The Plant Cell, Vol. 26: 151–163, January 2014, www.plantcell.org

RESEARCH ARTICLES

DNA Replicons for Plant Genome Engineering[W][OPEN]

Nicholas J. Baltes, Javier Gil-Humanes, Tomas Cermak, Paul A. Atkins, and Daniel F. Voytas[1]

Department of Genetics, Cell Biology, and Development, Center for Genome Engineering, University of Minnesota, Minneapolis, Minnesota 55455

Sequence-specific nucleases enable facile editing of higher eukaryotic genomic DNA; however, targeted modification of plant genomes remains challenging due to ineffective methods for delivering reagents for genome engineering to plant cells. Here, we use geminivirus-based replicons for transient expression of sequence-specific nucleases (zinc-finger nucleases, transcription activator–like effector nucleases, and the clustered, regularly interspaced, short palindromic repeat/Cas system) and delivery of DNA repair templates. In tobacco (*Nicotiana tabacum*), replicons based on the bean yellow dwarf virus enhanced gene targeting frequencies one to two orders of magnitude over conventional *Agrobacterium tumefaciens* T-DNA. In addition to the nuclease-mediated DNA double-strand breaks, gene targeting was promoted by replication of the repair template and pleiotropic activity of the geminivirus replication initiator proteins. We demonstrate the feasibility of using geminivirus replicons to generate plants with a desired DNA sequence modification. By adopting a general plant transformation method, plantlets with a desired DNA change were regenerated in <6 weeks. These results, in addition to the large host range of geminiviruses, advocate the use of replicons for plant genome engineering.

INTRODUCTION

Precise modification of plant DNA is of value for basic and applied biology because it facilitates gene function studies and crop improvement. One method to modify DNA involves introducing targeted double-strand breaks. Double-strand breaks activate two main repair pathways: nonhomologous end joining (NHEJ) and homologous recombination. These two pathways can be exploited to introduce sequence changes within the plant genome. Repair by NHEJ is often imprecise, leading to insertions or deletions of DNA at the cut site. Consequently, double-strand breaks directed to endogenous genes can facilitate targeted gene disruption or targeted mutagenesis (Lloyd et al., 2005; Zhang et al., 2010). Double-strand breaks can also be repaired with high fidelity by homologous recombination. Homologous recombination normally involves the use of the sister chromatid or homologous chromosome for template-directed repair. If an exogenously supplied repair template is present, gene replacement or targeted gene insertion can be performed (referred to hereafter as gene targeting; Puchta et al., 1996; Bibikova et al., 2003).

Targeted double-strand breaks can be generated using sequence-specific nucleases. These nucleases include meganucleases (Smith et al., 2006), zinc-finger nucleases (ZFNs; Kim et al., 1996), transcription activator–like effector nucleases (TALENs; Christian et al., 2010; Miller et al., 2011), and the clustered, regularly interspaced, short palindromic repeat (CRISPR)–associated protein (Cas) system (Cong et al., 2013; Mali et al., 2013). Using sequence-specific nucleases, plant genomes have been modified in many different ways, ranging from several nucleotide substitutions (Townsend et al., 2009) to the targeted deletion of megabases of DNA (Qi et al., 2013).

In plants, delivery of genome engineering reagents, including sequence-specific nucleases and repair templates, presents a barrier to efficiently achieving targeted genome modifications. For gene targeting, there are a few reports using *Agrobacterium tumefaciens* or physical means to deliver genome engineering reagents (Cai et al., 2009; Shukla et al., 2009); however, the highest frequencies of gene targeting have been achieved using protoplasts, plant cells lacking cell walls (Wright et al., 2005; Townsend et al., 2009; Zhang et al., 2010). Protoplasts can be transformed at high efficiency, but only a handful of plants can be effectively regenerated from protoplasts. Alternatively, constructs encoding nucleases and repair templates can be integrated into plant genomes (Fauser et al., 2012; Ayar et al., 2013). This so-called in planta genome engineering strategy relies on the gene targeting to occur during the life of the plant, with the anticipation that germline cells will be modified and ultimately give rise to seed with the desired sequence alterations.

In mammalian systems, effective delivery of genome engineering reagents has been achieved using viruses, including retroviruses (Ellis and Bernstein, 1989; Lombardo et al., 2007), adenoviruses (Wang and Taylor, 1993), and adeno-associated viruses (Russell and Hirata, 1998). In plants, the RNA virus *Tobacco rattle virus* was used to deliver a ZFN pair to petunia (*Petunia hybrida*) and tobacco (*Nicotiana tabacum*) leaves, resulting in targeted modification of an integrated reporter gene in somatic cells (Marton et al., 2010). Whereas the cargo capacity of plant RNA viruses enables delivery of a pair of ZFNs or a meganuclease, it is not sufficient to deliver larger proteins, such as a pair of TALENs or Cas9 for the CRISPR/Cas system. Furthermore, RNA viruses cannot deliver DNA repair templates for gene targeting.

