Pea early-browning virus-mediated genome editing via the CRISPR/Cas9 system in *Nicotiana benthamiana* and Arabidopsis

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Abstract

The clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated (Cas9) system has enabled efficient genome engineering in diverse plant species. However, delivery of genome engineering reagents, such as the single guide RNA (sgRNA), into plant cells remains challenging. Here, we report the engineering of *Tobacco rattle virus* (TRV) and *Pea early browning virus* (PEBV) to deliver one or multiple sgRNAs into *Nicotiana benthamiana* and *Arabidopsis thaliana* (Col-0) plants that overexpress a nuclear localization signal containing *Cas9*. Our data showed that TRV and PEBV can deliver sgRNAs into inoculated and systemic leaves, and this resulted in mutagenesis of the targeted genomic loci. Moreover, in *N. benthamiana*, PEBV-based sgRNA delivery resulted in more targeted mutations than TRV-based delivery. Our data indicate that TRV and PEBV can facilitate plant genome engineering and can be used to produce targeted mutations for functional analysis and other biotechnological applications across diverse plant species.

Key message: Delivery of genome engineering reagents into plant cells is challenging and inefficient and this limit the applications of this technology in many plant species. RNA viruses such as TRV and PEBV provide an efficient tool to systemically deliver sgRNAs for targeted genome modification.

Introduction

The ability to precisely manipulate eukaryotic genomes and transcriptomes can enable studies of protein function and the production of mutants that improve our understanding of the molecular underpinnings of growth, development, and disease states. These approaches require efficient genome engineering technologies that enable control of the genetic material at the single-base level (Zong *et al.*, 2017). Such technologies can be used for targeted mutagenesis, generation of gene knockouts, and precise integration of regulatory sequences or gene fusions. Site-specific nucleases can be engineered to bind a user-defined genomic sequence and generate a double strand break (DSB), which can be subsequently repaired by the imprecise non-homologous end joining (NHEJ) repair, or the precise homology dependent repair (HDR). Therefore, NHEJ is

useful for the generation of functional knockouts, and HDR is useful for the generation of gene variants, gene addition, fusions, and replacement (Mahfouz *et al.*, 2011; Zong *et al.*, 2017).

The clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated (Cas9) system uses the Cas9 endonuclease and a single guide RNA (sgRNA); Cas9 specificity depends on Watson-Crick base pairing between the 20 nucleotides of the sgRNA and the genomic target (Jinek *et al.*, 2012). Therefore, the CRISPR/Cas9 system can be easily engineered to target any sequence by the simple engineering of the 20-bp sequence of the sgRNA (Aouida *et al.*, 2015; Barrangou, 2014). CRISPR/Cas9 has been used for genome editing in diverse plant species and can be applied to all transformable plant species (Ali *et al.*, 2015b).

Efficient genome editing requires transient or stable expression of Cas9 and the sgRNA; improvements in delivery of these two components of the CRISPR/Cas9 machinery can facilitate genome editing (Ali *et al.*, 2015a). Delivery of transgenes producing Cas9 and the sgRNA for the generation of transmissible, germline mutations has traditionally involved the generation of transgenic plants; however, plant transformation and regeneration require time-consuming, resource-intensive work and occur with different efficiencies in different plant species. Because the CRISPR/Cas9 system requires only the 20-nt sgRNA to confer sequence specificity, transient delivery of different sgRNAs into a *Cas9* over-expressing line would enable editing of many different loci, thus enabling a variety of functional studies as well as plant trait engineering (Ali *et al.*, 2015c).

Studies in vertebrate cells have used viruses, including retroviruses and adenoviruses, to deliver genome-engineering reagents (Lombardo *et al.*, 2007). Studies in plants have used *Tobacco rattle virus* (TRV) for virus-induced gene silencing (VIGS), as an efficient tool to facilitate functional genomics in diverse plant species. TRV possesses a bipartite genome, composed of two positive-sense single-stranded RNAs, designated RNA1 and RNA2. The RNA2 genome can be engineered to carry gene fragments for functional genomics applications via VIGS (Senthil-Kumar & Mysore, 2014).

