a 5.8-kb linearized fragment without the lacZ region.
240 iii. Simulate the homology-based assembly reaction including the linearized
241 vector and the linear sgRNA-tFT fragment designed in step 1f.
242 ***Note:*** sgPDS3-TRV2 is used for *PDS* targeting
243
244 3. Chemically synthesize a linear double-stranded DNA fragment corresponding to the sgRNA-tFT
245 sequence designed in step 1f and resuspend it in MilliQ water as per manufacturer's
246 instructions.
247 ***Note:*** Chemically synthesized fragments are purchased as gBlocks™ Gene Fragment (Integrated
248 DNA Technologies).
249 ***Alternatives:*** Custom DNA fragments from alternative commercial providers may be used (e.g.
250 GeneArt™ Strings DNA Fragments, Thermo Fisher Scientific; Twist Gene Fragments without
251 adapters, Twist Bioscience; etc.).
252 ***Pause point:*** Resuspended DNA fragments can be frozen and stored at -20°C several days
253 before proceeding with the next step.
254
255
256 4. pLX vector backbone preparation:
257 a. Linearize the corresponding binary vectors by restriction digest in a final volume of 20
258 µL and incubation at 37°C (1 h):

259 i. For pLX-PVX linearization, add 0.5 µL 10 U MluI (Thermo Fisher Scientific) in 1x
260 Buffer R™.
261 ii. For pLX-TRV2 linearization, add 0.5 µL 20 U BsaI (BsaIHF®v2, New England
262 Biolabs) in 1x rCutSmart™ Buffer.
263 ***Pause point:*** Reaction can be frozen and stored at -20°C several days before proceeding
264 with the next step.
265 b. Prepare agarose gel in 1× TAE buffer and run electrophoresis of digestion reactions.
266 ***Note:*** Agarose D1 Medium EEO is used for gel preparation; 0.5-1% agarose gels provide
267 good electrophoresis resolution of the large (5-10 kb) DNA fragments.
268 c. Cut bands on a blue light transilluminator using single-use disposable scalpels.
269 ***Note:*** If a blue light transilluminator is not available a UV light transilluminator can be
270 used but ensure to avoid prolonged exposure to UV light as it damages DNA samples
271 and dramatically reduces assembly efficiency. If UV light transilluminator is used, wear
272 suitable protection personal protective equipment to avoid UV-associated hazards.
273 ***Pause point:*** Harvested gel bands can be frozen and stored at -20°C several days before
274 proceeding with the next step.
275 d. Purify DNA from gel bands using DNA Clean & Concentrator™-5 (Zymo Research) kit
276 according to the manufacturer's instructions:
277 i. Weight the gel bands, add 2-5 volumes of Agarose Dissolving Buffer to each
278 volume of DNA sample and incubate at 50°C (5-10 min)
279 ii. Transfer mixture to a Zymo-Spin™ column and centrifuge to discard the flow-
280 through
281 iii. Wash columns twice with Wash Buffer
282 iv. Centrifuge columns again to remove the residual liquid, add 10 µL MilliQ water
283 and incubate at room temperature for 1 min prior to centrifugation for final
284 elution
285 ***Note:*** Other commercial gel purification kits can also be used.
286 ***Pause point:*** Gel-purified DNA can be frozen and stored at -20°C several days before
287 proceeding with the next step.
288
289 5. Check 1 µL of the synthetic DNA fragment from step 3 and the gel-purified linearized vector
290 from step 4 by agarose gel electrophoresis alongside DNA standards and estimate DNA
291 fragment concentration.
292 ***Note:*** Spectrophotometer measurements may also be used for quality check and
293 quantification of DNA.
294
295 6. *In vitro* homology-based assembly:
296 a. Prepare a reaction mix including equimolar amounts of the gel-purified linearized
297 vector from step 4, a synthetic DNA fragment from step 3 and NEBuilder® HiFi DNA

298 Assembly Master Mix according to manufacturer's instructions, except that a final
299 reaction volume of 3–5 µL is used.
300 ***Note:*** The NEBiocalculator® Tool (https://nebiocalculator.neb.com/) can be used for
301 molarity calculations.
302 b. Incubate the mix at 50°C (1h). Once the reaction ends, place tubes on ice.
303 c. Purify the assembly reaction using DNA Clean & Concentrator™-5 (Zymo Research) kit
304 according to the manufacturer's instructions.
305 i. Add 100 µL of DNA Binding Buffer, transfer mixture to a Zymo-Spin™ column
306 and centrifuge to discard the flow-through
307 ii. Wash columns twice with Wash Buffer
308 iii. Centrifuge columns again to remove the residual liquid, add 10 µL MilliQ water
309 and incubate at room temperature for 1 min prior to centrifugation for final
310 elution.
311
312 7. Transformation of *E. coli* cells:
313 a. Thaw a 40-µL aliquot of electrocompetent cells on ice.
314 b. Mix gently by pipetting up and down the cell aliquot with the purified assembly reaction
315 from step 6 and transfer the mixture to a pre-chilled electroporation cuvette with 1
316 mm gap.
317 c. Electroporate cells (1500 V) and immediately after add 1 mL of room-temperature SOC
318 media to the cuvette and transfer the cells to a 1.5-mL tube.
319 ***Note:*** Once DNA is added to cells electroporation can be carried out immediately,
320 without any incubation.
321 d. Incubate the cells at 37°C, shaking vigorously at 200-250 rpm (1 h).
322 ***Note:*** Warm selection plates at 37°C while cells are recovering.
323 e. Pellet bacteria by centrifuging at 15 000 x g for 1 min, resuspend and plate 100 µL of
324 the bacterial suspension onto LB agar plates supplemented with 50 mg/L kanamycin
325 and 40 µL X-Gal (40 mg/mL). Incubate at 37°C (overnight).
326 ***Note:*** Plate the X-Gal solution on agar plates before use for white-blue screen of
327 recombinant clones.
328 ***Note:*** Bacterial cells harboring binary vectors with full-length virus clones generally
329 display low growth rates; extended plate incubation time may be necessary.
330 ***Pause point:*** After colony appearance, plates can be stored 1-3 days at 4°C before
331 proceeding with the next step.
332
333 8. Confirm the presence of recombinant binary vectors in *E. coli* transformants:
334 a. Pick a white colony obtained by X-Gal selection and inoculate 5-10 mL liquid LB with 50
335 mg/L kanamycin in 50 mL tubes. Incubate the tubes at 37°C, shaking vigorously at 200-
336 250 rpm (overnight).

