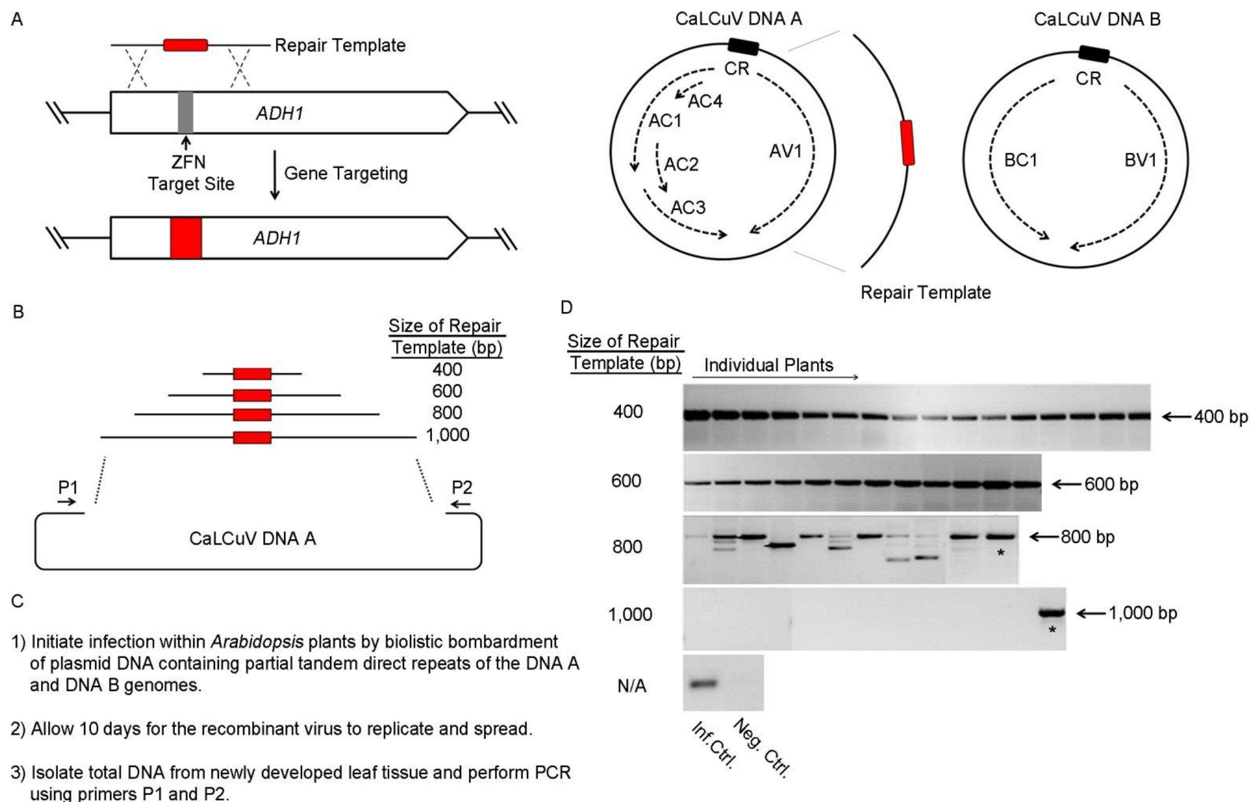


Supplemental Figure 1. Engineering Cabbage Leaf Curl Virus for Delivery of Repair Templates in *Arabidopsis*.

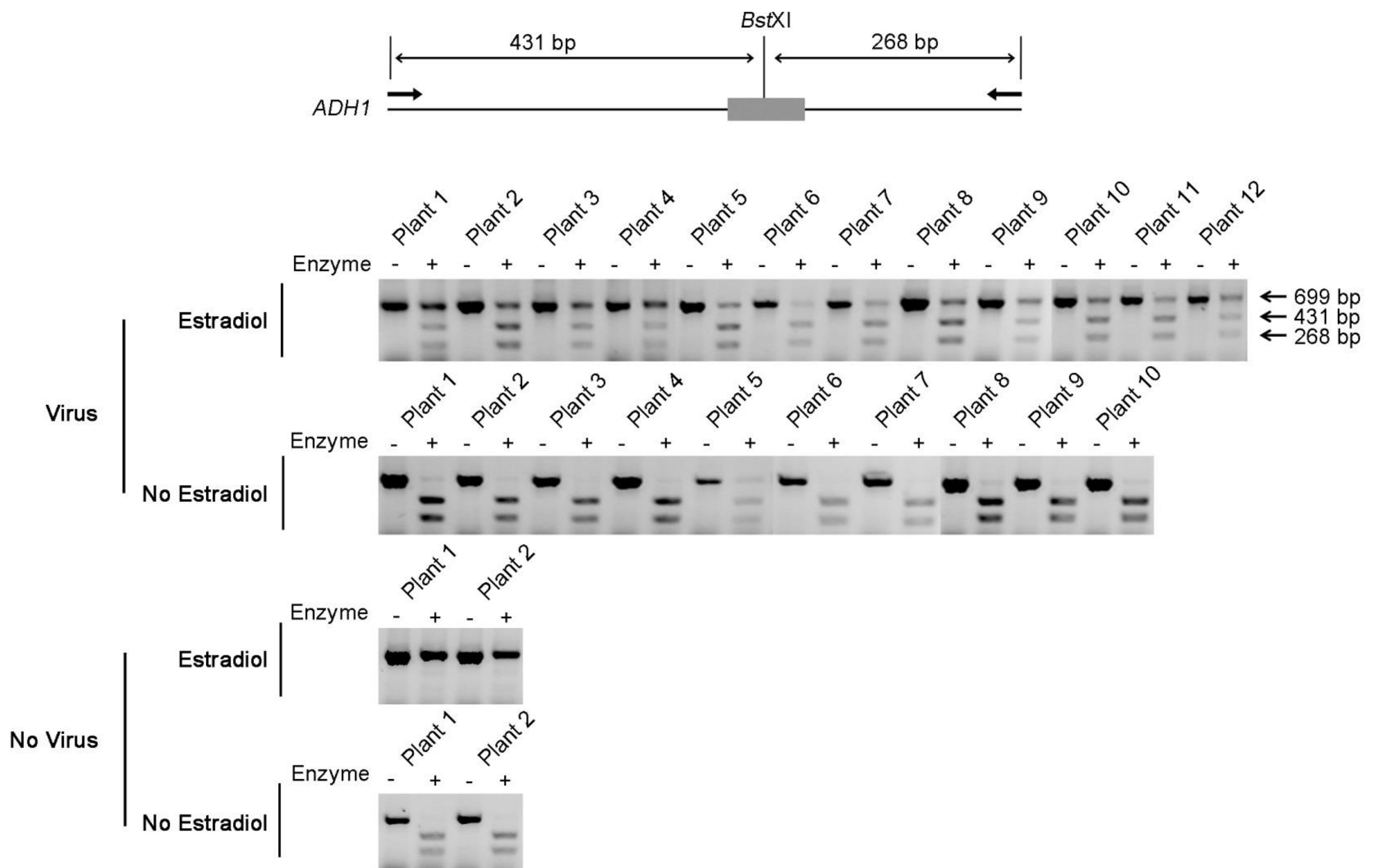
(A) The bipartite geminivirus CaLCuV was modified to deliver repair templates to *Arabidopsis* plants. The target for modification was the alcohol dehydrogenase gene (*ADH1*). Repair templates were designed to introduce a unique 18 bp sequence within the *ADH1* coding sequence (left illustration). The red box represents the 18 bp modification sequence. To generate double-strand breaks within the *ADH1* coding sequence, we stably integrated an *ADH1*-targeting ZFN pair into the *Arabidopsis* genome. The target site for this ZFN pair is illustrated with a gray box. To enable the delivery of repair template sequence, the coat protein gene from CaLCuV DNA A was replaced with repair template sequence (right illustration).CR, common region.
(B) To determine the maximum-size repair template that CaLCuV can stably replicate and spread throughout *Arabidopsis* plants, repair templates ranging from 400 to 1,000 bp in total length were cloned into the DNA A genome. P1 and P2 refer to primers designed to amplify repair templates.
(C) Methodology for assessing the stability of repair template sequences carried by CaLCuV.
(D) Molecular analysis of repair templates carried by infectious viruses. We observed repair-template instability when the total length was 800 bp or greater. This is indicated by the additional shorter bands relative to our positive control (plasmid DNA). Each lane is a different plant that was bombarded with CaLCuV vectors. Asterisks indicate the PCR-positive controls (plasmid DNA was used in replace of total DNA). The infection control (Inf. Ctrl) is from plants bombarded with control vectors pCPCbLVCA.008and pCPCbLCVB.002 (Muangsan and Robertson, 2004). This PCR was expected to yield a single product of approximately 400 bp. The negative control (Neg. Ctrl) is from plants bombarded with gold beads not containing plasmid DNA.