[1] Address correspondence to voytas@umn.edu.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Daniel F. Voytas (voytas@umn.edu).
[W] Online version contains Web-only data.
[OPEN] Articles can be viewed online without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.113.119792

To develop an efficient and facile vector system for plant genome engineering, we focused our efforts on the geminiviruses. Geminiviruses are a large family of plant viruses with single-stranded, circular DNA genomes of ~2.5 to 3.0 kb. Once inside a host cell's nucleus, their single-stranded genome is converted to a double-stranded intermediate by host DNA polymerases. This double-stranded genome is then used as a template for transcription of virus genes and for rolling-circle replication. The only geminivirus protein necessary for replication is the replication initiator protein (Rep). Rep initiates rolling-circle replication by binding to a site within the large intergenic region (LIR; *Mastreviruses*), intergenic region (*Curtoviruses* and *Topocuviruses*), or common region (*Begomoviruses* with bipartite genomes). There, it creates a single-strand nick within an invariant 9-nucleotide sequence (TAATATTAC) located at the apex of a conserved hairpin structure. Following rolling-circle replication, single-stranded genomes are either converted back to a double-stranded intermediate or encapsidated by coat protein to produce virions. These virions are then transported to adjacent cells through the plant's endogenous plasmodesmata pathways.

Methods to engineer geminiviruses for protein expression include the full virus and deconstructed virus strategies (Gleba et al., 2004). Under the full virus strategy, geminiviruses retain most or all of the features required for replication and systemic infection of the host plant. Heterologous sequence can be introduced within the genome by replacing the coat protein gene (coat protein is not required for cell-to-cell movement in some bipartite begomoviruses; Gardiner et al., 1988). Using this approach, tomato golden mosaic virus and African cassava mosaic virus were modified to express neomycin phosphotransferase (Hayes et al., 1988) and chloramphenicol acetyltransferase (Ward et al., 1988), respectively.

Unfortunately, it may not be possible to deliver relatively large heterologous sequences while maintaining cell-to-cell movement and rolling-circle replication. Cell-to-cell movement through the endogenous plasmodesmata pathways imposes strict requirements on the geminivirus genome size (Etessami et al., 1989; Hayes et al., 1989; Elmer and Rogers, 1990; Gilbertson et al., 2003). Larger than wild-type genomes frequently recombine to smaller sizes and, as a result, are outcompeted during the infection process. Whereas most studies exploring genome size restrictions use bipartite begomoviruses, these constraints may also be experienced with other geminiviruses, including mastreviruses. For example, using the maize streak virus, ~3.2 kb of heterologous sequence was inserted into the small intergenic region. Insertion of sequence into this region does not disrupt virus replication or expression of virus proteins (Shen and Hohn, 1991). After delivery to leaf cells, the chimeric virus replicated efficiently; however, no systemic movement was observed (Shen and Hohn, 1994).

Under the deconstructed virus strategy, limiting or undesired virus functions are removed, and only the useful blocks are kept. For example, to circumvent genome size constraints imposed by the plasmodesmata, movement protein and coat protein sequences are removed, whereas the *cis*- and *trans*-acting replicational elements are retained. Lack of cell-to-cell movement can be compensated using *Agrobacterium* or particle bombardment to deliver viral vectors to cells. This approach has been successfully used to generate replicons based on the bean yellow dwarf virus (BeYDV) for expression of reporter proteins, vaccine proteins and monoclonal antibodies (Mor et al., 2003; Zhang and Mason, 2006; Huang et al., 2009, 2010; Regnard et al., 2010). Currently, there is no obvious upper size limit for replicons that do not move from cell to cell (Laufs et al., 1990; Matzeit et al., 1991; Shen and Hohn, 1994; Huang et al., 2009). However, it may be that as replicon size increases, replication efficiency decreases (Suárez-López and Gutiérrez, 1997).

The aim of this study was to explore the use of geminiviruses for plant genome engineering. We demonstrate that DNA carried by geminiviruses can be used as a template for homologous recombination. We further demonstrate that geminivirus-based replicons enable highly efficient genome engineering. We show that this technology can be used to produce calli and plantlets with precise DNA sequence changes. Finally, we develop a simplified geminivirus vector system for efficient plant genome engineering.

RESULTS

Full Viruses for the Delivery of Repair Templates in *Arabidopsis*

We began our study by exploring the use of full viruses for the delivery of repair templates. Our target locus was the *Arabidopsis thaliana* alcohol dehydrogenase (*ADH1*) gene. We modified the bipartite cabbage leaf curl virus (CaLCuV) DNA A genome to contain repair templates designed to introduce a unique 18-bp sequence within *ADH1* (Supplemental Figure 1A). After optimizing repair template length (Supplemental Figures 1B to 1D), *Arabidopsis* plants were infected with CaLCuV (harboring a repair template with 600 bp of total sequence) by particle bombardment (Supplemental Figure 2). Within the genome of the infected plants was a ZFN pair that targets a site within the *ADH1* gene (Zhang et al., 2010). This ZFN pair was under the transcriptional control of an estrogen-inducible promoter (Zuo et al., 2000). As a result, double-strand breaks could be temporally controlled by spraying *Arabidopsis* plants with a solution containing estradiol (Supplemental Figure 3). Recombinant CaLCuV genomes were given 14 d to replicate and spread before ZFN expression was induced. Seven days after spraying, genomic DNA was isolated and the *ADH1* gene was assessed for the presence of the unique 18-bp sequence. From 23 experiments, we observed a single plant where the gene targeting product could be detected by PCR (Supplemental Figure 4). While these results suggest gene targeting with full viruses is possible, further optimization of this technology was warranted.

