Received: 15 September 2021 | Revised: 17 February 2022 | Accepted: 17 March 2022

DOI: 10.1002/biot.202100504

Biotechnology Journal

RESEARCH ARTICLE

Simplifying plant gene silencing and genome editing logistics by a one-*Agrobacterium* system for simultaneous delivery of multipartite virus vectors

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Abstract

Viral vectors provide a quick and effective way to express exogenous sequences in eukaryotic cells and to engineer eukaryotic genomes through the delivery of CRISPR/Cas components. Here, we present JoinTRV, an improved vector system based on tobacco rattle virus (TRV) that simplifies gene silencing and genome editing logistics. Our system consists of two mini T-DNA vectors from which TRV RNA1 (pLX-TRV1) and an engineered version of TRV RNA2 (pLX-TRV2) are expressed. The two vectors have compatible origins that allow their cotransformation and maintenance into a single *Agrobacterium* cell, as well as their simultaneous delivery to plants by a one-*Agrobacterium*/two-vector approach. The JoinTRV vectors are substantially smaller than those of any known TRV vector system, and pLX-TRV2 can be easily customized to express desired sequences by one-step digestion-ligation and homology-based cloning. The system was successfully used in *Nicotiana benthamiana* for launching TRV infection, for recombinant protein production, as well as for robust virus-induced gene silencing (VIGS) of endogenous transcripts using bacterial suspensions at low optical densities. JoinTRV-mediated delivery of single-guide RNAs in a Cas9 transgenic host allowed somatic cell editing efficiencies of ≈90%; editing events were heritable and >50% of the progeny seedlings showed mutations at the targeted loci.

KEYWORDS
CRISPR/Cas9, heritable gene editing, pLX binary vector multiplexing, tobacco rattle virus, virus-induced gene silencing (VIGS), virus-induced genome editing (VIGE)

1 | INTRODUCTION

Viral vectors provide a flexible, high-yield means for exogenous sequence delivery into eukaryotic cells and whole organisms. This feature has been exploited in multiple biotechnology and synthetic biology applications as varied as clinical gene therapy, vaccine development, pharmaceutical production, or accelerated plant breeding.[1–6]

Viral vectors have enabled delivery into human and plant cells of genome editing reaction components based on the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein systems.[7,8] Plant virus replicons were used for local expression of single-guide RNA (sgRNA) molecules and Cas nucleases,[9] and viral vectors were used for sgRNA delivery into Cas-expressing transgenic hosts.[10] Recent studies have reported viral vector-mediated delivery of both Cas nucleases and sgRNAs to

ABBREVIATIONS: CaMV, cauliflower mosaic virus; Cas, CRISPR-associated; *CHLI*, magnesium protoporphyrin chelatase subunit I; CP, coat protein; CRISPR, clustered regularly interspaced short palindromic repeats; crtB, phytoene synthase; *FT*, flowering locus T; GFP, green fluorescent protein; PDS, phytoene desaturase; PEBV, pea early browning virus; RT, reverse transcription; sgRNA, single guide RNA; TRV, tobacco rattle virus; VIGE, virus-induced gene editing; VIGS, virus-induced gene silencing

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plants.[11–13] Viral vectors or their encoded elements can systemically move from inoculated tissues to reach uninoculated plant organs. Apical meristem entry of genome editing reaction components delivered by viral vectors allows for germinal transmission of plant genome modifications and subsequence recovery of edited progeny without the need for tissue culture selection or regeneration steps.[14]

Genomes of ≈24% of plant DNA and RNA virus species are distributed on two or more genomic components.[4] Among multipartite viruses, the genus *Tobravirus* comprises an important group of plant viruses that have been repurposed as tools widely used by plant scientists for basic research as well as for biotechnology.[15] Strains of *Agrobacterium* species can be used for efficient delivery of virus genomic components and viral vectors to plant cells in an approach (agroinoculation) that requires the assembly of virus genome copies or viral vector sequences into T-DNA vectors.[4] Agroinoculation followed by *in planta* reconstitution of multipartite viruses is usually achieved by pooling *Agrobacterium* strains each hosting a binary vector for delivery of an individual virus genome segment.[16] This approach, although effective, may limit assay scalability given the need for selection, propagation, subculturing, and inoculation of multiple bacterial strains.

Here we explored the use of T-DNA vectors with compatible replication origins for simultaneous inoculation of multipartite virus components by a single *Agrobacterium* strain. We designed and developed JoinTRV, a one-strain/two-vector system for agroinoculation of engineered genomic components of the bipartite tobacco rattle virus (TRV). The robustness of the system presented and its usefulness for plant biotechnology applications was evaluated in the proof-of-concept experiments that included gene silencing, recombinant protein production, as well as in CRISPR/Cas9 genome editing assays that allowed tissue culture-free rescue of progeny with mutations at targeted genomic loci.

2 | EXPERIMENTAL SECTION

2.1 | Plasmid construction and bacterial strains

Plasmids were built by standard molecular biology techniques including PCR using Phusion high-fidelity DNA polymerase (Thermo Scientific) and Gibson assembly.[17] pLX-B2 (GenBank: KY825137; Addgene: 160636), pLX-Z4 (GenBank: KY825158; Addgene: 160648), pTRV1 (GenBank: AF406990) and pTRV2 (GenBank: AF406991) were described.[18,19] pLX-Z4 and pLX-B2 were linearized with *Bsa*I (*Bsa*I-HFv2, New England Biolabs) and used as the backbones of pLX-TRV1 (GenBank: OM372495; Addgene: 180515)and pLX-TRV2 (GenBank: OM372496; Addgene: 180516), respectively. TRV1 and TRV2 cDNA fragments were amplified from pTRV1 and pTRV2 and assembled along with the cauliflower mosaic virus (CaMV) 35S promoter and terminator, and a hepatitis delta virus-derived ribozyme.[20] pLX-TRV2 includes the pea early browning virus (PEBV) coat protein (CP) promoter to drive insert expression *in planta*, and the *Escherichia coli lacZ* alpha peptide for white-blue screens of recombinant clones. pLX-TRV2 was linearized with *Bsa*I and was used to build pLX-TRV2-GFP, pLX-TRV2-crtB, pLX-TRV2-*CHLI*, and pLX-TRV2-sgPDS by Gibson assembly of the corresponding inserts (Supplementary Figure S1, Supporting Information); the used crtB and sgPDS sequences were already reported.[21–23] White colonies were selected on plates supplemented with kanamycin and X-gal.