Pos. Ctrl.

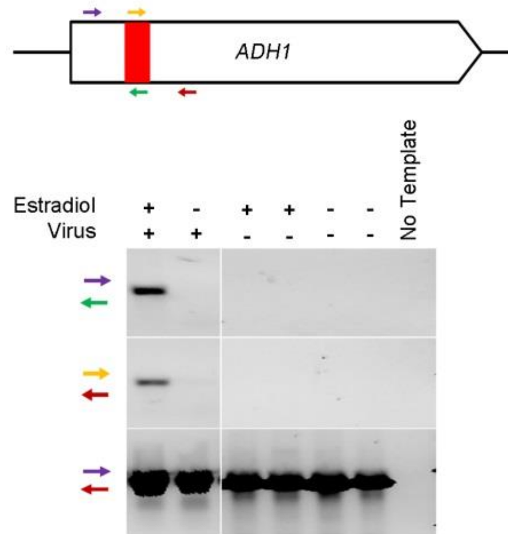
CaLCuV with *ADH1* repair template

Neg. Ctrl.

Supplemental Figure 2. Phenotype of *Arabidopsis* Plants Infected with Cabbage Leaf Curl Virus. *Arabidopsis* plants were bombarded with CaLCuV vectors and infection was monitored. The images are of plants approximately three weeks post bombardment. The positive control (Pos. Ctrl.) is of plants that were bombarded with control vectors pCPCbLCVA.008 and pCPCbLCVB.002 (Muangsan and Robertson, 2004). The DNA A vector pCPCbLCVA.008 contains homologous sequence to the *ChlI* gene as a coat protein gene replacment. As expected, plants infected with this virus displayed yellow or white in newly developed tissue. Additionally, plants displayed symptoms including leaf curling and uneven leaf surface. Plants bombarded with CaLCuV vectors harboring repair template sequences also displayed symptoms of virus infection. This phenotype was used as a marker for successful infection. The negative control (Neg. Ctrl.) image is of a plant bombarded with gold beads.



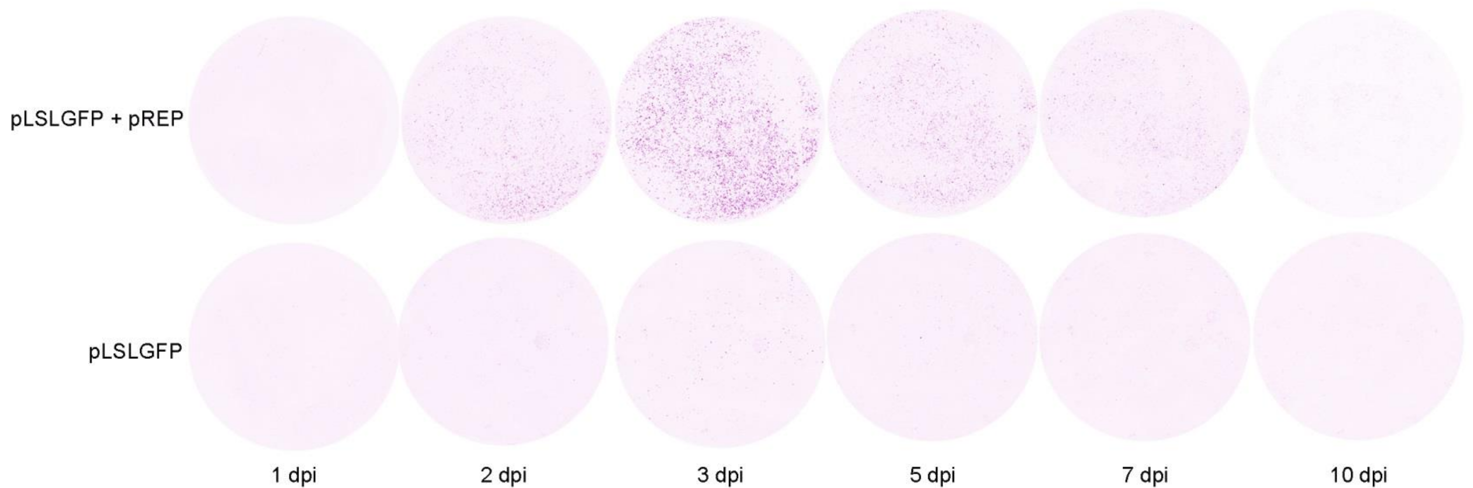
Supplemental Figure 3. Temporal Control of Targeted Double-Strand Breaks in Whole Plants. The estrogen-inducible ZFN pair targeting *ADH1* (Zhang et al., 2010) was integrated into the *Arabidopsis* genome and was assessed for its gene expression to be temporally controlled with estradiol. Three to four week old *Arabidopsis* plants were bombarded with CaLCuV vectors containing 400 or 600 bp repair templates. Plants displaying symptoms of CaLCuV infection (14 days post bombardment) were sprayed with a solution containing estradiol. Seven days post induction, genomic DNA was extracted and the *ADH1* loci were assessed for non-homologous end joining mutations. Genomic DNA was first predigested with *Bst*XI. The digested DNA was then used in a PCR reaction with primers designed to amplify the ZFN target site within *ADH1*. The resulting amplicons were digested with *Bst*XI and resolved by gel electrophoresis. Cleavage-resistant product in samples where the plant was exposed to estradiol indicates that ZFN expression was successfully induced. The No Virus samples were of plants bombarded with gold beads without CaLCuV vectors. Plants not exposed to estradiol were not sprayed with the estradiol solution. Genomic DNA from the control experiments (plants without virus infection and plants not exposed to estradiol) were sampled (and sprayed, if applicable) on the same day as the experimental samples.