337 ***Note:*** Bacterial cells harboring binary vectors with full-length virus clones generally
338 display low growth rates; extended culturing time may be necessary.
339 ***Note:*** It is recommended to purify DNA plasmid from 2–4 colonies per construct.
340 b. Harvest bacteria from the culture by centrifugation at 15 000 x g for 2 min; discard the
341 medium, centrifuge again for 30 sec and remove medium residues by pipetting.
342 ***Note:*** Harvested bacterial pellets can be frozen and stored at -80°C several days before
343 proceeding with the next step.
344 c. Purify DNA plasmids from the harvested bacterial pellets using NucleoSpin® Plasmid
345 (Macherey Nagel) Minipreps DNA Purification System follow kit manufacturer's
346 instructions, except that:
347 i. Use double volumes of resuspension, lysis and neutralization solutions supplied
348 within the kit to improve clearing of bacterial lysates and increase the amount of
349 purified plasmid.
350 ***Note:*** After addition of the lysis solution incubate until the cell suspension clears
351 (5 min).
352 ii. Transfer ~800 µL of cleared lysate to the column, centrifuge, discard the
353 flowthrough and load again the same column with the remainder of the lysate,
354 ***Note:*** The volume of the cleared bacterial lysate will exceed the spin column
355 capacity.
356 ***Note:*** Other commercial DNA plasmid purification kits can also be used.
357 ***Pause point:*** Purified DNA plasmids can be frozen and stored at -20°C indefinitely
358 before proceeding with the next step.
359
360 9. Perform restriction enzyme digestion of purified DNA plasmids to identify the recombinant viral
361 vectors.
362 ***Note:*** A double enzyme digestion mix including NheI and BsaI in 1× Tango yellow buffer
363 (Thermo Fisher Scientific) are used for pLX-PVX verification.
364 ***Note:*** A double enzyme digestion mix including EcoRI and BsaI in 2× Tango Yellow buffer
365 (Thermo Fisher Scientific) are used for pLX-TRV2 verification.
366 ***Pause point:*** DNA plasmids with the correct digestion profiles can be frozen and stored at -20°C
367 indefinitely before proceeding with the next step.
368
369 10. Confirm the sequence of recombinant binary vectors by submitting clones with the expected
370 digestion profiles to Sanger sequencing.
371 ***Note:*** It is recommended to check the sequences of 2 clones per construct.
372
373 ## *Agrobacterium*-mediated inoculation of plants
374
375 **Timing: 4-7 days**

In this section, binary vectors with the correct sequence confirmed by sequencing are transformed in electrocompetent *Agrobacterium* C58C1 cells including a disarmed pTi. Selected *A. tumefaciens* cells harboring the binary vectors are then cultured, prepared for inoculation as described (Bedoya et al., 2010; Uranga et al., 2021a) and used to inoculate *N. benthamiana* Cas9 plants.

CRITICAL: Ensure to coordinate preparation of *N. benthamiana* plants and *A. tumefaciens* strains hosting the viral vectors.

11. Transformation of *A. tumefaciens* cells:
    a. Thaw aliquots of electrocompetent *Agrobacterium* C58C1 cells including a disarmed pTi on ice.

       Alternatives: This protocol uses binary T-DNA vectors of the pLX series, which have been tested in a variety of *Agrobacterium* strains (Pasin et al., 2018). Strain alternatives include C58C1-313, EHA105, AGL1, CryX (Pasin et al., 2017; Roemer et al., 2013; de Saeger et al., 2021), as well as any *Agrobacterium* strain capable of plant cell transformation and sensitive to kanamycin and gentamicin, the antibiotic used for pLX-PVX, pLX-TRV1 and pLX-TRV2 selection.

       CRITICAL: GV3101::pMP90 is resistant to gentamicin and cannot be used with the JoinTRV system.
    b. Mix gently by pipetting up and down the cell aliquot with ~0.5 µg DNA plasmid and transfer the mixture to a pre-chilled electroporation cuvette with 1 mm gap.

       Note: It is recommended to independently transform *A. tumefaciens* with DNA plasmids obtained from two *E. coli* colonies per construct.
    c. Electroporate cells (1500 V) and immediately after add 1 mL of room-temperature SOC media to the cuvette and transfer the cells to a 1.5-mL tube.

       Note: Once DNA is added to cells electroporation can be carried out immediately, without any incubation.
    d. Incubate the cells at 28ºC, shaking vigorously at 200-250 rpm (2-4 h).

       Note: Warm selection plates at 28ºC while cells are recovering.
    e. Pellet bacteria by centrifuging at 15 000 x g for 1 min, resuspend and plate 100 µL of the bacterial suspension onto LB agar plates supplemented with 50 mg/L rifampicin plus antibiotic(s) for vector selection. Incubate at 28°C (48-72 h).

       CRITICAL: Supplement LB agar plates with 50 mg/L kanamycin for pLX-PVX selection.

       CRITICAL: For obtaining an infective TRV viral vector, *A. tumefaciens* need to be first electroporated with pLX-TRV1 and selected on plates supplemented with 20 mg/L gentamycin and 50 mg/L rifampicin. Competent cells of the recovered strain C58C1 (pLX-TRV1) are then electroporated with pLX-TRV2, and transformants simultaneously hosting the two vectors of TRV genomic components are selected on plates supplemented with 20 mg/L gentamycin, 50 mg/L kanamycin and 50 mg/L rifampicin.