Our approach using full viruses has several potential drawbacks, including (1) the uncertainty of having both gene targeting reagents (ZFN protein and geminivirus DNA) in a nucleus at the same time and (2) the variability in the concentration of these reagents within the same cell. For example, because we did not observe NHEJ-induced mutations within all *ADH1* loci (Supplemental Figure 3), we could not ensure that double-strand breaks were being produced in all cells after spraying with estradiol. Furthermore, leaky expression of the ZFN pair from the XVE promoter may result in premature double-strand breaks and subsequent NHEJ mutations that destroy the ZFN binding domain. Due to these uncertainties, we sought to develop a system where (1) geminivirus vectors could be efficiently delivered to target cells of interest and (2) sequence-specific nucleases and repair templates are simultaneously delivered.

Deconstructed Viruses for the Delivery of Sequence-Specific Nucleases and Repair Templates

We chose to deconstruct the mild strain of the BeYDV (Halley-Stott et al., 2007) because it had previously been used to express heterologous proteins in plant cells (Regnard et al., 2010). BeYDV replication requires three viral elements: the *cis*-acting LIR, the short intergenic region (SIR), and the *trans*-acting replication-initiation protein (Rep) and RepA. Rep and RepA are expressed from the same precursor mRNA: Rep is produced from the spliced mRNA, whereas the unspliced mRNA yields RepA (Wright et al., 1997). Eliminating coding sequences for the coat and movement proteins abolishes cell-to-cell movement but effectively eliminates genome-size constraints and makes it possible to increase vector cargo capacity. To compensate for loss of cell-to-cell movement, we used *Agrobacterium* to deliver geminivirus constructs to cells. The *Agrobacterium* T-DNA was modified to harbor *cis*-acting sequences needed for replication in a LIR-SIR-LIR orientation (hereafter referred to as the LSL T-DNA; Figure 1A). Concomitant delivery of Rep protein should result in replicational release and amplification of circular geminivirus replicons (GVRs; Stenger et al., 1991).

Delivery of conventional T-DNA to plant cells by *Agrobacterium* can result in transient gene expression. By modifying T-DNA to harbor sequences for genome engineering reagents (sequence-specific nucleases and repair templates), both targeted mutagenesis and gene targeting can be performed (Reiss et al., 2000; Li et al., 2013). To better distinguish between the effects of GVRs and the input T-DNA, we sought to design the LSL T-DNA such that expression of heterologous sequence is minimized when virus proteins are not present. We chose to position heterologous sequence downstream of the LIR in the virion-sense direction. The LIR functions as a bidirectional promoter and promoter strength in the virion-sense direction is weak in the absence of Rep and RepA proteins (Hefferon et al., 2006). To promote high gene expression within circularized GVRs, we positioned a duplicated 35S promoter (P35S) upstream of the LIR in the virion-sense direction. Splice donor and acceptor sequences were positioned upstream and downstream, respectively, of the LIR sequence that is predicted to form after circularization. High-protein expression may also occur from the transactivation of the virion-sense LIR promoter by the viral Rep proteins (Hefferon et al., 2006).

To investigate whether our GVRs replicate and express protein in plant cells, we modified LSL T-DNA plasmid to encode green fluorescent protein (GFP; pLSLGFP) or β-glucuronidase (GUS; pLSLGUS; Figure 1B). GVRs were delivered to tobacco leaf tissue by infiltrating a mixture of *Agrobacterium* strains containing pLSLGFP or pLSLGUS and a separate T-DNA expression plasmid encoding Rep and RepA (pREP). Both GUS and GFP expression was enhanced when pREP T-DNA was delivered (Figure 1B; Supplemental Figure 5). Background GUS activity seen in the tissue transformed with pLSLGUS is most likely due to activity of the virion-sense LIR promoter within *Agrobacterium* and plant cells.

To correlate the enhanced protein expression with GVR production, replicational release was evaluated by PCR using primers that would only amplify a product if circularization occurred. Strong amplification of the LIR sequence was observed only from samples in which pREP T-DNA was delivered, confirming successful replicational release of replicons (Figure 1C; Supplemental Figure 6). In a separate experiment, we quantified the relative GVR gene copy number over a 2-week time course using quantitative PCR (Supplemental Figure 7). Five days after infiltration, we

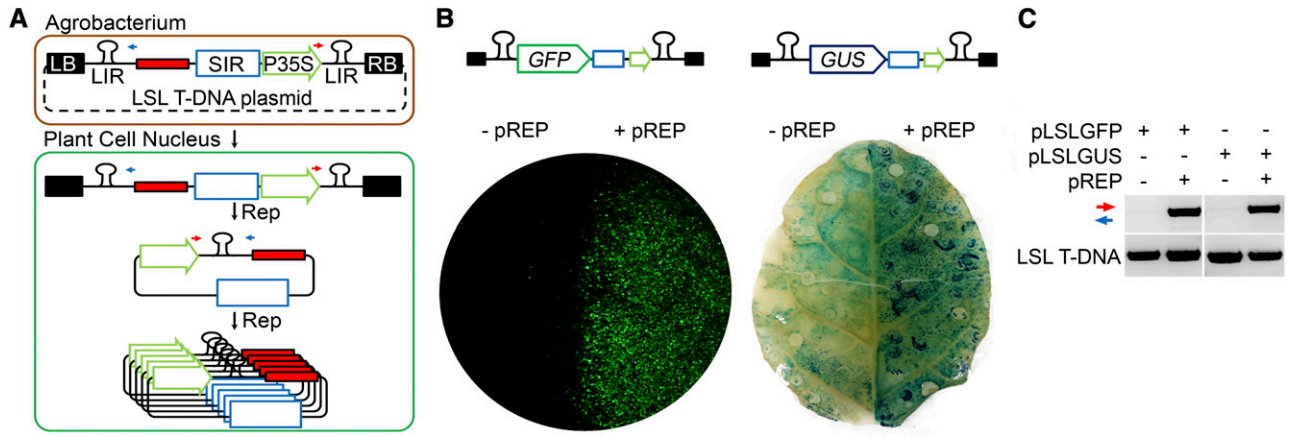

Figure 1. Development of GVRs for Protein Expression in Tobacco.