Number of PCR-detection events/total number of samples

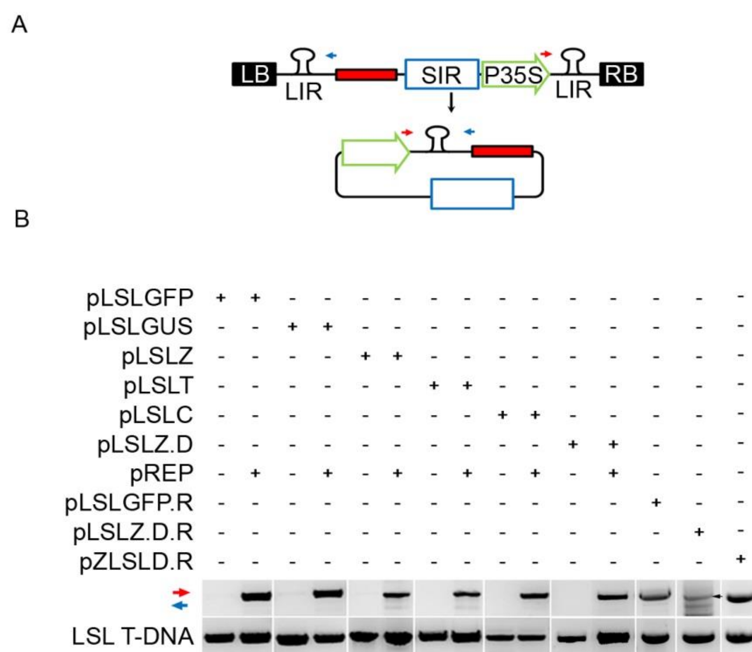
Estradiol and Virus: 1/23
No Estradiol and Virus: 0/18

Supplemental Figure 4. PCR-Based Detection of Geminivirus-Mediated Gene Targeting. Genomic DNA from *Arabidopsis* plants infected with CaLCuV harboring repair templates was assessed for the presence of the 18 bp modification within the *ADH1* gene. Illustration of the *ADH1* gene and the primers designed to detect loci that underwent gene targeting (top). No template, water was added to the PCR solution.



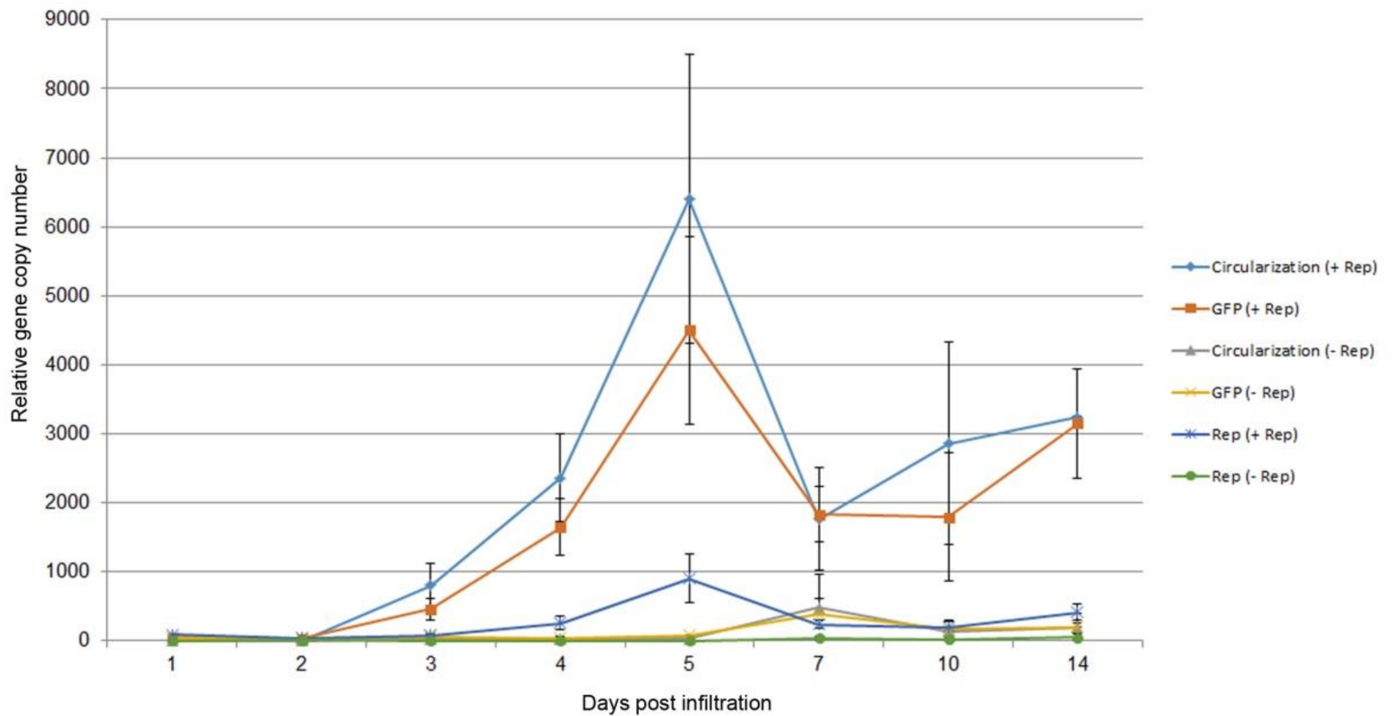
Supplemental Figure 5. Time Course of Geminivirus Replicon-Mediated Expression of Green Fluorescent Protein.