415 ***Note:*** Bacterial cells harboring binary vectors with viral vectors may display low growth
416 rates; extended plate incubation time may be necessary.
417 ***Pause point:*** After colony appearance, plates can be stored 1-3 days at 4°C before
418 proceeding with the next step.
419
420 12. Pick individual colonies harboring the corresponding binary vectors and use them to inoculate
421 3 mL liquid LB with 50 mg/L kanamycin and 50 mg/L rifampicin (for pLX-PVX) or with 20 mg/L
422 gentamycin, 50 mg/L kanamycin and 50 mg/L rifampicin (for pLX-TRV1/pLX-TRV2) in 12 mL
423 tubes. Incubate tubes at 28°C, shaking at 200-250 rpm (24-48 h).
424 ***CRITICAL:*** *A. tumefaciens* cells harboring viral vector plasmids may display low growth rates. If
425 a mixture of small and large colonies appears during agar plate selection of transformed
426 bacteria pick small colonies for subsequent analysis.
427
428 13. Use 0.7-mL culture aliquots from the previous step to prepare 20% glycerol bacterial stocks and
429 store them at -80°C indefinitely.
430
431 14. Use 0.1-mL culture aliquots from step 12 to inoculate 10 mL liquid LB with (for pLX-PVX) or with
432 20 mg/L gentamycin and 50 mg/L kanamycin (for pLX-TRV1/pLX-TRV2) in 50 mL tubes. Incubate
433 tubes at 28°C, shaking at 200-250 rpm (overnight).
434
435 15. Preparation of bacterial suspensions for inoculation:
436 a. Measure in a spectrophotometer the optical density at 600 nm (OD_{600}) of the bacterial
437 suspensions until it reaches an OD_{600} of 1-2.
438 b. Pellet bacteria by centrifugation at 7200 × g for 5 min, discard the medium, centrifuge
439 again for 30 sec and remove medium residues by pipetting.
440 c. Resuspend the cell pellet to an OD_{600} of 0.5 in 4 mL Infiltration Buffer.
441 d. Incubate at 28°C in the dark (2 h) for induction of bacterial genes required for T-DNA
442 delivery to plant cells.
443
444 16. Plant inoculation:
445 a. Water plants before starting with the inoculation to ensure they are well hydrated and
446 induce stomata opening.
447 ***Note:*** Organize and label plants before starting the inoculation.
448 ***Note:*** Include mock-inoculated and wild-type plants as controls.
449 b. Inoculate two leaves per plant on the abaxial side using a 1-mL needleless syringe.
450 ***CRITICAL:*** Make sure not to press the needleless syringe so hard as to pierce the leaf
451 through.
452 ***Note:*** Inoculate the second and third extended upper, true leaves for optimal
453 propagation of virus infection.

454 c. Transfer the plants to a growth chamber set at 25°C and 12 h-light/12 h-dark cycle.
455 ***Note:*** To avoid cross-contaminations, ensure that plants infected with different
456 constructs do not touch each other and they do not share the same watering tray.
457 ***Note:*** Other growth chamber settings may work.
458
459
460 ## Analysis of plant virus infectivity
461
462 **Timing: 14-21 days**
463
464 In this section, plants inoculated with full-length virus clones are monitored for the appearance and
465 development of infection symptoms. Biochemical assays are used to confirm the presence of virus
466 within plant tissues and analyze CRISPR-Cas-mediated genome editing.
467
468 17. Inspect inoculated plants every 2-3 days and record appearance of infection symptoms.
469 ***Note:*** *N. benthamiana* plants inoculated with pLX-PVX will display symptoms characterized by
470 vein banding, ring spots and leaf atrophy starting after ~1 week. Virus infection started by
471 simultaneous inoculation of pLX-TRV1 and pLX-TRV2 can be very mild or symptomless.
472 ***Note:*** Inoculation of Cas9-transgenic plants with viral vectors with sgRNA-tFT targeting the *PDS*
473 homeologs will result in a leaf mosaic with green and photobleached sectors starting after ~2
474 weeks.
475
476 18. Analysis of virus infectivity:
477 a. Collect samples of the first symptomatic systemic leaf with a 1.2-cm cork borer (~100
478 mg of tissue) at 14 days post-infiltration (dpi) and immediately freeze in liquid nitrogen.
479 ***CRITICAL:*** Avoid the first two leaves located above the inoculated leaves and collect
480 samples from the third or younger leaves.
481 ***CRITICAL:*** Collect samples from mock-inoculated plants to be used as negative controls
482 of virus detection assays.
483 ***Pause point:*** After collection, samples can be stored at -80°C before proceeding with
484 the next step.
485 b. Total RNA extraction:
486 i. Homogenize frozen leaf samples using a ball mill (Star-Beater, WMR) for 1 min at
487 30 sec^{-1}.
488 ii. Add 1 mL Extraction Buffer, mix by vortexing and centrifuge at 15,000 × g for 5
489 min.
490 iii. Transfer the supernatant (0.6 mL) to a 2-mL tube and mix by vortexing with 0.65
491 volumes (0.39 mL) of 96% ethanol.
492 iv. Centrifuge at 15,000 × g for 1 min and wash twice with 0.5 mL Washing Buffer.

493 v. Centrifuge again for 2 min to remove completely the residual buffer.
494 vi. Transfer the column to a 1.5-mL tube, add 10 µL of 20 mM Tris-HCl pH 8.5, and
495 incubate at room temperature for 1 min prior to centrifugation for final elution.
496 c. For cDNA synthesis, subject 1-µL RNA aliquots to reverse transcription (RT) with
497 RevertAid™ Reverse Transcriptase (Thermo Fisher Scientific) following manufacturer's
498 instructions and virus-specific reverse primer.
499 ***Note:*** Primer D2409 (5'-ATTTATATTATTCATACAATCAAACC-3') is used for RT of PVX viral
500 RNA.
501 ***Note:*** Primer D4337 (5'-GAATATGGTATCACCCACCCTC-3') is used for RT of TRV viral
502 RNA.
503 d. Subject 1-µL cDNA aliquots to PCR amplification with *Thermus thermophilus* (Tth) DNA
504 polymerase (Biotools) using gene-specific primers in 20-µl reactions. Conditions were
505 as previously described (Bedoya et al., 2010).
506 **Tth PCR reaction master mix (Biotools)**

Reagent	Final concentration	Amount
10X Standard Reaction Buffer (with $MgCl_2$)	1X	2 µL
dNTPs (10 mM each)	200 µM of each	0.4 µL
Primer F	0.5 µM	X µL
Primer R	0.5 µM	X µL
Tth DNA Polymerase (1U/µL)	0.02 U/µL	0.2 µL
DNA template		1 µL
ddH_2O		Up to 20 µL