Agrobacterium C58C1 including a disarmed pTi was electroporated with pLX-TRV1; colonies were selected on plates supplemented with rifampicin and gentamicin. Competent cells of the recovered strain C58C1(pLX-TRV1) were then electroporated with pLX-TRV2, pLX-TRV2-GFP, pLX-TRV2-crtB, pLX-TRV2-*CHLI*, or pLX-TRV2-sgPDS. *Agrobacterium* transformants simultaneously hosting the two vectors of TRV genomic components were selected on plates supplemented with rifampicin, gentamicin, and kanamycin. Antibiotics were used at final concentrations of 20 mg l^{-1} gentamicin, 50 mg l^{-1} kanamycin, and 50 mg l^{-1} rifampicin.

2.2 | Plant inoculation

Wild-type or *Streptococcus pyogenes* Cas9-expressing *Nicotiana benthamiana* plants were used.[23] Individual colonies of *Agrobacterium* strains that simultaneously host pLX-TRV1 and pLX-TRV2 or derivatives of the latter were used to inoculate liquid cultures and grown for 16–48 h at 28°C; bacteria were collected and resuspended in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH, 10 mM $MgCl_2$, 150 μM acetosyringone, pH 5.6. Bacterial suspensions were adjusted at the indicated optical densities and infiltrated in the abaxial side of one leaf of 4-week-old plants using 1-ml needle-less syringes. Plants were grown in a chamber at ≈25°C with a 12 h day/night cycle. *Chenopodium quinoa* plants were used in necrotic lesion assays which were done as described.[24]

2.3 | RNA purification and analysis of virus infectivity

Samples of upper uninoculated leaves were frozen in liquid N_2 and ground using a ball mill (Star-Beater, VWR) for 1 min at 30 s^{-1}. Ground tissue was homogenized in 1 ml of 4 M guanidinium thiocyanate, 0.1 M sodium acetate, 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 M 2-mercaptoethanol, pH 5.5, and 0.39 ml (0.65 volumes) of 96% ethanol added. The mix was vortexed, clarified by centrifugation and 0.7 ml of the supernatant was loaded into a silica gel spin column (Zymo Research).[23] After washing twice with 0.5 ml of 70% ethanol, 10 mM acetic acid, pH 5.5, RNA was eluted in 10 μl of 20 mM Tris-HCl, pH 8.5. RNA aliquots were subjected to reverse transcription (RT) with RevertAid reverse transcriptase (Thermo Scientific) using the primer D4337 (5′-GAATATGGTATCACCCACCCTC-3′). RT aliquots were amplified by PCR using *Thermus thermophilus* DNA polymerase (Biotools) using the primers D4338 (5′-ATGGGAGATATGTACGATGAAT-3′) and D4339 (5′-GGGATTAGGACGTATCGGACC-3′). PCR products were analyzed by electrophoresis in 1% agarose gels in buffer TAE (40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, pH 7.2), and visualized by ethidium bromide staining.

2.4 | Western blot analysis

Systemic leaf samples (≈100 mg) were ground with a ball mill, homogenized in 300 μl of 60 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 10% w/v glycerol, and 0.01% bromophenol blue, and the extracts were incubated for 5 min at 95°C. After clarification by centrifugation, supernatant aliquots were separated by polyacrylamide gel electrophoresis (PAGE) in discontinuous 12.5% polyacrylamide gels (5% for the stacking gel) containing 1% SDS.[25] Proteins were then electroblotted to a polyvinylidene fluoride membrane (GE Healthcare) and stained with 0.1% w/v Ponceau S in 5% v/v acetic acid. The membrane was next blocked with 5% nonfat milk in 10 mM Tris-HCl, pH 7.5, 154 mM NaCl, and 0.1% w/v Nonidet P40, and incubated with a green fluorescent protein (GFP)-specific antibody conjugated to horseradish peroxidase (HRP; ab6663, Abcam) at a 1:10 000 dilution. HRP was revealed using the SuperSignal West Pico PLUS Chemiluminescent substrate (Thermo Scientific).

2.5 | Genome editing analysis

Samples were collected from upper uninoculated leaves and used in somatic tissue analysis. Mature fruits were collected from inoculated plants for progeny analysis; seeds from these plants were sown on soil and leaf samples were collected from forty randomly chosen seedlings. Genomic DNA was purified from the samples using silica gel spin columns (Zymo Research). *N. benthamiana* genome fragments corresponding to the *PDS* homeologs were amplified from DNA aliquots by homeolog-specific PCR amplification with Phusion high-fidelity DNA polymerase and the primers D3665 (5′-GTGGGACAATCTTCTTACACTG-3′) and D3666 (5′-TGGCGAAGAAGTAAGAACC-3′; Niben101Scf01283g02002.1), or D4444 (5′-GAACCAGAATATTGAAAAAAC-3′) and D4445 (D4445: 5′-CTCCTAATCTAATCAGTTGGG-3′; Niben101Scf14708g00023.1). PCR products were separated by electrophoresis in 1% agarose gels, eluted, and subjected to Sanger sequencing. Genome editing efficiency was quantified using the Interference of CRISPR Edits algorithm (Synthego);[26] in progeny analysis mutations at each *PDS* homeolog were scored using the following parameters: <35% indels, wild type; 35%–80% indels, heterozygous mutation; >80% indels, homozygous/biallelic mutation.