(A) Approach to deliver GVRs to plant cells. Rep is delivered using a separate T-DNA expression plasmid (pREP; data not shown). LB, left T-DNA border; RB, right T-DNA border. The red rectangle indicates the region where heterologous sequence is cloned. P35S, 2x35S promoter from the cauliflower mosaic virus. Splice donor and acceptor sequences flank the LIR within circularized replicons (data not shown). Blue and red arrows indicate primer binding sites for PCR to detect circularized GVRs.

(B) Images of leaf tissue expressing GFP and GUS. As a negative control for GVR-mediated protein expression, leaf tissue was infiltrated with a single strain of *Agrobacterium* containing the LSL T-DNA plasmid. Tissue delivered pLSLGFP was imaged 3 d after infiltration. Tissue-delivered pLSLGUS was stained in a solution with X-Gluc 7 d after infiltration.

(C) PCR-based detection of circularized GVRs within plant cells. The PCR control (LSL T-DNA) used primers designed to amplify sequence within linear LSL T-DNA.

observed a maximum copy number of ~6000 per single-copy gene. This is consistent with previous reports describing that mastreviruses replicate to very high copy number within plant cells (Timmermans et al., 1992). Together, these data illustrate that our GVRs replicate and express heterologous proteins in leaf cells. Furthermore, these results show that peak replicon and protein levels occur between 3 and 5 d after infiltration, which is consistent with previous reports using BeYDV-based replicons for protein expression (Zhang and Mason, 2006). In subsequent genome engineering experiments, we chose to sample tissues between 5 and 7 d after infiltration. We reasoned that sampling on these days would allow sufficient time for the GVRs to reach maximum copy number and for peak protein expression. Furthermore, as DNA modifications are stable events, a targeted change occurring at 3 d after infiltration, for example, should be detectable 7 d after infiltration.

Expression of ZFNs with GVRs

To demonstrate targeted mutagenesis using GVRs, an LSL T-DNA plasmid was modified to encode the Zif268:*Fok*I ZFN (pLSLZ; Figure 2A). Target sequence for this ZFN is present within a reporter gene that is stably integrated in tobacco plants (Wright et al., 2005). This reporter is a nonfunctional, translational fusion between GUS and neomycin phosphotransferase (NPTII; Datla et al., 1991). Leaf tissue from transgenic tobacco plants carrying the *gus:nptII* reporter was syringe infiltrated with an *Agrobacterium* strain containing pLSLZ (one side of a leaf) or coinfiltrated with a mixture of *Agrobacterium* strains containing pLSLZ and pREP (the other side of the leaf). Total DNA was extracted 7 d after infiltration, replicational release was verified by PCR (Figure 2A), and the Zif268 target sequence was analyzed for mutations introduced by NHEJ. To this end, a 484-bp DNA fragment encompassing the Zif268 target sequence was amplified by PCR. Amplicons were digested with *Mse*I (an *Mse*I site is present in the spacer sequence between the two Zif268 binding sites), and bands were resolved by agarose gel electrophoresis (Figure 2B). Cleavage-resistant amplicons from leaf tissue transformed with both pLSLZ and pREP T-DNA were cloned and sequenced (Figure 2C). Six out of eight sequenced clones contained mutations at the ZFN target site. Five out of the six sequences had distinct NHEJ-induced mutations, indicating that DNA in multiple leaf cells had been modified. We observed significantly higher levels of targeted mutagenesis in cells transformed with pLSLZ and pREP compared with pLSLZ alone (Figure 2D). Cleavage-resistant amplicons observed from tissue delivered pLSLZ T-DNA are most likely due to expression of Zif268:*Fok*I from the virion-sense LIR promoter. These results indicate that GVRs can express ZFNs for creating targeted DNA double-strand breaks.

Expression of TALENs and Components of the CRISPR/Cas System with GVRs

Having demonstrated that GVRs can deliver ZFNs to plant cells, we next tested if they were effective vehicles for delivering TALENs and CRISPR/Cas reagents. We constructed a pLSL vector that encodes the T30 TALEN pair (pLSLT; Supplemental Figure 8A; Zhang et al., 2013), which targets a site within the tobacco