To expand the possible host range, several other RNA viruses have been used to deliver different nucleic acids into various plant species. Under natural conditions, *Pea early browning virus* (PEBV) can infect at least 30 dicotyledonous species of 10 different plant families, including key crops such as *Pea*, *Faba bean*, and *Alfalfa*. The host range or PEBV can be extended under

laboratory conditions to include *Tomato*, *Potato*, and *Rapeseed* (Constantin *et al.*, 2004). The cargo capacity of plant RNA viruses, including TRV, can allow the engineering of the viral genome to carry and produce sgRNAs. Recently, we and other demonstrated the use of the viral system to deliver an sgRNA to target endogenous plant genomic sequences and the genome of invading DNA viruses (Ali *et al.*, 2015a; Cody *et al.*, 2017; Zaidi *et al.*, 2016). Here, we expanded the use RNA viruses by engineering the PEBV-RNA2 and TRV for genome editing of *Arabidopsis*. Engineering of the RNA2 genome to carry single or multiple sgRNAs resulted in efficient generation of plant genomic mutations and can be used for interference with DNA viruses, indicating the broad usefulness of these viruses for plant genome engineering and disease resistance.

Results and Discussion

To improve the delivery of genome engineering reagents to plant cells, we previously developed a TRV-mediated platform that can deliver sgRNAs into *Cas9*-overexpressing plants (Ali *et al.*, 2015a). To extend this platform to other plant species, we generated an *Arabidopsis Cas9* overexpression line. *Arabidopsis Cas9*-overexpressing (*Cas9*-OE) plants were generated by the floral dip method, with the *Agrobacterium tumefaciens* GV3101 strain harboring the pEarleyGate103 construct *35S::Cas9:GFP*, which produces functional Cas9 fused to green fluorescent protein (GFP) and tagged with the Flag epitope. Basta-resistant seedlings were analyzed for the production of Cas9 protein by immunoblotting with GFP and Flag antibodies (Supplementary Figure 1). Two independent lines expressing significant levels of Cas9 (lines 17 and 22) were selected for further experiments.

Next, two sgRNAs targeting the *Arabidopsis GLABRA1* (*GL1*) and *TRANSPARENT TESTA GLABRA4* (*TT4*) sequences were cloned under the PEBV promoter in the RNA2 genome of TRV. Mutants of these two loci results in lack trichomes and transparent testa, easily visible phenotypes. We then determined whether the engineered TRV can produce and deliver sgRNAs molecules in inoculated and systemic leaves of *Arabidopsis*. To this end, *Agrobacterium* strains harboring TRV (RNA1 and RNA2) were used to infect plants. For *Arabidopsis*, TRV was first reconstituted in wild-type *N. benthamiana* leaves and then rub-inoculated into rosette leaves 3, 4, 5 of *Cas9*-OE *Arabidopsis* plants (Figure 1A). We then tested for mutations with the T7EI assay, which uses an endonuclease to detect mismatches, to test whether the sgRNAs had induced

editing of the target sequences in systemic (non-inoculated) leaves. The modification rates were analyzed using ImageJ software, as described previously (http://imageJ.nih.gov/ij/). Fifteen days post-infiltration (dpi), genomic DNA was extracted from the rosette leaves 10, 11, 12 of TRV infected (line 17) *Arabidopsis* plants. T7EI assay of PCR products from the targeted genomic sequences demonstrated a high level of genome editing in *GL1* and *TT4* (Figure 1B).

To extend this efficient sgRNA delivery platform to RNA viruses that can be used in economically important plants such as legumes, we selected the *Pea early browning virus* (PEBV) (Constantin *et al.*, 2004). Similar to TRV, the RNA2 genome of PEBV can be engineered to deliver open reading frames or regulatory sequences. We cloned sgRNAs targeting the *N. benthamiana PDS* gene, which encodes phytoene desaturase, and generated a cassette including the pPEBV promoter and sgRNA (pPEBV::Nb-PDS-sgRNA). We cloned this cassette into RNA2 of PEBV. The PEBV RNA1 and engineered RNA2 were mixed in infiltration buffer and agro-infiltrated into the lower leaves of *Cas9*-OE *N. benthamiana* plants. Genomic editing of *PDS* in both inoculated and systemic leaves was assessed by T7EI assays in three independent plants using PCR amplicons of a 797 bp fragment flanking the *PDS* target sequence. The T7EI assay and Sanger sequencing indicated robust genome editing efficiencies both in inoculated and systemic leaves (Figure 1C).