3 | RESULTS

3.1 | Generation of mini binary T-DNA vectors for *Agrobacterium*-mediated delivery of TRV genomic components

We used TRV, a member of the genus *Tobravirus*, in the proof-of-concept assays for plant delivery of multiple viral components from a single *Agrobacterium* strain. TRV has a positive, single-stranded RNA genome that comprises the two components RNA1 and RNA2 (Figure 1A). Guided by previous TRV work,[15,27] we generated

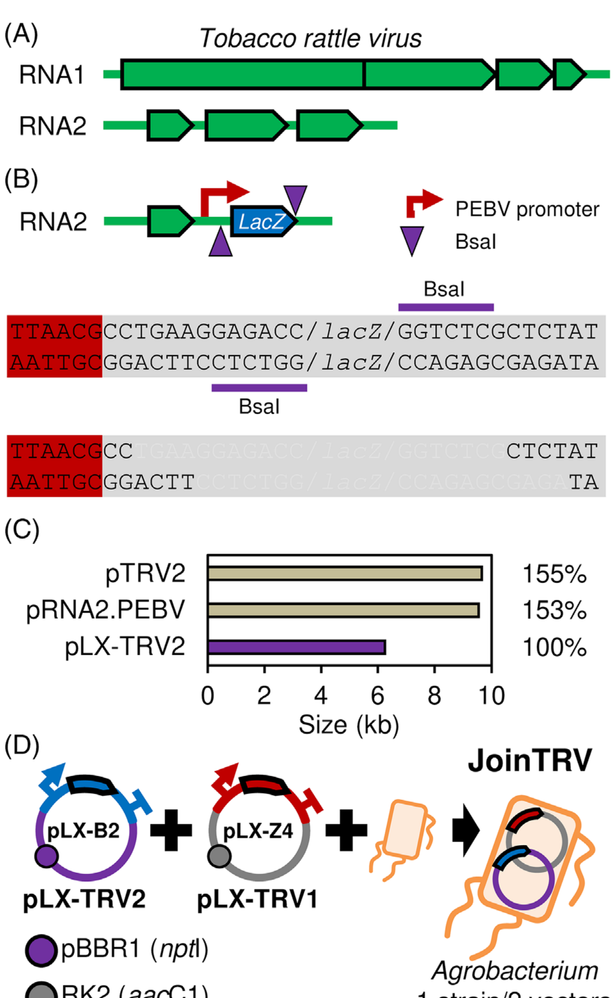

FIGURE 1 Design of JoinTRV, a TRV expression system based on mini T-DNA vectors with compatible origins. (A) Genomic organization of tobacco rattle virus (TRV). (B) TRV RNA2 engineering for sequence cloning and expression. Diagram of the cloning cassette of pLX-TRV2 is shown along with the *Bsa*I recognition sites and *Bsa*I-generated overhangs (bottom). The *LacZ* reporter allows visual selection of recombinant vectors; plant expression of the insert is driven by the pea early browning virus (PEBV) coat protein (CP) promoter. (C) Size comparisons of pLX-TRV2 and TRV RNA2-based vectors described for VIGS (pTRV2) and VIGE (pRNA2.PEBV). (D) Diagram of the JoinTRV system. Two T-DNA vectors are multiplexed into a single *Agrobacterium* cell for simultaneous delivery of TRV genomic components. pLX-TRV2 is a pLX-B2 derivative with the pBBR1 origin and a kanamycin resistance gene (*nptI*), pLX-TRV1 is a pLX-Z4 derivative with the RK2 origin and a gentamicin resistance gene (*aacC1*); the two T-DNA vectors can be simultaneously hosted by the same bacterial cell since they have compatible origins and independent antibiotic selection mechanisms

pLX-TRV2, an engineered cDNA clone of TRV RNA2 with a cloning cassette that includes divergent *Bsa*I sites flanking the *E. coli lacZ* reporter for white-blue screens of recombinant clones (Figure 1B). The *Bsa*I-linearized pLX-TRV2 is suitable for one-step digestion-ligation reactions as well as homology-based cloning methods, such as Golden Gate and Gibson assembly,[28,17] respectively. The vector further includes an additional promoter from PEBV to drive plant

expression of heterologous sequences inserted in its cloning cassette (Figure 1B). pLX-TRV2 is based on pLX-B2, a mini binary vector that includes the pBBR1 origin and autonomously replicates in *E. coli* and *Agrobacterium*.[19] pLX-B2 has been used as the backbone for plant virus clone assembly as well as for agroinoculation of diverse RNA and DNA plant viruses.[29–31] The compact size of pLX-TRV2 is below those of TRV RNA2 vectors previously reported for virus-induced gene silencing (VIGS) and virus-induced genome editing (VIGE) assays;[18,10] for instance pTRV2 and pRNA2.PEBV are 55% and 53% larger than pLX-TRV2, respectively (Figure 1C).