Tobacco leaves were infiltrated with two strains of *Agrobacterium* containing pLSLGFP and pREP (top row) or a single strain of *Agrobacterium* containing pLSLGFP. Following infiltration, leaves were cut into discs and placed in a water. Images were taken using a camera mounted on a confocal microscope (Nikon A1R-A1). Images are of a single leaf disc. Image colors were inverted to better visualize cells expressing GFP.



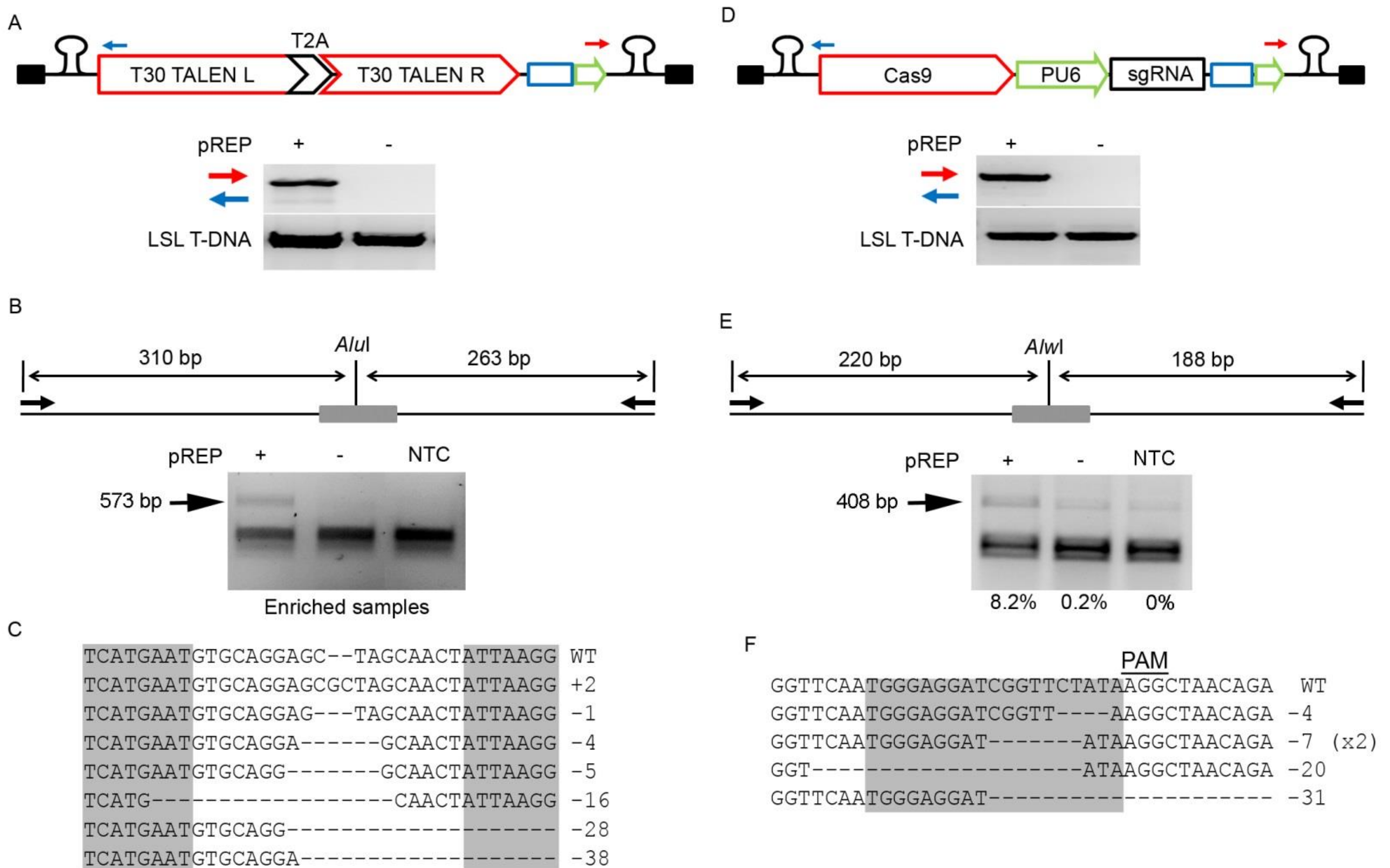
Supplemental Figure 6. PCR-Based Detection of Circularized Geminivirus Replicons.

(A) Illustration of replicational release. LSL T-DNA (top), and circularized replicons (bottom).
(B) PCR-based detection of circularized replicons. LSL T-DNA refers to a control PCR designed to detect sequence present within LSL T-DNA. Replicational release was assessed in tissue three to seven days post infiltration. Detection of circular GVRs with samples pLSLGFP.R, pLSLZ.D.R and pZLSLD.R used primers homologous to sequence within Rep and RepA and to sequence within *GFP*, *Zif268:FokI* or the *us:NPTII* repair template, respectively,



Supplemental Figure 7. Time Course of Geminivirus Replicon Gene Copy Number.

Quantitative PCR (qPCR) was performed to determine the relative gene copy number of GVRs over two weeks. Tobacco leaf tissue was infiltrated with two strains of *Agrobacterium* containing pLSLGFP and pREP (these samples are indicated in the figure legend as + Rep) or a single strain of *Agrobacterium* containing pLSLGFP (- Rep). Total DNA was isolated from different leaves at days 1, 2, 3, 4, 5, 7, 10 and 14 days post infiltration. Gene copy numbers were normalized to an internal control (*F-Box*). Error bars represent s.e.m. of at least two biological replicates. Primers were used that detect circularized GVRs, GFP coding sequence and Rep coding sequence. These samples are indicated in the figure legend as circularization, GFP and Rep, respectively.



Supplemental Figure 8. Expression of TALENs and Components of the CRISPR/Cas System using Geminivirus Replicons.

(A) Illustration of an LSL T-DNA encoding the T30 TALEN pair (top; pLSLT). PCR-based detection of circularized GVRs within plant cells (bottom). T2A, translational skipping sequence.