Next, we compared the gene editing efficiencies of the TRV and PEBV systems in *N. benthamiana*. Our results confirmed more-efficient genome editing by PEBV (57 to 63%) compared to that of TRV (27 to 35%) (Figure 1D and supplementary Table 1). To determine whether PEBV-mediated gene editing system exhibit off-target activities, we identified potential off-target sites in the *N. benthamiana* genome and PCR amplified eight different off-target loci to assess the off-target activities. Our results showed that the PEBV-mediated gene editing exhibited no off-target activities at these loci, or the off-target activities were too low to be detected (data not shown).

Here, we showed that PEBV can be used for plant genome engineering similar to TRV, and that PEBV exhibited robust efficiency for targeted mutagenesis of multiple targets. Furthermore, we extended the application of the TRV to *Arabidopsis*, showing that TRV can be used to deliver sgRNA into *Arabidopsis* for targeted mutagenesis. This work expands the utility of TRV and PEBV for various genome engineering applications in *N. benthamiana* and *Arabidopsis* model

plants. Therefore, this work establishes TRV and PEBV as efficient sgRNA delivery tools for CRISPR/Cas9-based genome engineering in model and non-model plant species. Indeed, more 200 plant species are natural hosts for TRV, and this increases 400 under laboratory conditions (Senthil-Kumar & Mysore, 2014). Moreover, viral-mediated genome editing is not limited to vectors derived from TRV and PEBV; other RNA viruses including *Tobacco mosaic virus* (TMV) and *Potato Virus X* (PVX) could also be used to deliver the sgRNA molecules to various plant species.

Our data demonstrated that the TRV can produce sgRNA molecules in inoculated and systemic *Arabidopsis* leaves leading to high frequencies of targeted sequence modification. Theoretically, the modified tissues could then be regenerated into whole plants and screened for the presence of the modification. PEBV and TRV can also infect meristematic tissues (Constantin *et al.*, 2004; Senthil-Kumar & Mysore, 2014); therefore, germinal transmission of the genomic modifications may be feasible and seed progeny can be screened for the modification. This approach of sgRNA delivery and genome editing might provide a general method for the recovery of seeds with the desired targeted modifications, and may obviate the need for classical tissue culture and subsequent transformation to generate heritable targeted modifications.

This viral-mediated genome editing system exhibits several important features: 1) TRV and PEBV can systematically infect a large number of plant species, under natural and laboratory conditions; 2) these viruses are easily introduced into plants via *Agrobacterium*; 3) the small genome size of these viruses facilitates sgRNA cloning, library construction, and subsequent agroinfection; and 4) importantly, the viral RNA genomes of TRV and PEBV do not integrate into plant genomes. Therefore, this approach will likely have broad utility for producing targeted mutations.

In conclusion, this work expands the utility of plant RNA viruses for CRISPR/Cas9-based genome engineering for functional genomics and biotechnology applications.

Material and Methods

Plant material and growth conditions

All experiments were carried out on T3 progeny plants of Cas9 over-expressing Arabidopsis or *N.benthamiana* plants. *N. benthamiana* line B14, (Ali *et al.*, 2015a) and

Arabidopsis line 17. Seeds (100-200) were sown in a single pot on soil directly in green house for 7days and were transferred individual pots.

sgRNA expressing plasmid construction

The sgRNAs against Arabidopsis genomic DNA targets GL1 and TT4 were custom synthesized. The fragment containing XbaI recognition site, 20 nucleotides guide sequence, 84bp Cas9 binding scaffold and terminator (9 – T) followed by XmaI recognition were inserted in to the XbaI – XmaI of TRV-RNA2 under pPEBV promoter.