Additionally, we generated pLX-TRV1 for plant delivery of a full-length cDNA copy of TRV RNA1. The vector is based on pLX-Z4, a mini binary vector that includes the RK2 origin which autonomously replicates in *E. coli* and *Agrobacterium* and is compatible with the pBBR1 origin of pLX-TRV2.[19] pTRV1, whose full sequence is not publicly available, is widely used for TRV RNA1 delivery to plants,[18,24,10] and it is based on pBIN19 (GenBank: U09365.1), which is ≈3 fold larger than pLX-Z4. Compared to described TRV vectors, stable propagation of pLX-TRV2 and pLX-TRV1 in bacterial cells is predicted to be enhanced by the substantially reduced size of plasmid DNA, as well as by the presence of bacterial terminators up- and downstream of T-DNA to prevent transcriptional read-through from adjacent sequences.[19]

In both pLX-TRV2 and pLX-TRV1, plant expression of the TRV genomic components is regulated by the CaMV 35S promoter and terminator, and release of exact 3′ end of viral RNAs is mediated by a ribozyme derived from the hepatitis delta virus antigenome.

3.2 | Binary vector multiplexing in a single *Agrobacterium* cell allows simultaneous delivery of the TRV genomic components and systemic plant infection

Given their compatible replication origins and the different antibiotic resistance mechanisms, transformation of pLX-TRV2 and pLX-TRV1 into *Agrobacterium* cells in the presence of gentamicin and kanamycin is predicted to allow selection of a strain that simultaneously hosts the two binary vectors. This strain would allow multiplexed delivery of the TRV genomic components in a one-strain/two-vector strategy (JoinTRV; Figure 1D).

As anticipated, growth of *Agrobacterium* cells transformed with the individual vectors pLX-TRV1 or pLX-TRV2 could be detected on plates supplemented with either gentamicin or kanamycin, respectively, but it was inhibited in the presence of the two antibiotics (Figure 2A). Transformed cells that simultaneously harbored pLX-TRV1 and pLX-TRV2 showed resistance to kanamycin, gentamicin as well as the combination of the two antibiotics (Figure 2A); this allowed the selection of C58C1(pLX-TRV1, pLX-TRV2), an *Agrobacterium* strain suitable for simultaneous delivery of the TRV genomic components. *N. benthamiana* plants were inoculated with C58C1(pLX-TRV1, pLX-TRV2) to assess the functionality of the JoinTRV system. Symptoms of TRV infection appeared in newly emerged, uninoculated leaves as soon as three days post-inoculation (dpi) (Figure 2B). Viral RNA accumulation in upper uninoculated leaves was confirmed by RT-PCR detection of the TRV CP gene (Figure 2C). pTRV1 and pTRV2 are widely used vectors for TRV inoculation that require mixing of two *Agrobacterium* strains for their use.[18] *N. benthamiana* delivery of a bacterial suspension mix of two *Agrobacterium* strains hosting the vectors, that is, C58C1(pTRV1) plus C58C1(pTRV2), caused necrosis of the infiltrated area; the necrotic phenotype was not evident in JoinTRV-infiltrated leaves (Figure 2D). At three dpi, samples were collected from upper uninoculated leaves of plants infiltrated with JoinTRV or with the pTRV1 and pTRV2 suspension mix. Crude extracts were prepared and inoculated to *C. quinoa*, a plant species that shows a local hypersensitive response to TRV infection.[32] At four dpi, necrotic lesions indicating TRV infection were visible in the inoculated leaves of tested conditions; no necrosis was detected in control plants (Figure 2E). These results confirm the usefulness of pLX-B2 and pLX-Z4 for plant cell delivery of components of a bipartite RNA virus, as well as suitability of our JoinTRV system for launching systemic infection by simultaneous agroinoculation of the TRV genomic components. Compared to the pTRV1/pTRV2 system, JoinTRV does not elicit cell death in infiltrated *N. benthamiana* leaves and show similar speed of systemic infection.

3.3 | Robust virus-induced gene silencing (VIGS) by the JoinTRV system

TRV-based vectors are widely used in VIGS assays that have been reported for functional characterization of genes from a wide variety of angiosperm species.[33] Magnesium protoporphyrin chelatase participates in the biosynthesis of chlorophyll in plants, and its subunit gene *CHLI* is a reliable VIGS marker since its silencing invokes a yellowing phenotype owing to disruption of chlorophyll biosynthesis.[34]

To evaluate the VIGS suitability of the JoinTRV system a 300-bp cDNA fragment of *N. benthamiana CHLI* (Niben101Scf16898g00001.1) was inserted in the antisense orientation into the cloning cassette of pLX-TRV2. The obtained vector pLX-TRV2-*CHLI* was transformed into *Agrobacterium* competent cells hosting pLX-TRV1. Bacteria that simultaneously harbored pLX-TRV1 and pLX-TRV2-*CHLI* were selected in plates supplemented with kanamycin and gentamicin. The isolated *Agrobacterium* strain C58C1(pLX-TRV1, pLX-TRV2-*CHLI*) was infiltrated into *N. benthamiana* leaves. At seven dpi, uniform discoloration consistent with extensive chlorophyll loss was visible in upper uninoculated leaves of plants inoculated with C58C1(pLX-TRV1, pLX-TRV2-*CHLI*) (Figure 3A). Serial dilution assays showed that infiltration of a bacterial suspension with an optical density at 600 nm (OD_{600}) of $1{\cdot}10^{-5}$ was sufficient to generate robust *CHLI* silencing (Figure 3B). No photobleaching was detected in control plants (Mock, Figure 3A and B).