507
508 **Tth PCR cycling conditions (Biotools)**

Steps	Temperature	Time	Cycles
Initial Denaturation	94 °C	2 min	1
Denaturation	94 °C	1 min	25-35 cycles
Annealing	55 °C	30 sec	
Extension	72 °C	60 sec/kb	
Final extension	72 °C	10 min	1
Hold	4 °C	∞	

509
510 ***Note:*** Primers D3436 (5'-ATGTCAGGCCTGTTCACTATCC-3') and D2410 (5'-
511 TGGTGGTGGTAGAGTGACAAC-3') are designed to amplify a 627-nt region of PVX
512 genome corresponding to coat protein (CP).
513 ***Note:*** Primers D4338 (5'-ATGGGAGATATGTACGATGAAT-3') and D4339
514 (5'-GGGATTAGGACGTATCGGACC-3') are designed to amplify a 612-nt region of TRV
515 genome corresponding to CP.
516 e. Analyze PCR products by electrophoresis in 1% agarose gels in 1 x TAE buffer.
517 ***Note:*** Agrobacteria are present in inoculated leaves, which host plasmid DNA of the assembled
518 virus clones. Viral RNA molecules are generated in inoculated cells from T-DNA transcription in

519 absence of viral replication. To prevent false positives, only upper uninoculated plant samples
520 should be analyzed and agrobacteria contamination avoided.
521 ***Note:*** Carefully run RT-PCR assays to prevent false positive results due to contaminations with
522 plasmid DNA of the assembled cloned.
523

524 ## Analysis genome editing of somatic cells and recovery of mutant progeny

525
526 **Timing: 0.5-2.5 months**
527
528 In this section, biochemical assays are used to analyze CRISPR-Cas-mediated genome editing in
529 inoculated plants and progeny thereof.
530
531 19. Detection of Cas9-mediated genome editing in somatic cells of inoculated plants (Figure 4):
532 a. Collect samples of the first symptomatic systemic leaf with a 1.2-cm cork borer (~100
533 mg of tissue) at 14 and 21 dpi and immediately freeze in liquid nitrogen.
534 ***CRITICAL:*** Collect samples from mock-inoculated or wild-type plants to be used as
535 negative controls of editing detection assays.
536 ***Pause point:*** After collection, samples can be stored at -80°C before proceeding with
537 the next step.
538 b. Purify genomic DNA by the procedure of step 18b, except for not adding ethanol to the
539 extract before loading the silica gel column.
540 c. Subject 1-µL genomic DNA aliquots to PCR amplification with Phusion™ High-fidelity
541 DNA polymerase (Thermo Fisher Scientific) using gene-specific primers in 20-µl
542 reactions.
543
544 **Phusion™ High-fidelity PCR reaction master mix**

Reagent	Final concentration	Amount
5X Phusion HF Buffer	1X	4 µL
dNTPs (10 mM each)	200 µM of each	0.4 µL
Primer F	0.5 µM	X µL
Primer R	0.5 µM	X µL
Phusion DNA Polymerase (2U/µL)	0.02 U/µL	0.2 µL
DNA template		1 µL
ddH_2O		Up to 20 µL

545 20.
546 **Phusion™ High-fidelity PCR cycling conditions**

Steps	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 sec	1
Denaturation	98 °C	10 sec	25-35 cycles
Annealing	55 °C	30 sec	

Extension	72 °C	15-30 sec/kb	
Final extension	72 °C	10 min	1
Hold	4 °C	∞	

547
548 ***CRITICAL:*** Use Phusion™ or alternative high-fidelity DNA polymerases to reduce the PCR
549 error rate.
550 ***Note:*** Use the primer pair D4444/D4445 to amplify a genomic fragment of the *NbPDS*
551 homeolog Niben101Scf14708g00023.1, which spans the region targeted by sgPDS3-tFT.
552
553 e. Separate PCR products by electrophoresis in 1% agarose gels in 1 x TAE buffer.
554 f. Purify PCR products from gel bands using DNA Clean & Concentrator™-5 (Zymo
555 Research) kit following the procedure explained on step 4.d.
556 g. Subject the purified PCR products to Sanger sequencing.
557 ***CRITICAL:*** Ensure the good quality of sequencing reads by checking the
558 electropherograms.
559 h. Analyze the presence of targeted modifications within the sequencing reads using the
560 Inference of CRISPR Edits (ICE) online tool (https://ice.synthego.com) (Figure 4C).
561 ***CRITICAL:*** Include PCR product reads from uninoculated or mock-inoculated plants as
562 controls.
563 ***Note:*** Samples from infected plants are somatic mosaics of the wild-type genomic
564 sequence and multiple edited alleles (Figure 4D).
565 ***Note:*** Use TTGGTAGTAGCGACTCCATG as the guide target sequence in the analysis of
566 samples obtained using sgPDS3-tFT.
567 ***Alternative:*** Tracking of Indels by Decomposition (TIDE) online tool (https://tide.nki.nl)
568 can also be used for analysis of genome editing; batch analysis may be used
569 (http://shinyapps.datacurators.nl/tide-batch/).
570
571 20. Recovery of mutant progeny (Figure 5).
572 a. Collect seeds from inoculated plants.
573 ***CRITICAL:*** Collect seeds from capsules from the top of the parent plant since high
574 fraction of progeny with mutations was reported in capsules distal to the point of
575 infiltration (Ellison et al., 2020).
576 b. Sow seeds on soil and inspect every day for appearance of seedlings with mutant
577 phenotypes.
578 ***CRITICAL:*** Plants with genetic knock-out or chemical inhibition of *PDS* do not survive
579 when grown in soil (Norris et al., 1995; Guo et al., 2003; Brausemann et al., 2017). Sow
580 seeds on ½ MS medium agar plates supplemented with 3% sucrose to facilitate
581 identification of progeny with homozygous loss-of-function mutants of *PDS*.
582 c. Collect samples from seedlings and proceed with DNA analysis as described in step 18.