To target PDS through PEBV system, the whole cassette pPEBV::NB-PDS-sgRNA of 309bp (Ali *et al.*, 2015a) (SpeI recognition site, 193bp PEBV promoter, 20 nucleotide target guide sequence, 84 bp Cas9 binding scafold loop followed by poly T (9 – T) termination and PstI site) was PCR amplified using the respective forward, PCAP2-PEBV-sgRNA-F, ATGGACTAGTGAGCATCTTGTTCTGGGGTTTCA and reverse PCAP2-PEBV-sgRNA-R, GATCTGCAGAAAAAAAGCACCGACTCGG primers containing SpeI and PstI recognition sites respectively. The PCR product was digested with respective enzymes and 309bp fragment was gel purified. PEBV RNA1 (pCAPE1, PEBV1) and RNA2 (pCAPE2, PEBV2) were used in these clones (Constantin *et al.*, 2004). The PsPDS fragment between SpeI and PstI in pCAPE2-PsPDS (RNA2 vector of PEBV system) (Constantin *et al.*, 2004) was replaced with the PEBV::sgRNA cassette. All clones were confirmed with SpeI and PstI digestion and Sanger sequencing for the presence of pPEBV-sgRNA fragment.

Agrobacterium transformation and culture growth

Electro-competent cells (50ul) of GV3101 were transformed with the vectors containing engineered TRV and PEBV RNA1 and RNA2 genomes and spread on to 50mg/L Kanamycin, 25mg/L Rifampicin and 30mg/L Gentamycin LB agar plates. Single colony was picked and grown in 5ml LB media with kanamycin, gentamicin and rifampicin at 28^0C shaking incubator for 36-48 hours. At OD_{600} 1 – 1.2 cells were harvested by centrifuging at 3500rpm at room temperature. Cell pellets were re-suspended in infiltration buffer (10mM MgCl2, 10mM MES and 100uM acetosyringone). For all agrobacterium cells TRV (RNA1, RNA2) or PEBV (RNA1, RNA2) containing sgRNA constructs, OD_{600} was adjusted to 0.3. Cells were placed at room temperature for 2 hours. Before infiltration Agro cell containing RNA1 and RNA2 were mixed in 1:1 ratio.

Inoculation of PEBV AND TRV to Cas9-OE tobacco and Arabidopsis plants

For infecting Arabidopsis, first *N.benthamiana* wild type was agro-infiltrated to enrich engineered TRV. After 10 days inoculation plant sap was collected in 100mM K_2PO_4 (pH8) buffer and 10ul was rub inoculated through carborandon (mish size 250-400) to the leave of two weeks old soil grown Cas9-OE Arabidopsis plants. Samples were collected from systemic leaves 15 day post inoculation (DPI).

For PEBV experiments, 3 weeks old Cas9-OE *N.benthamiana* plants were agro-infiltrated with PEBV, RNA1 and RNA2 to the lower epidermis of two fully expanded leaves by 1ml syringe without needle. Plants were kept covered for two days in green house. After 10 days of infiltration samples from both infiltrated and systemic leaves were collected separately.

DNA extraction from *N. benthamiana* and Arabidopsis

DNA was extracted from grinded samples of both infiltrated and systemic leaves with DNA extraction buffer (100mM Tris-Cl, 1mM EDTA, 100mM NaCl, 100mM LiCl, 100mM B-mercaptoethanol, 0.4% RNase), PCI (Phenol :Chloroform: Isoamyl alcohol), Chloroform and ethanol.