3.4 | Use of JoinTRV system for protein overexpression

Besides the use in VIGS applications, tobravirus-based vectors have been engineered for protein overexpression in plants.[15] Genes

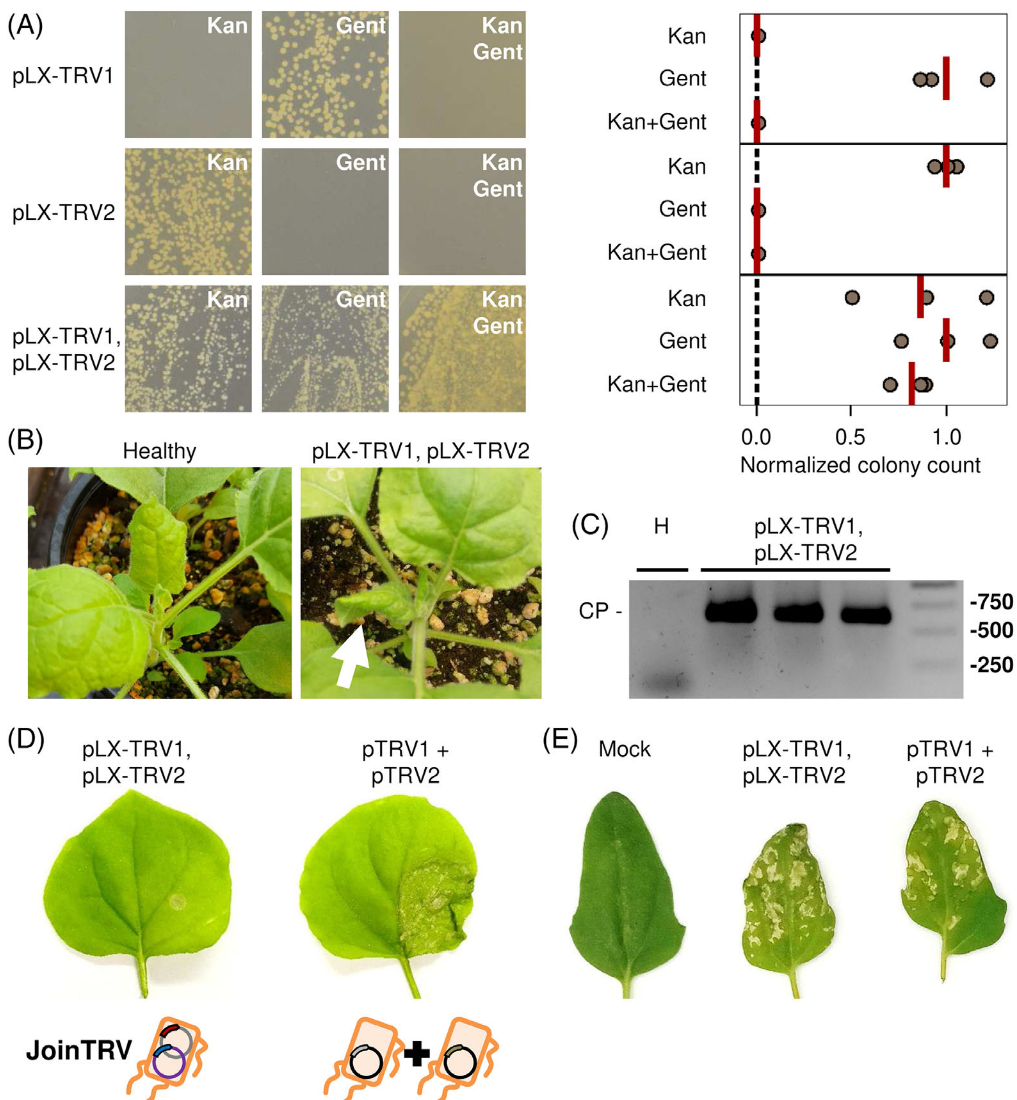

FIGURE 2 Multiplexing and delivery to plants of TRV genomic components using the JoinTRV system. pLX-TRV1, pLX-TRV2, or the vector pair were transformed into *Agrobacterium* cells. (A) Growth of *Agrobacterium* strains that harbor a single or multiple T-DNA vectors for TRV component delivery. Colony images and normalized counts ($n = 3$) using plates supplemented with the indicated antibiotics are shown. (B) Systemic TRV infection using JoinTRV. *Nicotiana benthamiana* plants were inoculated with C58C1(pLX-TRV1, pLX-TRV2), an *Agrobacterium* strain that simultaneously hosts pLX-TRV1 and pLX-TRV2. Symptoms of upper uninoculated leaves at three dpi are shown (the arrow indicates leaf curling). (C) Viral RNA detection in upper uninoculated leaves collected at three dpi from the assay described in panel B. RT-PCR amplification results of the TRV CP gene are shown; H, healthy. (D) Symptoms of *N. benthamiana* leaves infiltrated with C58C1(pLX-TRV1, pLX-TRV2) and a bacterial suspension mix of two *Agrobacterium* strains hosting pTRV1 or pTRV2, that is, C58C1(pTRV1) plus C58C1(pTRV2); pictures were taken at four dpi. (E) Lesions of *Chenopodium quinoa* leaves mechanically inoculated with a mock solution or extracts prepared from upper uninoculated leaves collected at three dpi from the *N. benthamiana* assay described in panel D; pictures were taken at four dpi

encoding *Pantoea ananatis* phytoene synthase (crtB) or GFP were inserted into pLX-TRV2 under the PEBV promoter to assess the JoinTRV usefulness for protein production in plants. The correct translation of these reporter genes from the obtained vectors pLX-TRV2-crtB and pLX-TRV2-GFP can be visually assessed. The crtB overexpression triggers chloroplast-to-chromoplast differentiation, which results in a bright yellow pigmentation,[21,35] whereas GFP accumulation can be easily tracked by fluorescence imaging. *Agrobacterium* strains that simultaneously harbored pLX-TRV1 and one of the overexpression constructs were selected and infiltrated into *N. benthamiana* leaves. In agreement with the phenotype reported upon virus-mediated overexpression of crtB,[21] leaf yellowing was detected in upper uninoculated leaves of plants inoculated with C58C1(pLX-TRV1, pLX-TRV2-crtB); no color alteration was visible under natural light illumination in the mock or C58C1(pLX-TRV1, pLX-TRV2-GFP) conditions (Figure 3C). Fluorescencestereoscope inspection of leaf samples of the latter revealed green signal in plant cells that was consistent with GFP accumulation (Figure 3D). The result was further confirmed by immunoblot detection of GFP in total protein extracts (Figure 3D), which supports the suitability of assembled vec-