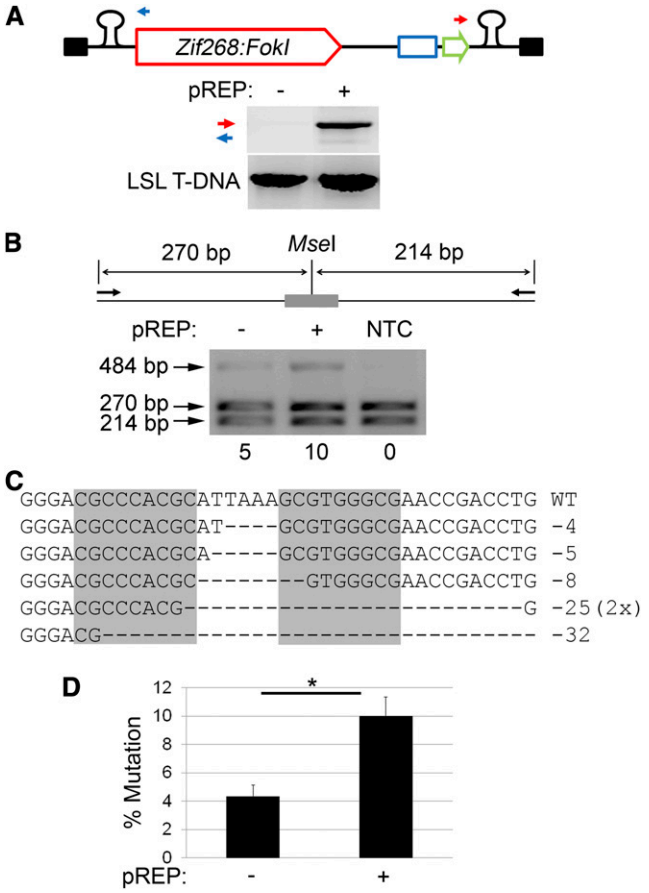

Figure 2. GVR-Mediated Expression of ZFNs for Targeted Mutagenesis.

(A) LSL T-DNA encoding Zif268:*Fok*I (top). PCR-based detection of circularized GVRs within plant cells (bottom).

(B) PCR-based detection of ZFN-induced mutations at the Zif268 target sequence. Numbers beneath the gel image indicate the percentage of cleavage-resistant amplicons. NTC, nontransformed control.

(C) Sequences of individual amplicons containing NHEJ-induced mutations. Letters in front of gray background indicate the Zif268 binding sequence.

(D) Quantification of NHEJ-induced mutations. Error bars represent SE of three experiments. *$P < 0.05$.

acetolactate synthase (*ALS*) genes. A second vector was constructed that encodes Cas9 and synthetic guide RNA (sgRNA), also targeting *ALS* (pLSLC; Supplemental Figure 8D). After delivery to plant cells, mutagenesis of endogenous targets was monitored by loss of a restriction enzyme site due to NHEJ-induced mutation at the site of cleavage. We observed an enhancement in mutagenesis when pREP was cotransformed, suggesting GVR-mediated expression of TALENs and CRISPR/Cas elements can be achieved (Supplemental Figures 8B, 8C, 8E, and 8F).

GVRs Enhance Frequencies of Gene Targeting

GVRs were next assessed for their ability to achieve gene targeting through the delivery of sequence-specific nucleases and repair templates. The target for modification was the defective

gus:nptII gene, which can be repaired by correcting a 600-bp deletion that removes part of the coding sequences for both GUS and NPTII (Wright et al., 2005). An LSL T-DNA plasmid was constructed that encodes the Zif268:*Fok*I ZFN followed immediately by repair template sequence (designated *us:NPTII*) designed to restore *gus:nptII* function (pLSLZ.D; Figure 3A). Cells having undergone gene targeting will express a functional GUS protein and, consequently, will stain blue when incubated in a solution with the GUS substrate X-Gluc. Random integration of the repair template or read-through transcription from virus promoters should not produce functional GUS protein due to 500 bp that is missing from the 5′ end of the coding sequence. This was experimentally confirmed by transforming nontransgenic leaf tissue with pLSLZ.D and pREP T-DNA; no GUS activity was observed 5 d after infiltration (Figure 3B, Neg. Ctrl.). To compare the performance of GVRs with conventional T-DNA, we engineered a T-DNA vector with Zif268:*Fok*I sequence followed immediately by the *us:NPTII* repair template (p35SZ.D). Five days after infiltration, leaf tissue was stained in X-Gluc and assessed for cells having undergone gene targeting. We observed an enhancement in GUS activity in leaf tissue transformed with pLSLZ.D and pREP T-DNA (Figure 3B) relative to the p35SZ.D and pLSLZ.D controls. To verify repair of the reporter gene, PCR was performed on total DNA extracted from leaf tissue transformed with pLSLZ.D and pREP T-DNA. Primers were used that recognize sequence within the repaired reporter gene and ~1 kb downstream of the homology carried by the repair template (Supplemental Figure 9). Amplification of a 2.3-kb product from 9 of the 11 leaf samples indicates the presence of the repaired *GUS:NPTII* transgene. Sequence analysis confirmed the presence of an *NPTII* repair template-transgene junction, consistent with repair of the reporter by gene targeting.

The relative enhancement in gene targeting was calculated by quantifying the density of blue sectors within transformed leaf tissue. A significant enhancement in gene targeting was observed in tissue transformed with pLSLZ.D and pREP T-DNA across multiple transgenic plant lines where the reporter gene is integrated at different chromosomal positions (Figure 3C; Wright et al., 2005). Notably, in plant line 1, we observed an enhancement in gene targeting greater than two orders of magnitude. Taken together, these results demonstrate that GVRs can deliver sequence-specific nucleases and repair templates for gene targeting and that GVRs contribute features that result in the enhancement of gene targeting in somatic cells relative to conventional T-DNA delivery.