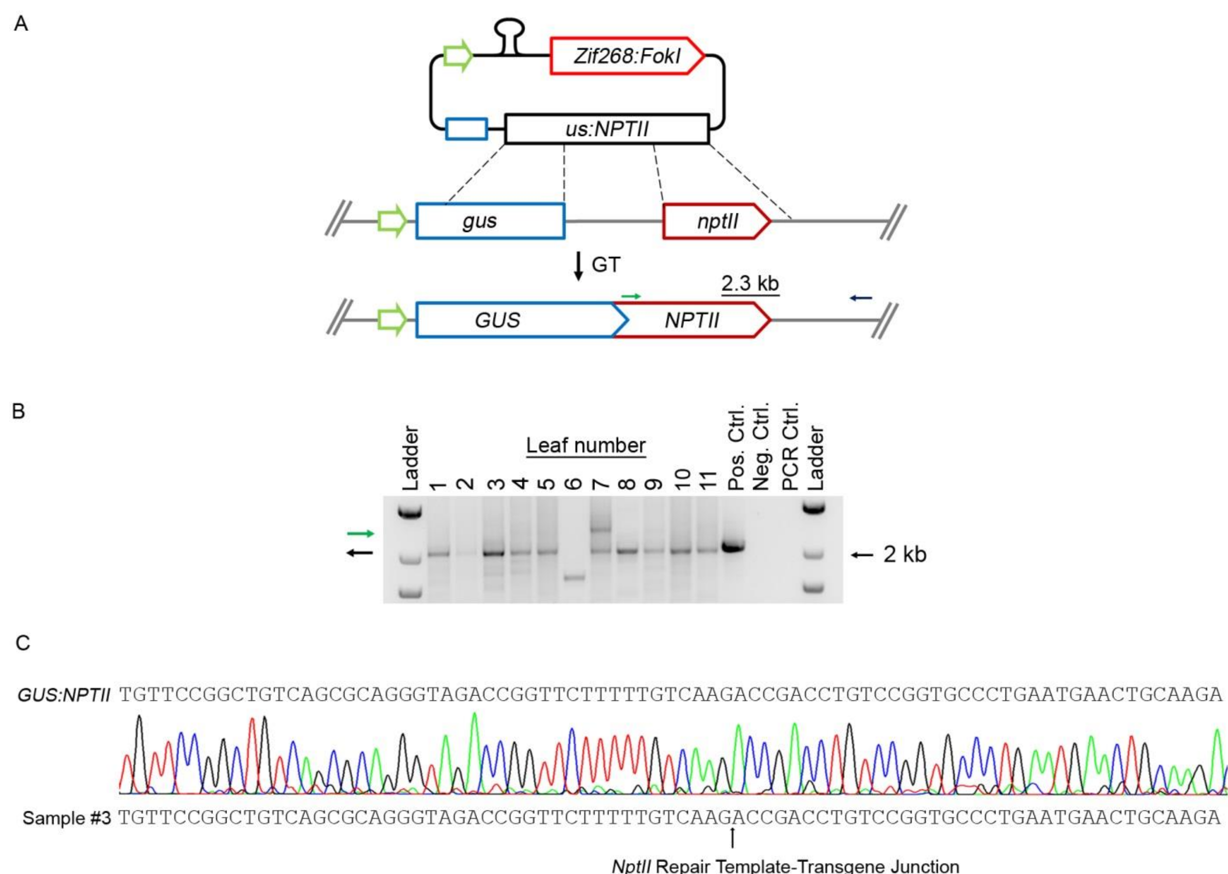
(B) Detection of TALEN-induced mutations at the acetolactate synthase (*ALS*) genes. Tobacco leaf tissue was syringe infiltrated with two strains of *Agrobacterium* containing pLSLT and pREP or a single strain of *Agrobacterium* containing pLSLT. Five days post iniltration, genomic DNA was isolated digested with *Alu*I. Digested DNA was used as a template in a PCR designed to amplify the T30 target site within *SurB*. The resulting amplicons were digested with *Alu*I and bands were seperated by gel electrophoresis. NTC, non-transformed control.

(C) Sequence results from cleavage-resistant amplicons in the sample transformed with pLSLT and pREP T-DNA.

(D) Illustration of an LSL T-DNA harboring a plant codon optimized Cas9 (*Streptococcus pyogenes*). Synthetic guide RNA (sgRNA) was placed downstream of the *Arabidopsis* U6 promoter (PU6; top; pLSLC). PCR-based detection of circularized GVRs within plant cells.

(E) Detection of Cas9-induced mutations at *ALS*. Tobacco leaf tissue was syringe infiltrated with two strains of *Agrobacterium* containing pLSLC and pREP or a single strain of *Agrobacterium* containing pLSLC. Five days post iniltration, genomic DNA was isolated and used as a template in a PCR designed to amplify the Cas9/sgRNA target site within *ALS*. The resulting amplicons were digested with *Alw*I and bands were seperated by gel electrophoresis. The percent of cleavage resistant product seen in the NTC was subtracted from all three samples.

(F) Sequence results from cleavage-resistant amplicons in the sample transformed with pLSLC and pREP T-DNA. PAM, protospacer adjacent motif.