583 ***Note:*** In progeny analysis, score mutations at each *PDS* homeolog using the following
584 parameters: <35% indels, wild type; 35%–80% indels, heterozygous mutation; >80%
585 indels, homozygous/biallelic mutation (Figure 5C).
586

587 ## Expected outcomes

588

589 The procedures described in this protocol enable the assembly of recombinant viral vectors based on
590 binary T-DNA vectors and suitable for *Agrobacterium*-mediated delivery to plants. It is expected that
591 the generated PVX and TRV vectors will be infective and able to spread systemically through the plant,
592 leading to fast (14 dpi) and efficient (up to 80% indels) CRISPR-Cas-mediated genome editing in somatic
593 cells (Uranga et al., 2021; Aragonés et al., 2022). This approach conforms an ideal screening tool to
594 rapidly assess sgRNA design and can still be used when editing of the target gene alters plant fitness.
595 Finally, targeted modifications are successfully inherited by the progeny of infected plants.
596 Besides the reported mutagenesis of targeted genomic loci via double-stranded DNA break induction,
597 the described viral vectors can be used to deliver sgRNA constructs that guide Cas9 fusion proteins for
598 a variety of applications including base editing and transcriptional reprogramming (Cody and Scholthof,
599 2019; Ghoshal et al., 2020; Liu et al., 2022).
600 Furthermore, the described viral vector pLX-PVX and the pLX-TRV1/pLX-TRV2 pair can be used for
601 additional applications such as virus-mediated gene silencing (VIGS) or recombinant protein
602 overexpression (Uranga et al., 2021; Aragonés et al., 2022).
603

604 ## Limitations

605

606 PVX and TRV possess a wide host range specially in the Solanaceae family (Macfarlane, 2010; Verchot,
607 2022), however the viral vectors assembled according to this protocol have only been tested in *N.*
608 *benthamiana*. To apply this protocol to other plant species, generation of a transgenic line stably
609 expressing the *S. pyogenes* Cas9 nuclease is required. A variability in virus infectivity can be observed
610 when inoculating other plant species that may need an optimization of the protocol for each case.
611 Gene knockout may cause severe growth defects and lethality. Analysis of genes whose loss-of-function
612 phenotypes are lethal may require condition optimization and special selection scheme.
613 Progeny plants recovered from editing assays are transgenic since they include the Cas9 expression
614 cassette.
615

616 ## Troubleshooting

617

618 ## Problem 1:

619 High background of empty vector colonies precludes identification of positive clones (steps 7-8); no
620 vector is recovered with the correct digestion profile (step 9).
621

622 ## Potential solutions:

623 Get familiar with procedures for plasmid construct generation and specifically with principles of in vitro
624 homology-based (Gibson et al., 2009).
625 Ensure that the cloning strategy and the used materials (i.e. primers, synthetic DNA fragments…) are
626 correct.
627 Screen more colonies.
628 Work in sterile conditions and avoid plasmid or bacterial contaminations.
629

630 ## Problem 2:

631 Low DNA plasmid yield from *E. coli* cultures.
632

633 ## Potential solutions:

634 Binary vectors used herein include a medium copy origin by design to enhance stability (Pasin et al.,
635 2017), higher culture volumes (step 8) should be used to reach yields obtained using with high-copy
636 plasmids. The DNA plasmid amount recovered from a single miniprep is nonetheless sufficient for the
637 procedures detailed in this protocol, that is restriction enzyme digestion verification (step 9), clone
638 verification by sequencing (step 10), and transformation of *A. tumefaciens* cells (step 11).
639

640 ## Problem 3:

641 Slow growth rates of bacteria harboring binary vectors with viral vectors.
642

643 ## Potential solution:

644 For most applications, extended bacterial culturing time may solve the problem.
645

646 ## Problem 4:

647 Sequence alteration and degeneration of viral vectors after propagation in bacteria.
648

649 ## Potential solution:

650 Start experiments using a controlled plasmid aliquot from a backup stock.

651 Bacterial cells harboring binary vectors with full-length viral vectors may display low growth rates. Avoid
652 contaminations with bacterial strains with fast growth rates. If a mixture of small and large colonies
653 appears during agar plate selection pick small colonies for subsequent analysis.
654 Reduce of *E. coli* culturing temperatures from 37°C to 25-30°C to improve recovery and propagation of
655 full-length viral vectors (Pasin et al., 2019).
656

657 ## Problem 5:

658 *N. benthamiana* leaves are hard to infiltrate (step 16).
659

660 ## Potential solution:

661 Create a small nick with a needle or blade in the epidermis on the adaxial side of the leaf. Make sure
662 not to scratch so hard as to pierce the leaf through, otherwise the *A. tumefaciens* inoculum will pass
663 through the puncture instead of penetrating into leaf tissue.
664

665 ## Problem 6:

666 Analysis of virus infectivity is inconsistent between plants infected with the same viral vector.
667

668 ## Potential solution:

669 Use freshly prepared Extraction buffer when performing RNA extractions to prevent false positive
670 results due to contaminations.
671 Carefully run RT-PCR assays to prevent false positive results due to contaminations with plasmid DNA
672 of the assembled cloned.
673

674 ## Problem 7:

675 No somatic editing is detected.
676

677 ## Potential solution:

678 Ensure to use Cas9-transgenic plants.
679 Ensure that the sgRNA design and the used materials are correct.
680 Test protospacer sequences designed at difference regions of the target genes.
681

682 ## Problem 8:

683 PCR products of target genomic regions show poor quality electropherograms (i.e. short reads, multiple
684 peaks) in control, unedited samples.
685

686 ## Potential solution:

687 *N. benthamiana* is an allotetraploid. For genome editing analysis of specific homeologs ensure to use
688 primer pairs designed and validated by dedicated tools, e.g. BLAST
689 (https://solgenomics.net/tools/blast/) and In Silico PCR (https://solgenomics.net/tools/in_silico_pcr).
690 Carefully run PCR products on 2% gels for obtaining good electrophoresis resolution of short (0.2-1 kb)
691 DNA fragments.
692 Purify PCR products using silica gel columns as explained in step 4.d.
693 If the problem persists, clone PCR amplicons into suitable plasmid vectors and perform Sanger
694 sequencing of DNA plasmids from selected colonies.
695

696 ## Problem 9:

697 No mutant progeny is recovered.
698

699 ## Potential solution:

700 Somatic editing phenotypes increase in intensity as the plants mature, extending the assay duration
701 and seed collection from late flowers may be required to recover mutant progeny.
702 Growth conditions of the parental and progeny plants may require empirical optimization to ensure
703 high levels of hereditable editing.
704 Gene knockout may cause severe growth defects and lethality, which would require large screen efforts
705 or *ad hoc* rescue strategies to identify mutant progeny.
706

707 # Resource availability

708

709 ***Lead contact***

710 Further information and requests for resources and reagents should be directed to and will be fulfilled
711 by the lead contact, José-Antonio Daròs (jadaros@ibmcp.upv.es).
712

713 ***Materials availability***

714 pLX-TRV1 and pLX-TRV2 are available at Addgene with product number 180515
715 (https://www.addgene.org/180515) and 180516 (https://www.addgene.org/180516), respectively.
716

717 ***Data and code availability***

718 This protocol does not disclose new data or code.
719

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721

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Author contributions

All authors participated in work conception and design. M.U., F.P. and J.A.D. wrote the manuscript with input from the rest of the authors.

Declaration of interests

The authors declare no competing interests.

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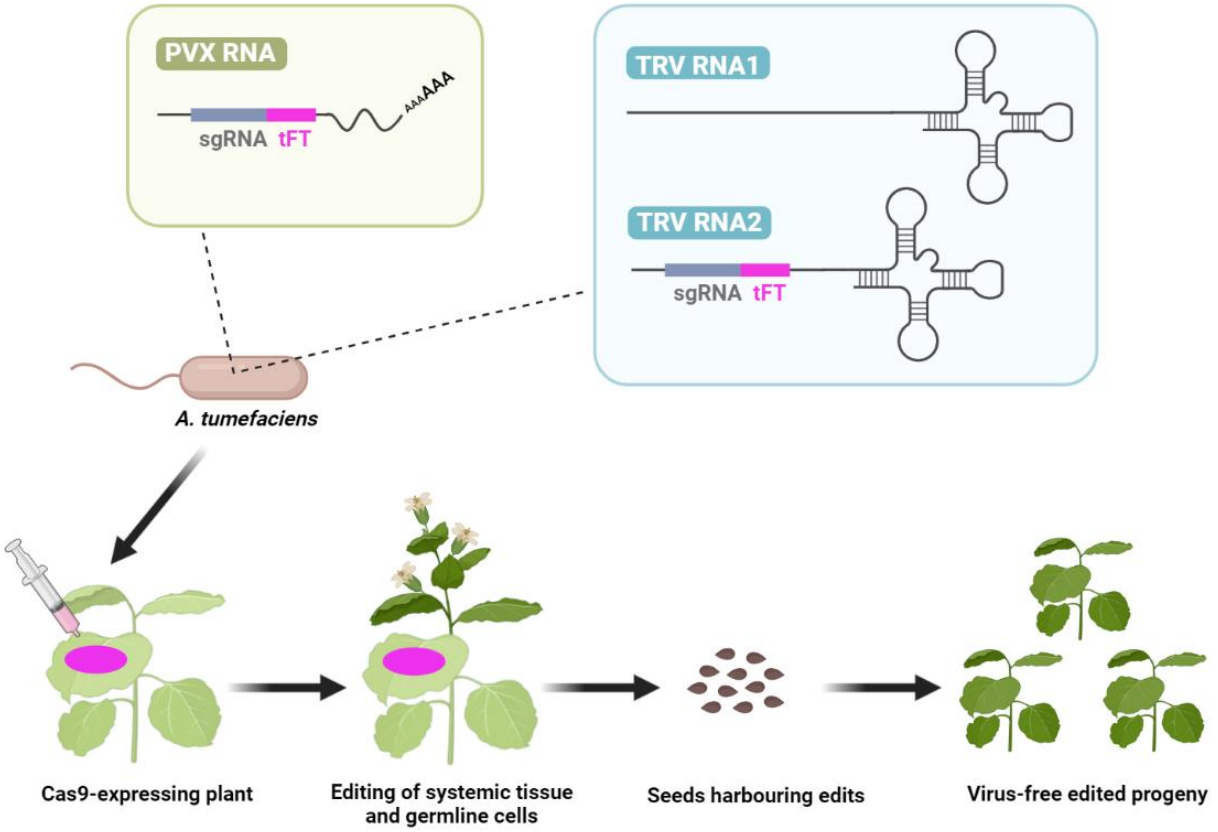
826 ## Figure legends



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829 **Figure 1: Protocol outline for CRISPR-Cas9-mediated genome editing in plants using viral vectors.**
830 Following agroinoculation of Cas9-expressing *N. benthamiana plants,* systemic infection with an RNA
831 viral vector carrying mobile sgRNAs can cause mutations in the germline. This results in a high rate of
832 edited seeds harbouring modifications that are inherited by progeny. sgRNA, single guide RNA; tFT,
833 truncated *A. thaliana* Flowering locus T.
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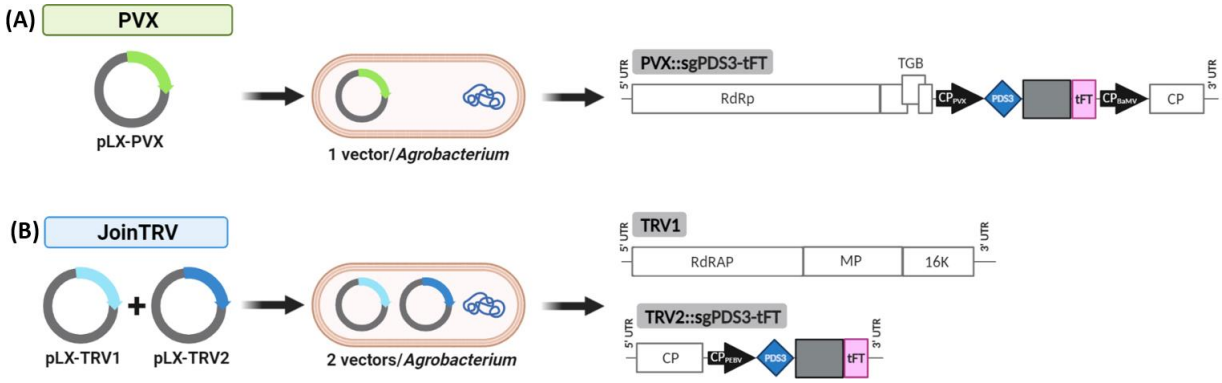