Double-Strand Breaks Enhance Gene Targeting

There are several features of GVRs that may promote gene targeting, including replication to high copy numbers, high protein expression and pleiotropic activities of Rep and RepA. To test these features, we transformed single leaves with two *Agrobacterium* samples and directly compared GUS activity resulting from gene targeting (Figure 4A). Pairing two samples on a single leaf reduced the variation in results due to environmental conditions (e.g., differences in leaf age, size, and health). As T-DNA size affects transfer efficiency, we ensured that vectors being directly compared had similar T-DNA sizes. To determine the contribution of double-strand breaks to gene targeting and to test the effectiveness of this assay, *Zif268:FokI* (897 bp) was replaced with *GFP* (912 bp). We observed a significant decrease (100-fold) in GUS activity when *Zif268:FokI* was removed (Figure 4B), which is consistent with previous reports describing a stimulatory effect of double-strand breaks on recombination (Puchta et al., 1996).

Replication of Repair Templates Enhances Gene Targeting

To determine if replication of GVRs contributes to gene targeting, we compared our GVR system (pLSLZ.D and pREP) to a replication-inactive system (p35SZ.D and pREP). We observed

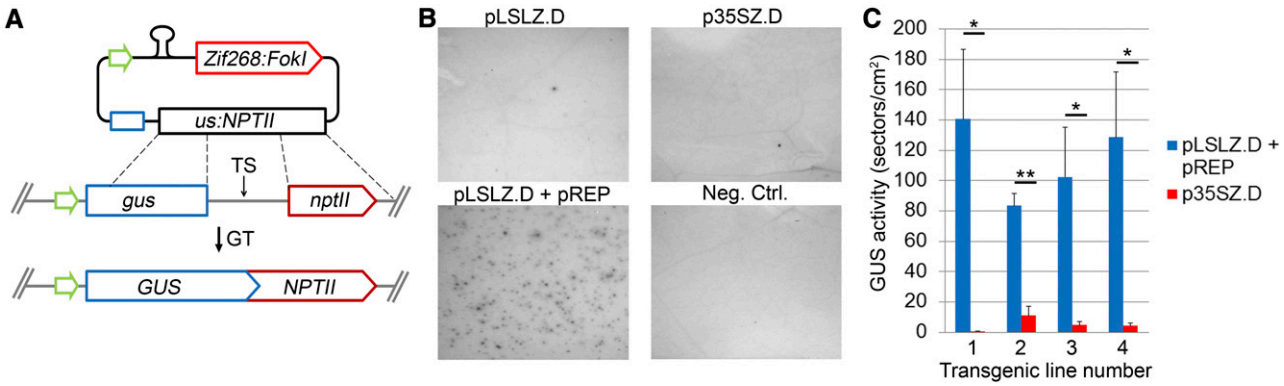

Figure 3. GVRs Promote High-Frequency Gene Targeting.

(A) Approach to repair a nonfunctional *gus:nptII* gene through homologous recombination. GT, gene targeting; TS, Zif268:*Fok*I target site.

(B) Images of leaf tissue stained in a solution with X-Gluc. To better visualize stained cells, chlorophyll was removed from leaf tissue, and the green and blue channels were removed from the image. The Neg. Ctrl. image is of nontransgenic leaf tissue transformed with two strains of *Agrobacterium* containing pLSLZ.D and pREP.

(C) Relative frequencies of gene targeting using GVRs (pLSLZ.D + pREP; blue bars) and conventional T-DNA (p35SZ.D; red bars). The *x* axis represents four different plant lines with the *gus:nptII* gene integrated at different chromosomal positions. Error bars represent SE of at least three biological replicates. *P < 0.05 and **P < 0.005.

a significant decrease in blue sectors when the *cis*-acting replication elements were removed (Figure 4C, left graph). These results indicate that replication enhances gene targeting; however, we could not conclude whether replicating sequence-specific nucleases or repair templates contributed more toward gene targeting. Therefore, to address this issue, we explored the role of sequence-specific nuclease expression on GVR-mediated gene targeting.

One explanation for the observed high frequency of gene targeting is that GVRs mediate high expression of sequence-specific nucleases, and, as a result, double-strand breaks are more frequent. To test this hypothesis, we compared the level of NHEJ-induced mutations in leaf tissue delivered Zif268:*Fok*I using GVRs (pLSLZ and pREP) or conventional T-DNA (p35SZ). We predicted that if expression of sequence-specific nuclease was enhanced using GVRs, we would observe higher levels of NHEJ-induced mutations. Our results refute this hypothesis (Supplemental Figure 10A): We observed a lower level of NHEJ-induced mutations with GVRs, suggesting that replication of sequence-specific nucleases does not augment levels of gene targeting. As an alternative approach to assess the impact of nuclease amplification, we modified Rep and RepA to carry a mutation in the conserved ATPase domain (K219H for BeYDV). This mutation impairs replication (Desbiez et al., 1995; Supplemental Figure 10B); however, other functions of Rep and RepA should remain active (e.g., nicking activity, ligation activity, and transactivation of virion-sense transcription). As expected, pREPK219H T-DNA delivered to leaf tissue was not replicated (Supplemental Figure 10C), and we observed similar levels of targeted mutagenesis with pREPK219H as with pREP (~10%; Supplemental Figures 10D and 10E). Collectively, the results suggest that replication of Zif268:*Fok*I coding sequence does not significantly contribute to enhancing targeted mutagenesis.