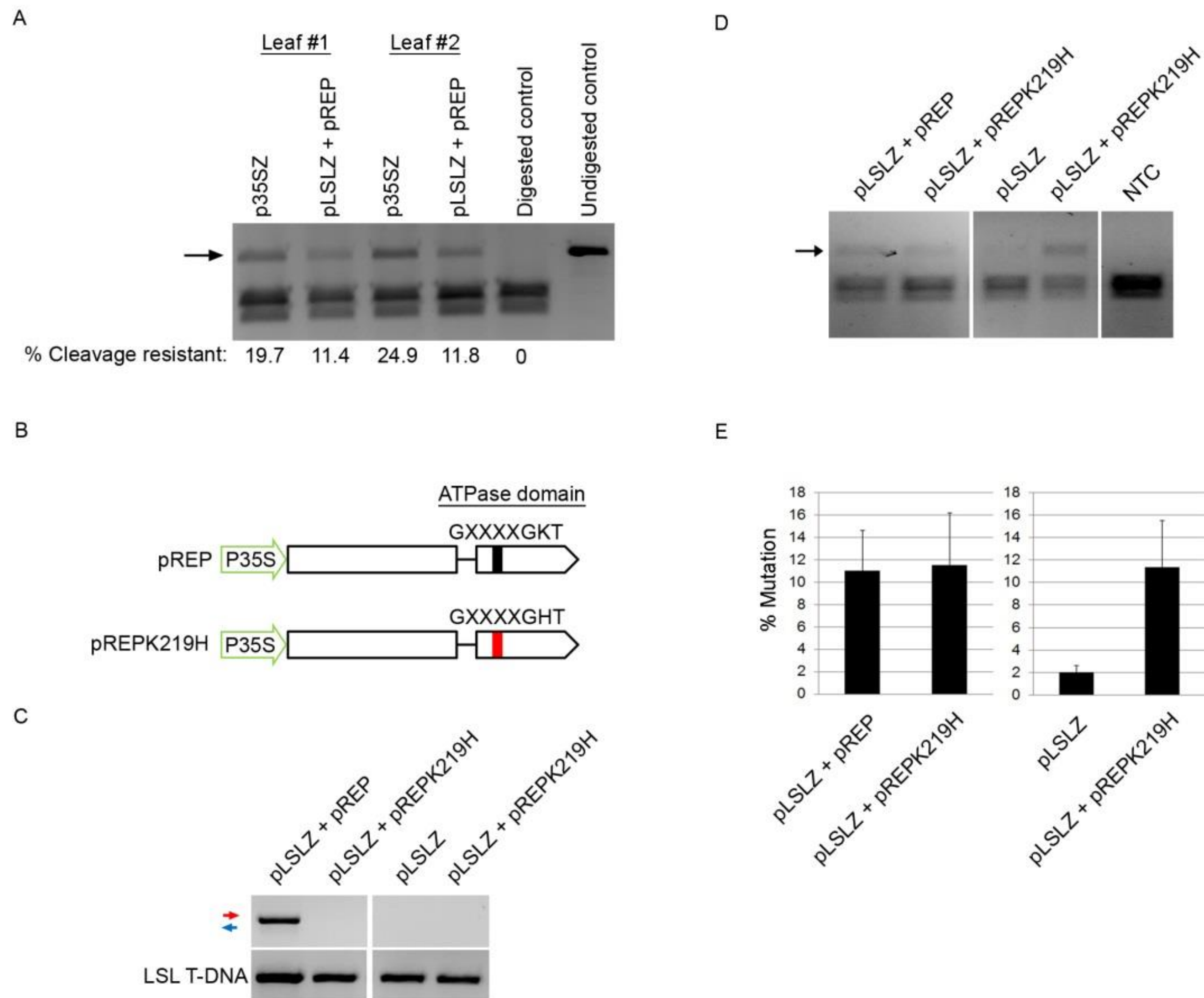


Supplemental Figure 9. PCR-Based Detection of the Repaired *GUS:NPTII* Transgene in Tobacco Leaf Cells.

(A) Illustration of the approach to repair the *gus:nptII* transgene. GT, gene targeting.

(B) Molecular analysis of leaf tissue delivered pLSLZ.D and pREP T-DNA for the repair of the *gus:nptII* transgene. Pos.Ctrl. used plasmid DNA containing the corrected *GUS:NPTII* transgene. Neg. Ctrl. used genomic DNA from non-infiltrated tissue from plants with the *gus:nptII* transgene. PCR ctrl. used water in replace of genomic DNA. Each lane represents a different leaf transformed with pLSLZ.D and pREP. Genomic DNA was extracted five days post infiltration.

(C) DNA sequence of amplicons from leaf number 3.



Supplemental Figure 10. Replication of Sequence-Specific Nucleases Does Not Enhance Targeted Mutagenesis.

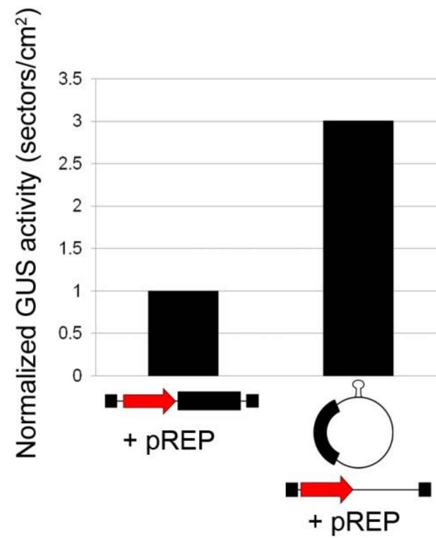
(A) PCR-based detection of ZFN-induced mutations at the Zif268 target site. See also Figure 2B for an illustration of the assay. Numbers beneath the gel image indicate the percentage of cleavage-resistant amplicons. p35SZ refers to a T-DNA plasmid harboring P35S:*Zif268*:*FokI*. The black arrow points to cleavage-resistant product.

(B) Illustration of the the Rep/RepA coding sequence within pREP. A mutation was introduced into the Rep and RepA coding sequence (K219H) within the conserved ATPase domain. This K219H mutation is predicted to result in the loss of replication activity (Desbiez et al., 1995).

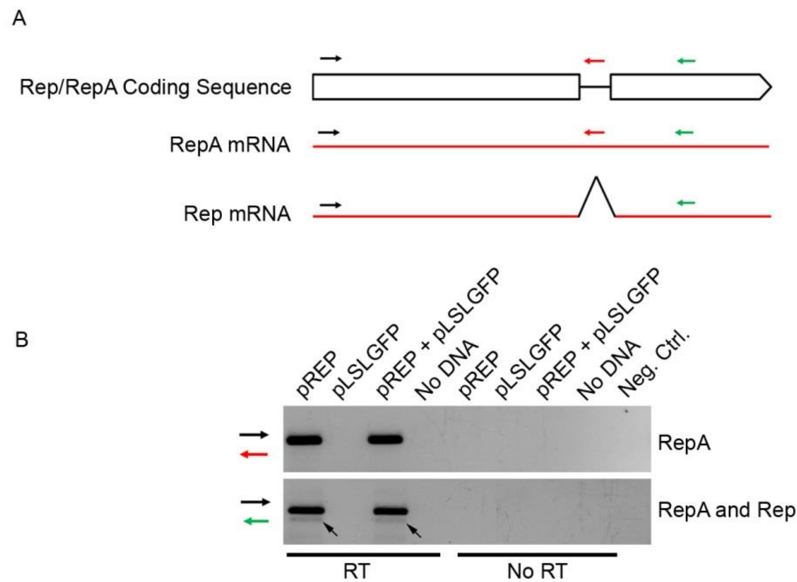
(C) Replicational release of GVRs was assessed following leaf infiltration with *Agrobacterium* containing pREP or pREPK219H. Leaves were infiltrated with a mixture of *Agrobacterium* containing pLSLZ and pREP (one side of a leaf), and pLSLZ and pREPK219H (the other side of the leaf). Additional leaves were infiltrated with *Agrobacterium* containing pLSLZ and a mixture of *Agrobacterium* containing pLSLZ and pREPK219H.

(D) Genomic DNA was assessed for the presence of NHEJ-induced mutations at the Zif268 target site.