FIGURE 3 Use of JoinTRV for plant gene silencing and protein overexpression. (A) A fragment of *N. benthamiana CHLI* (Niben101Scf16898g00001.1) was inserted into pLX-TRV2 to yield pLX-TRV2-*CHLI*. The vector was transformed into *Agrobacterium* along with pLX-TRV1, and the obtained strain C58C1(pLX-TRV1, pLX-TRV2-*CHLI*) was inoculated to *N. benthamiana* plants. Images of upper uninoculated leaves at seven dpi are shown; $OD_{600} = 0.5$ was used. (B) Images show photobleaching upon inoculation of different amounts of C58C1(pLX-TRV1, pLX-TRV2-*CHLI*); OD_{600} values of bacterial suspensions are indicated. (C) Gene sequences of *Pantoea ananatis* crtB or GFP were inserted into pLX-TRV2 to yield pLX-TRV2-crtB and pLX-TRV2-GFP, respectively. The vectors were transformed into *Agrobacterium* along with pLX-TRV1, and the obtained strains C58C1(pLX-TRV1, pLX-TRV2-crtB) or C58C1(pLX-TRV1, pLX-TRV2-GFP) were inoculated to *N. benthamiana* plants. Images of upper uninoculated leaves at seven dpi are shown. (D) GFP was detected by fluorescence imaging (left; at five dpi) and immunoblotting (right) of upper uninoculated leaf samples of the indicated conditions; M, mock; Ponceau stained membrane is shown as a loading control

tor pLX-TRV2 and the JoinTRV system for protein overexpression in plants.

3.5 | JoinTRV allows heritable CRISPR-Cas9-mediated genome editing

VIGE is a promising technology for obtaining genome-edited plants without the need for tissue culture.[8,14] Viral entry in shoot apical meristems occurs during tobravirus infection.[15] This feature has been exploited in plant engineering strategies for germinal transmission of desired genome modifications and recovery of mutant progeny.[36,37] Tobravirus vectors were used to deliver sgRNAs into Cas9-overexpressing hosts and to subsequently recover mutant plants.[10,22,38]

A sgRNA spacer was designed to target a 20-nt conserved region in the second exon of two *Phytoene desaturase 3* (*PDS*) homeologs present in the genome of the allotetraploid *N. benthamiana* (Niben101Scf01283g02002.1 and Niben101Scf14708g00023.1; Figure 4A). The sgRNA scaffold was extended with a truncated *Flowering locus T* (*FT*) sequence that was shown to promote long-distance mobility of sgRNAs and editing efficiency,[22,23] and inserted in pLX-TRV2 to yield pLX-TRV2-sgPDS (Figure 4A). C58C1(pLX-TRV1, pLX-TRV2-sgPDS), an *Agrobacterium* strain that simultaneously harbored pLX-TRV1 and pLX-TRV2-sgPDS, was selected and infiltrated into *N. benthamiana* plants. Agro-inoculation of a Cas9 transgenic line resulted in a leaf mosaic with green and photobleached sectors. The phenotype increased in intensity as the plants matured, and was absent in wild-type plants inoculated with the same bacterial strain (Figure 4B and C). We reasoned that the Cas9-dependent, photobleaching phenotype