T7 endonuclease I (T7EI) assay for targeted mutation detection

T7EI assay was performed as described by (Reyon *et al.*, 2012). The respective target loci were PCR amplified from the genomic DNA using AtGL1-TR1-gDNA-F GATTCGTTGATAGGGCTAAAGAGATGTGGG and AtGL1-TR1-gDNA-R GGTTGAGTTTCATTGAAGAGAAGACAAACT. For AtTT4, Forward primer AtTT4-TR1-gDNA-F cccgtgtcagtttgttatataagctctcac and reverse primers AtTT4-TR1-gDNA-R TCCGTCAGATGCATGTGACGTTTCCGAATT and for NB-PDS, NB-PDS-TR12-F: GAAACACAACTCCTTGGCGG and NB-PDS-TR12-R: GGGCGTGAGGAAGTACGAAA primers for NB-PDS. Phusion polymerase (NEB) was used for all PCR. The purified 200ng PCR product was re-annealed in NEB Buffer 2 in PCR machine (95^{o}C for 10 min, 85^{o}C for 2 min, 75^{o}C for 3 min, 65^{o}C for 3 min, 55^{o}C for 3 min, 45^{o}C for 3 min, 35^{o}C for 3 min, 25^{o}C for 3 min, 4^{o}C) to make hetero-duplex DNA. T7EI (New England Bioloab) 0.5ul was added to the samples and incubate for 1h at 37^{o}C. The samples were resolved on two (2%) Agarose TAE gel.

Sequencing of targeted loci

To confirm modification at targeted locus, all samples were analyzed by Sanger sequencing. PCR product of the respective loci were cloned in to pJet2.1 cloning vector. All plasmids were Sanger sequenced using T7 primer.

Mutation rate calculations

Mutation rates were estimated by using ImageJ software (http//rsb.info.nih.gov/ij) according to developers protocols and described by (Guschin *et al.*, 2010). The PCR amplified target flanking DNA (300ng) was treated with NcoI for complete digestion. To calculate mutation, rate the intensity of the resistant band was divide by total intensity of all different size DNA bands in a single lane of 2% Agarose gel.

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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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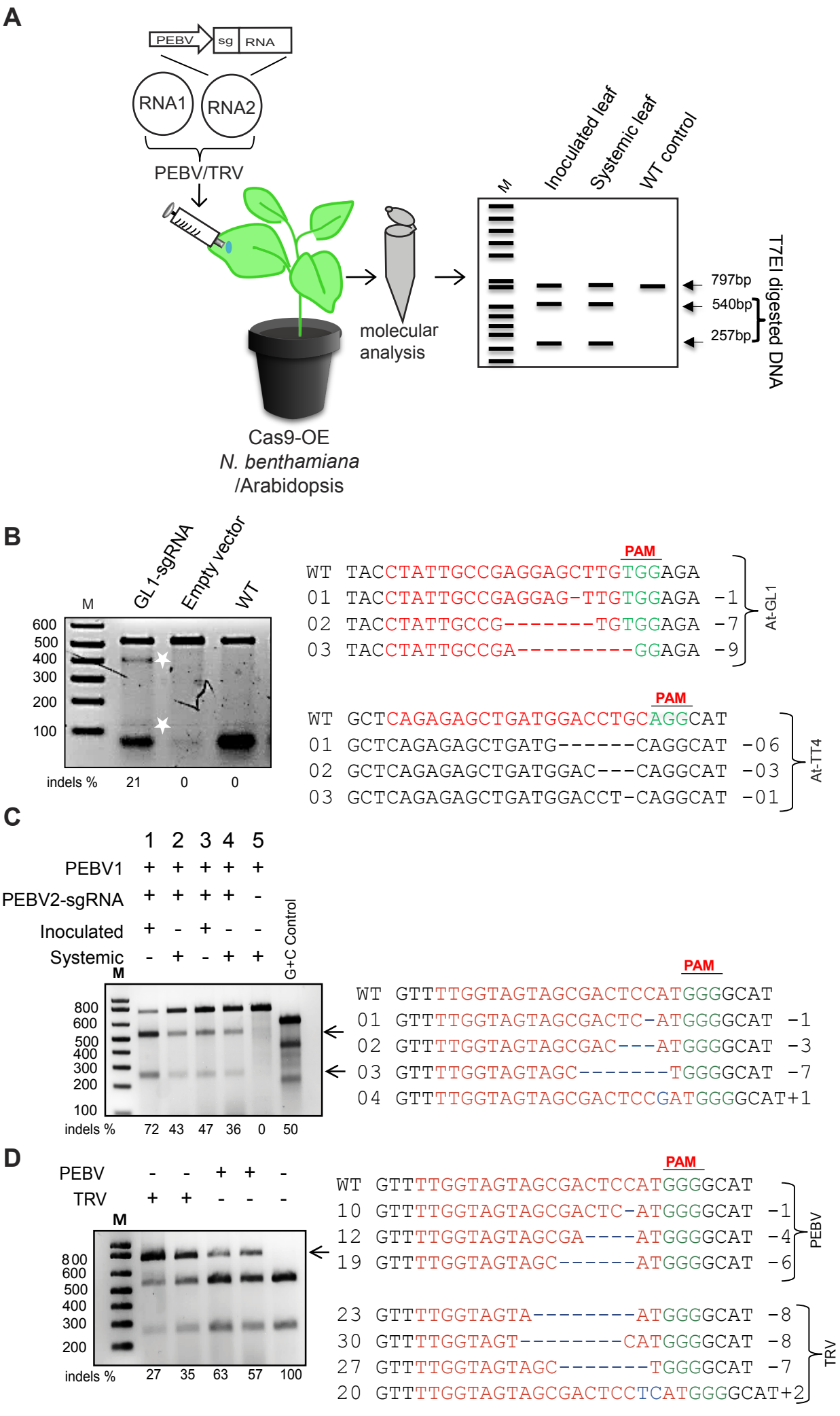
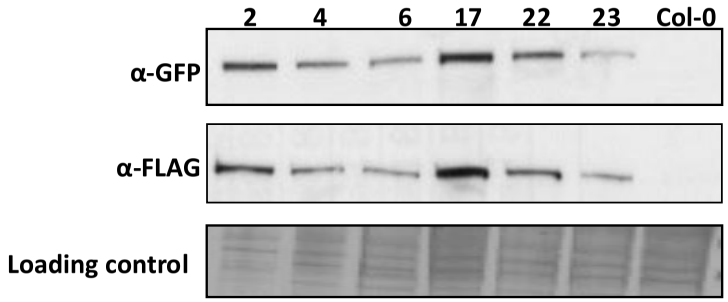
Figure Legend