Figure 2: Schematic representation of the viral vectors used. Viral vectors for delivery of sgPDS3-tFT, a single guide RNA (sgRNA) construct that comprises of a protospacer (blue diamond) targeting both *N. benthamiana PDS* homeologs, a conserved Cas9 scaffold (gray box) and a truncated *A. thaliana Flowering locus T* (tFT; a pink box). (**A**) Diagram of the PVX system. One T-DNA vector is hosted by *A. tumefaciens* for delivery of PVX genomic components. pLX-PVX is a derivative of pLX-B2, which includes the pBBR1 origin and a kanamycin resistance gene. (**B**) Diagram of the JoinTRV system. Two T-DNA vectors are hosted by a single *A. tumefaciens* cell for simultaneous delivery of TRV genomic components. pLX-TRV1 is derivative of pLX-Z4, which includes the RK2 origin and a gentamicin resistance gene; pLX-TRV2 is a pLX-B2 derivative with the pBBR1 origin and a kanamycin resistance gene. RdRp, RNA-dependent RNA polymerase; TGB, triple gene block; CP, coat protein; RdRAP, RNA-dependent replication-associated protein; MP, movement protein; 16K, 16 kDa protein. Heterologous bamboo mosaic virus (BaMV) and pea early browning virus (PEBV) coat protein promoters are represented by a black arrow. Promoters, sgRNA and tFT, not at scale.

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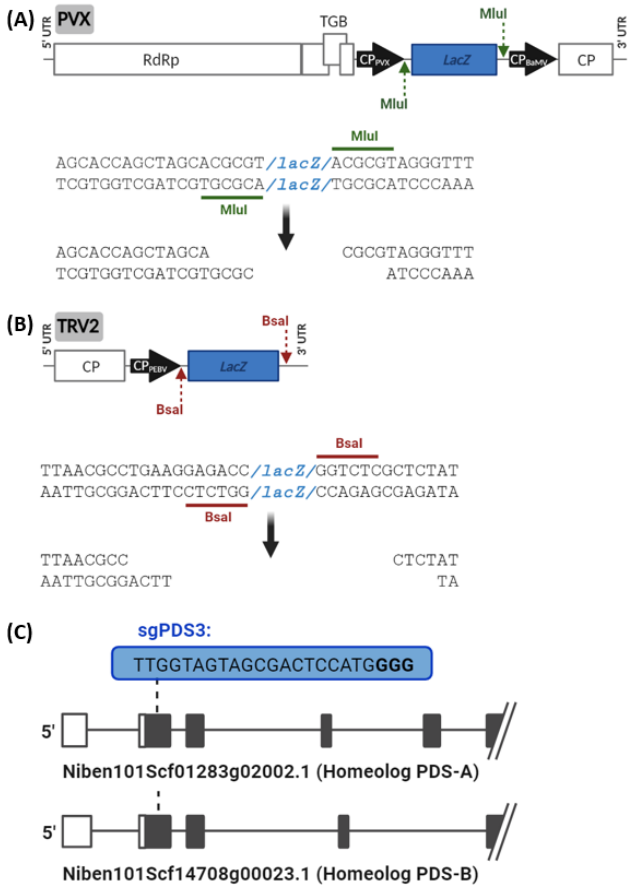

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853 **Figure 3: Features of pLX-PVX and pLX-TRV2 cloning cassettes for sgRNA delivery.** (**A**) Schematic
854 representation of pLX-PVX cloning cassette is shown along with the *Mlu*I recognition sites (green
855 underline) and *Mlu*I-generated overhangs (bottom). The *LacZ* reporter (blue box) allows white-blue
856 screen of recombinant vectors. Plant expression of the insert is driven by the endogenous coat protein
857 (CP) promoter. (**B**) Schematic representation of pLX-TRV2 cloning cassette is shown along with the *Bsa*I
858 recognition sites (red underline) and *Bsa*I-generated overhangs (bottom). Plant expression of the insert
859 is driven by the pea early browning virus (PEBV) CP promoter. Other details are as in panel A. (**C**) The
860 protospacer of sgPDS3-tFT is designed to target a 20-nt conserved region immediately downstream the
861 starting codon of two *N. benthamiana PDS* homeologs (Niben101Scf01283g02002.1 and
862 Niben101Scf14708g00023.1).