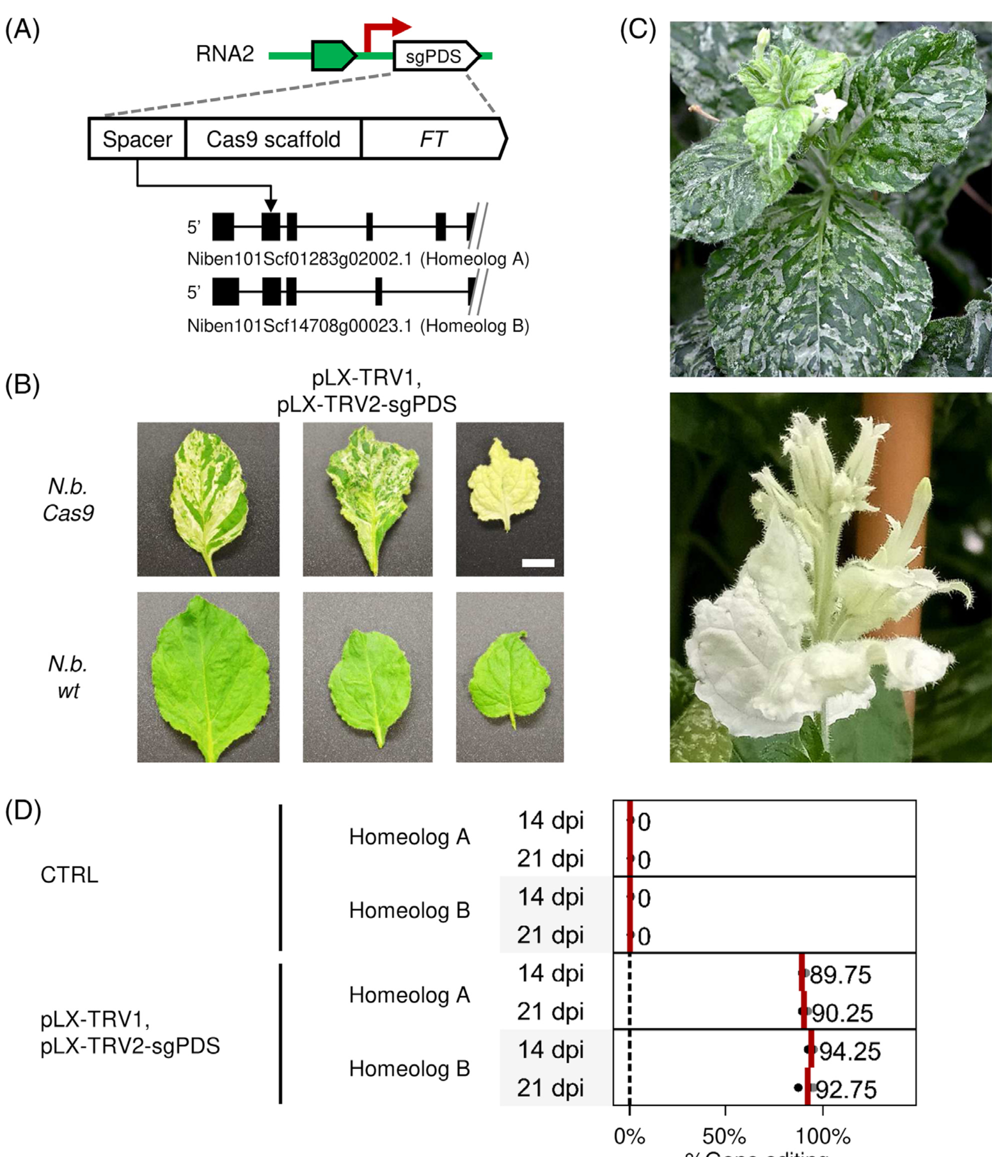

FIGURE 4 JoinTRV-mediated delivery of sgRNAs and genome editing of somatic tissue. A sgRNA targeting the *N. benthamiana* PDS homeologs Niben101Scf01283g02002.1 and Niben101Scf14708g00023.1, and fused to a *FT* fragment was inserted into pLX-TRV2. The vector was transformed into *Agrobacterium* along with pLX-TRV1, and the obtained strain C58C1(pLX-TRV1, pLX-TRV2-sgPDS) was inoculated to *N. benthamiana* plants. (A) Diagrams of the construct expressed by pLX-TRV2-sgPDS, and of the 5′ portion of targeted genes are shown. (B) Pictures show leaves of a *Streptococcus pyogenes* Cas9 transgenic line (*N.b. Cas9*) or wild-type (*N.b. wt*) plants inoculated with C58C1(pLX-TRV1, pLX-TRV2-sgPDS); scale bar = 1 cm. (C) Phenotypes of the inoculated, mature Cas9 plants. (D) Genomic editing in somatic tissue. Genomic DNA from upper uninoculated leaf samples was PCR amplified using homeolog-specific primers; Sanger sequencing data were computationally processed to quantify editing efficiencies; red lines indicate mean values ($n = 4$)

was due to editing and functional loss of the *PDS* homeologs in somatic cells. Genomic DNA was purified from upper uninoculated leaves and fragments spanning the predicted sgPDS targeting sites were amplified by homeolog-specific PCR. Sanger sequencing of the PCR products and interference of CRISPR edits by computational analysis indicated an editing efficiency of ≈90% at both homeologs (Figure 4D). No *PDS* mutagenesis could be detected in control plants (Figure 4D).

Seeds were collected from a Cas9 plant inoculated with C58C1(pLX-TRV1, pLX-TRV2-sgPDS) and sown onto soil. *PDS* sequences were analyzed using homeolog-specific PCR primers to assess the germinal transmission of genomic modifications. A small screen of 40 seedlings was sufficient to identify mutant progeny. Mutations at the targeted loci were identified in more than half of the seedlings (57.5%; Figure 5A), most of which had one mutant allele (heterozygous; Figure 5B). Five lines had homozygous/biallelic mutations at one of the two homeologs (Figure 5C). Two lines showed editing inheritance at both *PDS* homeologs, albeit only heterozygous mutations were identified (stars in Figure 5C). In summary, these results support the usefulness of JoinTRV for targeted CRISPR/Cas9-mediated genome mutagenesis and tissue culture-free rescue of mutant progeny.

FIGURE 5 JoinTRV-mediated heritable plant genome editing and tissue culture-free rescue of mutant progeny. Seeds were collected from a Cas9 plant inoculated with C58C1(pLX-TRV1, pLX-TRV2-gPDS), sown on soil, and the derived plantlets ($n = 40$) were genotyped by sequencing of the targeted *PDS* homeologs. (A) Percentage of the mutant plants recovered. (B) Percentages of genotypic classes identified for each homeolog. (C) Genotypes of the plantlets analyzed. Genotypic classes shown in B and C are as follows: wild-type, no mutation; heterozygous, progeny with one mutant allele at the indicated homeolog; homozygous/bi-allelic mutation, progeny with no wild-type allele at the indicated homeolog; stars indicate plantlets with at least a mutant allele for each of the two homeologs

4 | DISCUSSION

Traditional plant breeding is time- and cost-consuming and innovative strategies are needed for accelerated and tailored manipulation of crop traits.[39,40] Virus-mediated engineering of plant genomes and agronomic performance traits has great potential to be directly used for crop breeding and reprogramming.