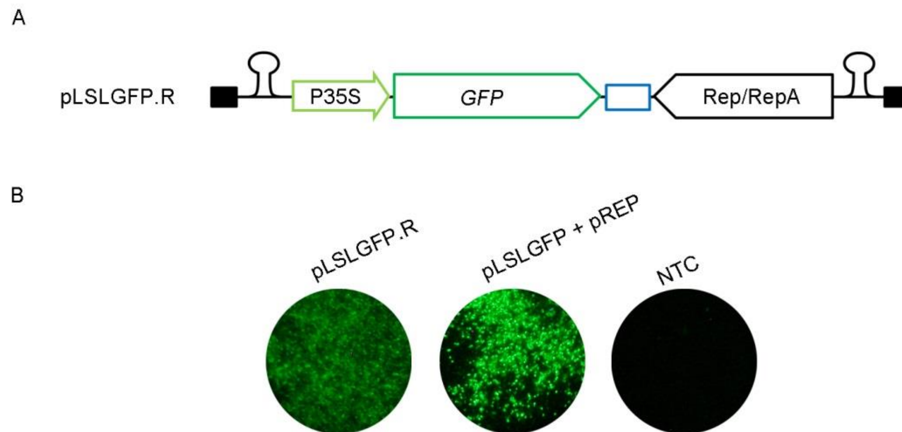
(E) Percentage of NHEJ-induced mutations at the Zif268 target. Error bars represent s.e.m. of three biological replicates. P values for the left and right graphs are 0.72 and 0.12, respectively.



Supplemental Figure 11. Exploring the Role of Repair Templates for Geminivirus Replicon-Mediated Gene Targeting.
The impact of replicating repair templates on gene targeting was assessed. Data were normalized to sample 1 (p35SZ.D and pREP). Data represent results from one leaf.



Supplemental Figure 12. Detection of Rep and RepA Transcripts in Tobacco Protoplasts.
(**A**) Illustration of the Rep and RepA coding sequence (top) and mRNA products. The intron within Rep and RepA coding sequence is depicted with a black line. Primer binding sites are indicated with colored arrows. Intron size is 86 bp.
(**B**) RT-PCR analysis of Rep and RepA transcripts. Protoplasts were transfected with plasmids pREP, pLSLGFP, pREP and pLSLGFP, or water (No DNA). RNA was extracted 24 hours post transfection. RT,reverse transcriptase. Neg. Ctrl., water was used in replace of template in the PCR solution. Two bands were present in the bottom gel image with samples pREP and pREP + pLSLGFP. The upper band is the unspliced mRNA (RepA) and the bottom band is the spliced mRNA (Rep). Arrows point to the spliced product.



Supplemental Figure 13. Expression of Green Fluorescent Protein with Single-Component Geminivirus Replicon Vectors.
(A) Illustration of the single-component geminivirus replicon vector for expression of GFP.
(B) Images of leaf tissue expressing GFP. One side of a leaf was infiltrated with *Agrobacterium* containing pLSLGFP.R and the other side was infiltrated with a mixture of *Agrobacterium* containing pLSLGFP and pREP. The non-transformed control (NTC) is an image of leaf tissue not infiltrated with *Agrobacterium*. Images were taken on the same day (three days post infiltration) and at the same exposure and magnification.

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Supplemental Figure 14. Sequence of Features within the Single-Component Geminivirus Vector pLSLZ.D.R.

Supplemental Methods :

Generating Cabbage Leaf Curl Virus Vectors for Gene Targeting

Repair templates with homology to the *ADH1* locus were cloned into pCPCbLCVA.007. pCPCbLCVA.007 is a plasmid containing a partial tandem direct repeat of the cabbage leaf curl virus (CaLCuV) genome (Muangsan and Robertson, 2004). The template for amplifying homology arms was genomic DNA from *Arabidopsis* (*Arabidopsis thaliana* ecotype Columbia). Repair templates were designed to introduce a unique 18 bp modification sequence (5'-GAGCTCAGTACTGCATGC) within the *ADH1*-ZFN binding site.

Biolistic Transformation of Cabbage Leaf Curl Virus Vectors

To prepare *Arabidopsis* plants (homozygous for a transgene encoding the ZFN pair targeting *ADH1*; Qi et al. 2013, Zhang et al. 2010) for biolistic bombardment, seeds were dispensed onto the surface of soil in each of the four corners of 2.5 x 2.5 inch pots. Plants were grown at 22°C for 2 weeks under a plastic dome, and then grown for an additional 1-2 weeks with watering when needed. Watering was stopped approximately 7 days before bombardment. Plants were bombarded when they reached the six- to nine-leaf-stage (approximately three to four weeks).

Biolistic bombardment was carried out closely following previously described protocols (Muangsan and Robertson, 2004). Briefly, to prepare microprojectile particles for five bombardments, 5 μg of each plasmid DNA was added to a tube containing 50 μl of 60 mg/mL gold beads and briefly vortexed. 50 μl of 2.5 M $CaCl_2$ was directly added to the samples and immediately pipetted in and out of a tip to break up conglomerates. 20 μl of 0.1 M spermidine was added and the samples were immediately vortexed for 5 min. The samples were centrifuged at 10,000 rpm for 10 seconds and the supernatant was removed. The gold-bead pellet was resuspended in 250 μl of 100% ethanol and then centrifuged at 10,000 rpm for 10 sec. Supernatant was removed and the samples were resuspended in 65 μl of 100% ethanol. The particles were then stored on ice until bombardment. To prepare the assembly for the microprojectile particles, macrocarrier

holders and macrocarriers were soaked in 95% ethanol, air-dried, and assembled. Resuspended particles (10 μl) was spotted onto the center of the macrocarrier and allowed to air dry. Biolistic bombardment was carried out using a PDS-1000 machine (Bio-Rad) in a laminar flow hood.

Sampling Parameters for Cabbage Leaf Curl Virus Experiments

After biolistic bombardment, plants were moved back to the growth chamber. For gene targeting experiments, plants displaying symptoms of virus infection at fourteen days post bombardment were sprayed with a solution containing estradiol (0.01% Silwet L-77, 20 μM β-estradiol). Plants were sprayed daily for one week. For experiments analyzing the stability of the repair templates, tissue was collected approximately 10 days post bombardment. For experiments analyzing the *ADH1* gene for targeted insertions, tissue was collected approximately seven days after beginning estradiol treatment. For both experiments, newly developed tissue was chosen for subsequent analysis; one rosette leaf and one cauline leaf were combined and total DNA was extracted from both tissues. In the cases where no cauline leaves were present, one rosette leaf was sampled.