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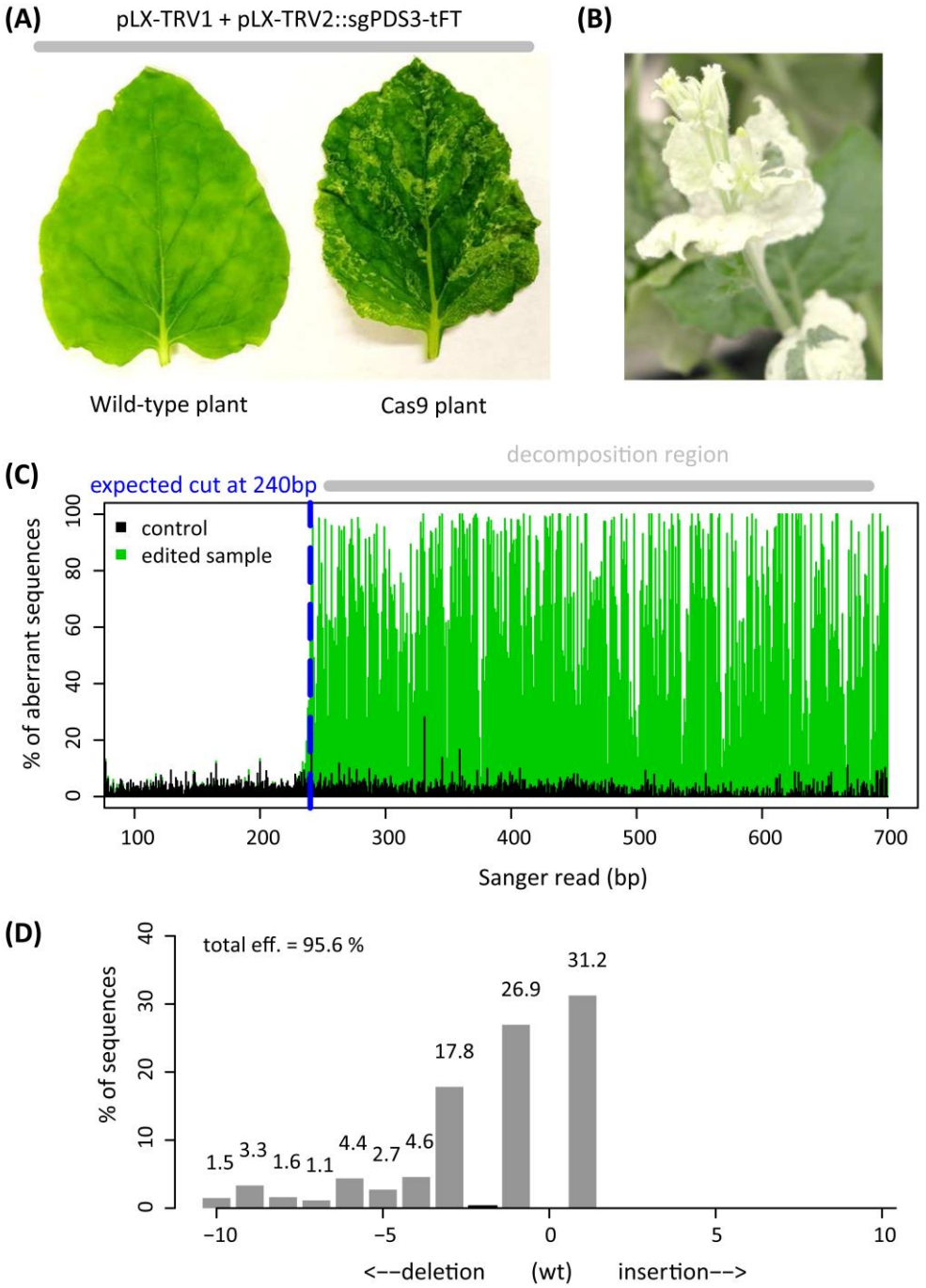

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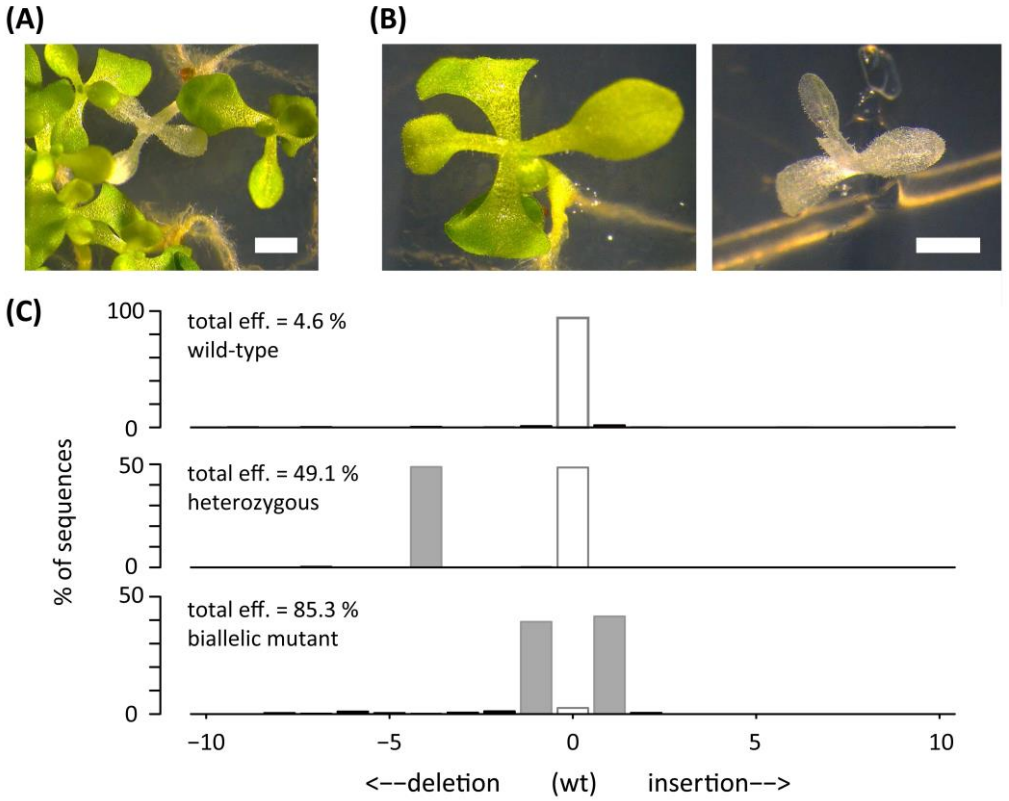
866 **Figure 4: Genome editing of somatic cells**. (**A**) Phenotypes of wild-type and Cas9 plants co-inoculated
867 with pLX-TRV1 and pLX-TRV2 including a sgRNA targeting two *N. benthamiana* PDS homeologs
868 (sgPDS3-tFT). (**B**) Severe photobleaching phenotype in Cas9 mature plants co-inoculated with pLX-
869 TRV1 and pLX-TRV2::sgPDS3-tFT. (**C**) Chromatogram deconvolution of edited and control samples
870 from plants infected with viral vectors for sgRNA delivery; note the high % of aberrant sequence

871 downstream the predicted Cas9 cleavage site (blue dashed line). (**D**) Total editing efficiency and indel
872 distribution at one *PDS* locus of an edited sample at 21 days post inoculation.



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874 **Figure 5:** Recovery of mutant progeny. (**A**) Phenotypes of seedlings obtained from seeds collected
875 from a Cas9 plants co-inoculated with pLX-TRV1 and pLX-TRV2::sgPDS3-tFT; scale bar = 2 mm. (**B**)
876 Details of green and albino seedlings; scale bar = 2 mm. (**C**) Total editing efficiency and indel
877 distribution at one *PDS* locus of individual progeny lines. Results of representative genotypes are
878 shown: wild-type line (top), line with a wild-type allele and a mutant one (middle), line with two
879 mutant alleles (bottom).
880