To test the hypothesis that gene targeting is enhanced by replication of repair templates and not sequence-specific nucleases, we compared gene targeting frequencies of two GVR systems that either replicate sequence-specific nucleases or repair templates (Figure 4C, right graph). Here, we observed significantly higher levels of gene targeting when repair templates were replicated. Similarly, when we translocated repair templates from conventional

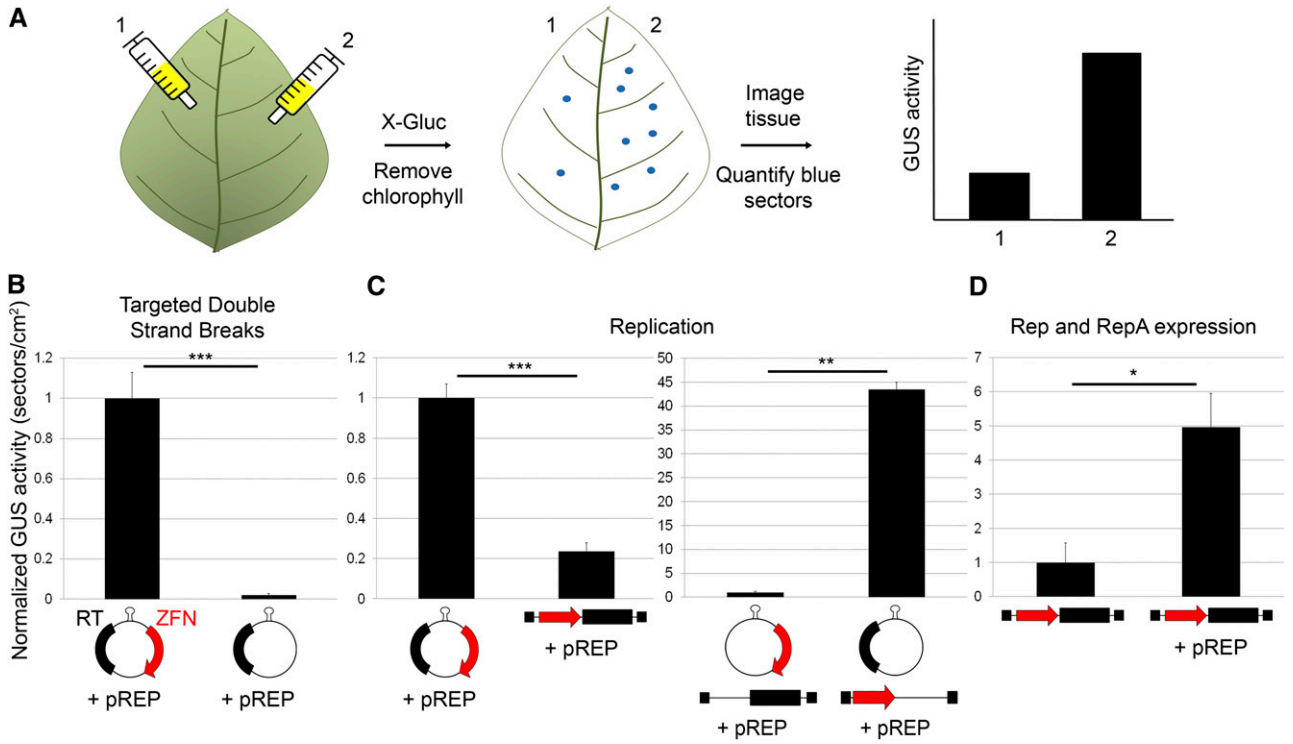

Figure 4. Synergism between Double-Strand Breaks, Replication of Repair Templates, and Pleiotropic Activity of Rep and RepA on Gene Targeting.

(A) Illustration of the approach to directly compare gene targeting frequencies between two *Agrobacterium* samples.

(B) Effect of targeted double-strand breaks on gene targeting. As indicated, red arrows represent coding sequence for Zif268:*Fok*I; black boxes depict the *us:NPTII* repair templates (RT); small black boxes represent the left and right T-DNA borders. Error bars represent SE of six experiments. ***P < 0.001. Data were normalized to sample 1.

(C) Effect of replicating ZFN and repair template sequence on gene targeting (left graph). Error bars represent SE of four experiments. ***P < 0.001. Effect of replicating repair template sequence on gene targeting (right graph). Error bars represent SE of three experiments. **P < 0.005. Data were normalized to sample 1 in both graphs.

(D) Effect of Rep and RepA expression on gene targeting. Measure of center is mean. Error bars represent SE of four experiments. *P < 0.05. Data were normalized to sample 1.

T-DNA to GVRs, we observed substantially higher levels of gene targeting (Supplemental Figure 11). These results indicate that although expression of sequence-specific nucleases is critical for gene targeting, replication of repair template sequence contributes more toward enhancing gene targeting.