Figure 1. Virus-mediated gene editing via CRISPR/Cas9 in *Arabidopsis* and *N. benthamiana*.

(A) Schematic representation of the establishment of CRISPR/Cas9 machinery (Cas9, sgRNA) for targeting genomic DNA of *Arabidopsis* / *N. benthamiana*. sgRNAs specific for the genomic sequences are engineered in RNA2 of either PEBV or TRV. *Cas9*-OE *Arabidopsis* or *N. benthamiana* plants were inoculated with RNA1 and RNA2 of PEBV/TRV. After the establishment of CRISPR/Cas9 machinery in plants, samples were collected at 7–10 days post-inoculation (dpi) through infiltration in N. benthamiana or mechanical inoculation sap in Arabidopsis. Molecular analysis, using T7EI assay is represented by a diagram. **(B)** CRISPR/Cas9-mediated targeted modification of the *Arabidopsis* genome. PCR amplicons flanking the target genomic loci of *GL1* and *TT4* were analyzed to detect the mutations. PCR amplicons of *GL1* subjected to T7EI endonuclease. The asterisk indicates the T7EI digested DNA fragment in samples of *GL1*-sgRNA, but not in control samples of TRV empty vector or wild-type plants. Alignment of Sanger sequencing reads of PCR amplicons cloned from GL1 and TT4 targeted regions, PAM and target/mutated sequence are represented. **(C)** PEBV-mediated CRISPR/Cas9-based gene modification of the *N. benthamiana* genome. The *PDS* target flanking genomic DNA was PCR amplified from the samples infiltrated with engineered PEBV. The amplified target region was analyzed for mutation detection with T7EI endonuclease. The arrow indicates T7EI digested DNA fragments in samples collected from both inoculated and systemic leaves of plants infected with *PDS*-sgRNA of PEBV, but not in samples inoculated with empty vector control. *G/C* fragment was used as T7EI experimental control. Alignment of Sanger sequencing reads of *PDS* PCR amplicons cloned into pJET2.1 are shown. Target/mutated sequence and PAM is represented. **(D)** Comparison of PEBV and TRV-mediated CRISPR/Cas9-based gene modification rate of *N. benthamiana* genome. The *PDS* target flanking genomic DNA PCR was analyzed for percentage mutation detection with NcoI endonuclease. The arrow indicates NcoI resistant DNA fragments. A high rate of modification was detected in plant samples collected from engineered PEBV compared to engineered TRV infected plant samples. Sanger sequencing reads alignment of *PDS* PCR amplicons from PEBV upper and TRV lower panel are shown. Target/mutated sequence and PAM are represented. For all experiments, mutation rates (%) were calculated using ImageJ software and represented below each panel.