Time points for DNA isolation, estradiol spraying and our leaf collection strategy were chosen based on data from previously reported studies analyzing CaLCuV infection in *Arabidopsis* (Ascencio-Ibáñez et al., 2008). It was demonstrated that *Arabidopsis* plants infected with CaLCuV have a strong presence of virus DNA in upper rosette leaves 12 days post infection. We chose to induce ZFN-expression in our plants at 14 days post bombardment in light of these observations, and also because symptoms of virus infection were more clear at 14 days post bombardment. Whereas our inoculation methods were very efficient (near 100% of bombarded plants were successfully infected as determined by symptoms that developed 10 – 14 days after bombardment) this latter condition allowed us to remove plants that were phenotypically normal (most likely not infected with CaLCuV). For each plant, we chose to sample one upper rosette leaf and one cauline leaf because CaLCuV was shown to accumulate within these tissues (Ascencio-Ibáñez et al., 2008). To induce ZFN expression, plants were exposed to estradiol for at least seven days. Seven days was chosen as it allows ample time for

high gene expression to occur using the XVE promoter system (e.g., in two week old *Arabidopsis* plants, gene expression reaches a maximum approximately 24 hours post induction and high expression continues 96 hours post induction; Zuo et al., 2000).

In the experiments assessing the stability of 400 bp repair templates, a total of 32 different plants were bombarded. Using primers P1 and P2 (Supplemental Figure 1), we detected a 400 bp sequence in 30 of the 32 bombarded plants. In two samples we did not detect sequence. In the experiments assessing 600 bp repair templates, a total of 24 different plants were bombarded. We detected a 600 bp sequence in 23 of the 24 plants. In one sample we did not detect sequence. In experiments assessing 800 bp repair templates, a total of 12 plants were bombarded. We obtained a single product with a size of 800 bp in 5 of the 12 plants. In the other 7 plants, we detected one or multiple products smaller than 800 bp. In the experiments assessing the stability of 1,000 bp repair templates, a total of 12 different plants were bombarded. In all twelve plants, we did not detect repair template sequence.

Time Course of GFP Expression in Tobacco Leaf Discs

Leaves from 4 week-old tobacco plants were infiltrated with *Agrobacterium* containing pLSLGFP (one side of a leaf) or a mixture of *Agrobacterium* containing pREP and pLSLGFP (the other side of the leaf). One hour after infiltration, discs were excised from the leaf tissue. Leaf discs were maintained in 6-well plates in approximately 4 mL of water at 26°C, with 12-h photoperiod. Images shown in Supplemental Figure 5 are of a single leaf disc from both treatments (pLSLGFP or pLSLGFP and pREP). Images of the entire leaf disc were taken at the indicated time points using a camera mounted on an AZ100 (Nikon) confocal microscope.

Quantitative PCR to Detect GVRs

Leaves from 4 week-old tobacco plants were infiltrated with *Agrobacterium* containing pLSLGFP and a mixture of *Agrobacterium* containing pREP and pLSLGFP. One leaf was infiltrated with one sample. At each of the indicated time points (see Supplemental

Figure 4), tissue was excised from at least two different leaves (from different plants) and total genomic DNA was extracted. Infiltrated leaves were sampled only once. Quantitative PCR was performed using the FastStart Universal SYBR Green Mix kit (Roche) on the LightCycler® 480 Instrument (Roche). Primers were designed to detect GFP coding sequence (5’- ATCCTCGGCCACAAGTTGGA and 5’- GTGGCGGGTCTTGAAGTTGG), circularization (5’- GAGATGAGCACTTGGGATAGGTAAGG and 5’- CTGCAAACAATACACAACAAAGACAATG) and Rep coding sequence (5’- TCCGACACCCCAGCCTCTAC and 5’- TTGCTTCCACAATGGGACGA). The *F-box* gene was used as reference gene to normalize the gene copy number (5’- GGCACTCACAAACGTCTATTTC and 5’- ACCTGGGAGGCATCCTGCTTAT). Three technical replications were performed for each sample. Expression data were standardized using the Microsoft Excel Qgene template as previously described (Muller et al., 2002). Error bars represent s.e.m. of at least two different biological replicates.

Protoplast Isolation and Transfection

Rep and RepA transcripts were detected by RT-PCR after transfection of protoplasts with pREP. Leaf tissue was digested and protoplasts were isolated followed protocols as previously described (Zhang et al., 2013) with slight modifications. Protoplasts from tobacco (*Nicotiana tabacum*) were isolated from approximately 4-week-old plants. Approximately 10 leaves were cut into 1- to 2-mm strips using a razor. Leaf strips were then incubated in an enzyme solution (1.0% cellulose R10, 0.25% macerozyme R10, 0.45 M mannitol, 20 mM MES, 20 mM KCl, 10 mM $CaCl_2$) for 14 hours at 25˚C with shaking (20 rpm). The enzyme-plant mixture was then passed through a cell strainer (70 μm) moistened with 2 ml of washing buffer (0.45 M mannitol and 10 mM $CaCl_2$, pH 5.8) and cells were collected in a perti dish. The protoplast solution was divided into two parts and each half was transferred to a 15 mL tube containing 8 mL of 0.55 M sucrose solution. The sample was centrifuged at 1,000 g for 5 min. The protoplasts, found floating above the sucrose solution were then transferred to a 50 mL tube containing 10 mL of washing buffer and centrifuged again (100 g for 5 min at room temperature). The supernatant was removed, and the protoplast pellet was resuspended in a solution

containing 0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES to a final density of 10^6 cells per mL.

Purified protoplasts were transfected with pREP T-DNA plasmid. To this end, 30 μL of plasmid (15 μg for each plasmid) was incubated with 200,000 cells (200 μL). This solution was gently mixed with 230 μL of 40% PEG-Calcium transformation buffer (40% PEG, 0.2 M Mannitol and 100 mM $CaCl_2$). After 20 min incubation at room temperature, 900 μL of washing buffer was added to the reaction. Protoplasts were pelleted by centrifuging at 250 g for 5 minutes. The cells were resuspended in 800 μL of washing buffer and centrifuged again at 150 g for 5 min. The cells were resuspended in 1 mL of washing buffer. The suspended protoplasts were stored in a six well plate for 24 hours at 25˚C in the dark.

RNA isolation and RT-PCR

Transfected cells (600,000 in total for each sample) were pelleted 24 hours post transfection by centrifuging at 13,000 g for 5 minutes. Total RNA was extracted with Trizol (Invitrogen) following manufacturer's protocols. Isolated RNA was treated with DNase I (ambion). RNA (500 ng) was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Rep and RepA transcripts were amplified using primers 5'-AACTCACACCTTTTCTTATTTTCT and 5'-TTTTCCATATTTAGGGTTGACAGT (Rep and RepA), and 5'-AACTCACACCTTTTCTTATTTTCT and 5'-ATTGAGCTTGTTGGTATGAG (RepA).

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