Here, we show that the use of binary vectors with compatible origins in a one-strain/two-vector system provides a robust approach for *Agrobacterium*-mediated delivery of engineered TRV components. The generated JoinTRV system was successfully used in a variety of assays that included plant gene silencing, recombinant protein overexpression, and CRISPR-Cas9-mediated genome editing.

The JoinTRV vectors are substantially smaller than those of any known TRV vector system, and pLX-TRV2 can be easily customized to express desired sequences by one-step digestion-ligation and homology-based cloning. The system presented is based on pLX backbones that have been tested in a variety of *Agrobacterium* strains,[29] and is expected to be functional in any strain sensitive to kanamycin and gentamicin, the antibiotics used for vector selection. Although not tested, JoinTRV efficiency could be possibly enhanced by the use of hypervirulent strains such as AGL-1, EHA105, or recently described derivatives of the chrysopine type.[41,42]

Recently, *Agrobacterium*-mediated delivery of CRISPR/Cas9 components was reported using a ternary vector system composed of a first, nonautonomous T-DNA vector and a second plasmid that provides the replication function for the first vector.[43] In contrast, JoinTRV relies on two mini T-DNA vectors that are autonomous and do not require coresident plasmids for their maintenance in *E. coli* and *Agrobacterium*. Our TRV vectors are compact and facilitate flexible experimental designs since they can be used in the one-strain/two-vector approach presented, as well as through a culture pool of two strains hosting a single T-DNA vector (i.e., one strain/one vector).

JoinTRV-mediated delivery of sgRNAs targeting the *PDS* homeologs resulted in the visual detection of somatic cells with a photobleaching phenotype typical of carotenoid biosynthetic defects. The sgRNA construct used included a 20-nt spacer homologous to the endogenous *PDS* genes as well as transcripts. Recent work showed that guide RNAs can induce gene silencing in plants in the absence of Cas effectors.[44] Our results nonetheless indicate the photobleaching phenotype associated with inoculation of C58C1(pLX-TRV1, pLX-TRV2-sgPDS) is strictly Cas9 dependent, and thus due to functional loss of the targeted genomic loci. In agreement with our finding, a TRV vector including a 54-nt *PDS* fragment, or a potato virus X vector with a 20-nt homology with a transgene did not trigger VIGS in previous surveys.[45,46]

Consistent with very recent reports,[22] we show that the use of JoinTRV for delivery of sgRNAs into Cas9-overpressing plants allows tissue culture-free rescue of mutant progeny. Of note, over 50% of the progeny seedlings showed mutations at the targeted loci. *N. benthamiana* is allotetraploid; no biallelic, homozygous mutations at both *PDS* homeologs (that is with four alleles simultaneously being edited) could be identified in our screen. This may be a consequence of a counterselective bias during germination or early development stages of progeny with *PDS* functional loss, which can implicate an underestimation of frequencies of mutagenesis heritability. Special selection schemes including embryo rescue or seed culture in sucrose-containing medium might be required to recover plants homozygous for defects in early steps of carotenoid biosynthesis.[47,48]

Given the known *Agrobacterium* promiscuity and the broad-host-range of TRV,[15,33] JoinTRV would likely be applicable with no or minor optimization for genome engineering of most of the dicot species for which transgenic lines expressing Cas nucleases are available. Additionally, miniature Cas12 versions, as well as the transposon-associated IscB and TnpB, are ≈400–500-amino acid-long DNA nucleases that were recently reported as effective tools for

eukaryotic genome editing.[49–53] Compact RNA-guided endonucleases provide an opportunity for enhanced viral-mediated delivery of editing reagents in transgenic-free plant cells.

Spraying crops with suspensions of *Agrobacterium* strains hosting viral vectors has been recently reported for fast, tunable reprogramming of agronomic traits.[41] Besides the positive results obtained in the proof-of-concept experiments of this study and given its simplified logistics, JoinTRV, and the presented one-strain/two-vector approach could be of interest for industrially scalable transfection of crops with multipartite viral vectors.

The binary vector multiplexing approach herein presented could be applied for other viral vector systems that require simultaneous plant inoculation of multiple components. For instance, systems amenable for multiplexing include those based on multipartite viruses,[48,54–59] combination of helper viruses/satellites,[60,61] as well as incomplete genomic fragments that reconstitute functional replicons upon *in planta* recombination.[62–64] In conclusion, this work expands the toolbox for plant virus vector engineering and adds a new flexibility level for plant cell delivery of multicomponent viral systems and genome editing reagents.

ACKNOWLEDGMENTS

This work was funded by Ministerio de Ciencia e Innovación, Spain, and co-financed by European Regional Development Fund (grant PID2020-114691RB-I00). F.P. is the recipient of a Juan de la Cierva Incorporación contract (grant IJC2019-039970-I) from Ministerio de Ciencia e Innovación (Spain). Ministerio de Ciencia e Innovación (Spain, co-financed European Regional Development Fund) grants: PID2020-114691RB-I00 and IJC2019-039970-I.

CONFLICT OF INTEREST

The author declares no financial or commercial conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available in article supplementary material. Other data are available on request from the authors. The JoinTRV vectors are available at Addgene with catalog numbers 180515 (pLX-TRV1) and 180516 (pLX TRV2). Vector sequences from this study can be found at NCBI under the GenBank accession numbers OM372495 (pLX-TRV1) and OM372496 (pLX-TRV2).

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SUPPORTING INFORMATION

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How to cite this article: Aragonés, V., Aliaga, F., Pasin, F., & Daròs, J.-A. (2022). Simplifying plant gene silencing and genome editing logistics by a one-*Agrobacterium* system for simultaneous delivery of multipartite virus vectors. *Biotechnology Journal*, *17*, e2100504. https://doi.org/10.1002/biot.202100504