A
PEBV
sg
RNA
RNA1
RNA2
PEBV/TRV
Cas9-OE
N. benthamiana
/Arabidopsis
molecular
analysis
M
Inoculated leaf
Systemic leaf
WT control
797bp
540bp
257bp
T7EI digested DNA
B
M
GL1-sgRNA
Empty vector
WT
600
500
400
300
200
100
indels %
21
0
0
PAM
WT TACCTATTGCCGAGGAGCTTGTGGAGA
01 TACCTATTGCCGAGGAG-TTGTGGAGA -1
02 TACCTATTGCCG-------TGTGGAGA -7
03 TACCTATTGCCGA---------GGAGA -9
At-GL1
PAM
WT GCTCAGAGAGCTGATGGACCTGCAGGCAT
01 GCTCAGAGAGCTGATG------CAGGCAT -06
02 GCTCAGAGAGCTGATGGAC---CAGGCAT -03
03 GCTCAGAGAGCTGATGGACCT-CAGGCAT -01
At-TT4
C
1 2 3 4 5
PEBV1 + + + + +
PEBV2-sgRNA + + + + -
Inoculated + - + - -
Systemic - + - + +
G+C Control
M
800
600
500
400
300
200
100
indels %
72 43 47 36 0 50
PAM
WT GTTTTGGTAGTAGCGACTCCATGGGGCAT
01 GTTTTGGTAGTAGCGACTC-ATGGGGCAT -1
02 GTTTTGGTAGTAGCGAC---ATGGGGCAT -3
03 GTTTTGGTAGTAGC-------TGGGGCAT -7
04 GTTTTGGTAGTAGCGACTCCGATGGGGCAT+1
D
PEBV - - + + -
TRV + + - - -
M
800
600
500
400
300
200
indels %
27 35 63 57 100
PAM
WT GTTTTGGTAGTAGCGACTCCATGGGGCAT
10 GTTTTGGTAGTAGCGACTC-ATGGGGCAT -1
12 GTTTTGGTAGTAGCGA----ATGGGGCAT -4
19 GTTTTGGTAGTAGC------ATGGGGCAT -6
PEBV
23 GTTTTGGTAGTA--------ATGGGGCAT -8
30 GTTTTGGTAGT---------CATGGGGCAT -8
27 GTTTTGGTAGTAGC-------TGGGGCAT -7
20 GTTTTGGTAGTAGCGACTCCTCATGGGGCAT+2
TRV



Supplementary Figure 1. Cas9 expression confirmation. Cas9 in transgenic Arabidopsis was confirmed by western blot in T3 lines. Flag-Cas9-GFP was detected with both anti-GFP and anti-FLAG antibody. Line 17 and 22 were used for further experiments. The lower panel shows the protein loading control.

Supplementary table 1. Genome editing efficiencies of PEBV and TRV.

Agro-Infection	No. of plants agro-inoculated	No. of leaves showing Indels		Efficiency of genome modification		Type of mutation in the genomic target
		Inoculated leaves	Systemic leaves	Inoculated leaves	Systemic leaves	
PEBV	12 (3 leaves each)	36 (100%)	31(86%)	47 - 72 %	43 - 65 %	1 - 7 nt deletion 1 nt addition substitutions
TRV	12 (3 leaves each)	36 (100%	27(75%)	27 - 49 %	27 - 35 %	1 - 8 nt deletion substitutions