Pleiotropic Activity of Rep and RepA Enhances Gene Targeting

Geminivirus Rep and RepA proteins interact with a multitude of host proteins. Specifically, RepA protein from mastreviruses interacts with Geminivirus RepA binding protein 1 (GRAB1) and GRAB2 (Xie et al., 1999) and the retinoblastoma-related protein (RBR; Horváth et al., 1998). It may be possible that expression of mastrevirus Rep and RepA in plant cells affects levels of homologous recombination. To test this hypothesis, we compared gene targeting levels in tissue-delivered p35SZ.D T-DNA or p35SZ.D and pREP T-DNA. Here, we observed a significant increase in blue sectors when pREP was delivered (Figure 4D). To confirm that delivery of pREP T-DNA to plant cells results in the expression of both Rep and RepA, we used RT-PCR to detect Rep and RepA transcripts. To avoid detecting RepA transcripts produced by *Agrobacterium*, we transfected protoplasts with the pREP T-DNA plasmid. Total RNA was extracted 24 h after transfection, and we detected unspliced (RepA) and spliced (Rep) transcripts (Supplemental Figure 12). These results demonstrate that pleiotropic activity of Rep and/or RepA promotes gene targeting (Figure 4D). Taken together with the results in the previous two sections, high-frequency gene targeting using GVRs is due to synergy between targeted double-strand breaks, replication of repair template sequence, and pleiotropic activity of Rep and RepA.

Recovery of Calli and Plantlets with a Precise DNA Change

To demonstrate the feasibility of GVRs for generating fully developed plants with a desired sequence change, we regenerated cells harboring the corrected *GUS:NPTII* transgene. We chose to regenerate leaf cells because our previous data demonstrate the usefulness of GVRs in whole leaves. Leaves were syringe infiltrated with two strains of *Agrobacterium* containing pLSLZ.D and pREP. Five days later, infiltrated leaves were removed from the plant, surface sterilized, and placed on regeneration media with kanamycin (Figure 5A). To track the progression of regenerating cells with the *GUS:NPTII* transgene, subsets of leaf discs were stained in X-Gluc solution 0, 7, 14, 21, 42, and 49 d after plating. We observed GUS expression from leaf discs stained between 0 and 21 d (Figures 5A to 5D). After day 42, we observed shoots with GUS expression (Figures 5E and 5F). To confirm that tissue staining blue harbors the corrected *GUS:NPTII* transgene, we extracted genomic DNA from blue calli and performed PCR with primers designed to amplify sequence from the repaired *GUS:NPTII* transgene. Because our negative control samples (noninfiltrated tissue) did not produce calli or shoots, we chose to analyze genomic DNA from callus tissue that did not stain blue in the pLSLZ.D and pREP samples. We observed amplification of sequence from all four blue-stained tissues and not from tissue that did not stain blue (Figure 5G). Sequencing of all four PCR products confirmed the presence of an *NPTII* repair template-transgene junction, consistent with repair of the reporter by gene targeting (Figure 5H). These findings demonstrate that GVRs can be used to introduce exact DNA changes in plant cells and that these cells maintain the ability to regenerate.

Development of a Single-Component GVR System

Having demonstrated the usefulness of GVRs for genome engineering and that there are virus-specific features that promote gene targeting, we sought to improve the design of the geminivirus vectors for efficient genome editing. To this end, we developed a single-component system that requires the delivery of only one T-DNA molecule to generate GVRs. Transfer of a single T-DNA to cells is more efficient than the transfer of two T-DNAs, which may result in a higher frequency of cells receiving a functional GVR. We modified the GVR sequence such that Rep and RepA coding sequences were placed downstream of the complementary-sense LIR promoter, the natural position of Rep and RepA coding sequences within mastrevirus genomes. Furthermore, we positioned the strong constitutive promoter (P35S) directly upstream of our reporter gene or sequence-specific nuclease of interest (Figure 6A).

To test the effectiveness of this vector system for protein expression, we placed GFP coding sequence downstream of P35S (pLSLGFP.R; Supplemental Figure 13A). We compared protein expression of pLSLGFP.R to the dual-component GVR system (pLSLGFP and pREP). In general, we observed more uniform GFP expression with pLSLGFP.R (Supplemental Figure 13B). This is most likely due to a higher number of cells receiving a functional GVR and from transient expression of GFP from the linear LSL T-DNA. To verify that replicational release had occurred, total genomic DNA was extracted from leaf tissue expressing GFP and PCR was performed using primers that were homologous to sequences within GFP and the Rep/RepA coding sequences. Strong amplification of LIR sequence indicated the presence of circular GVRs (Supplemental Figure 6).

To assess gene targeting with our single-component vector system, we placed *Zif268:FokI* and *us:NPTII* repair template sequence downstream of the 2x35S promoter and within the LIR borders (pLSLZ.D.R; Figure 6A; Supplemental Figure 14). One side of a leaf was syringe infiltrated with *Agrobacterium* containing pLSLZ.D.R and the other with was infiltrated with a mixture of *Agrobacterium* containing pLSLZ.D and pREP. As an additional control, a separate leaf was infiltrated with p35SZ.D. Five days after infiltration, leaf tissue was stained in a solution containing X-Gluc (Figure 6B) and the density of blue spots was quantified (Figure 6C). We observed comparable levels of GUS activity using our single-component vector relative to the two-component system. In general, we noticed that tissue delivered pLSLZ.D.R had more uniform GUS activity. This observation may reflect a difference in the transfer efficiency of a single T-DNA molecule to that of two T-DNA molecules.

Having previously demonstrated that gene targeting is stimulated by replication of repair templates, and not sequence-specific nucleases, we predicted that positioning Zif268*:Fok*I coding sequence outside of the LIR borders (but still within the same T-DNA; pZLSLD.R; Figure 6A) would result in comparable gene targeting frequencies to pLSLZ.D and pREP. Our results

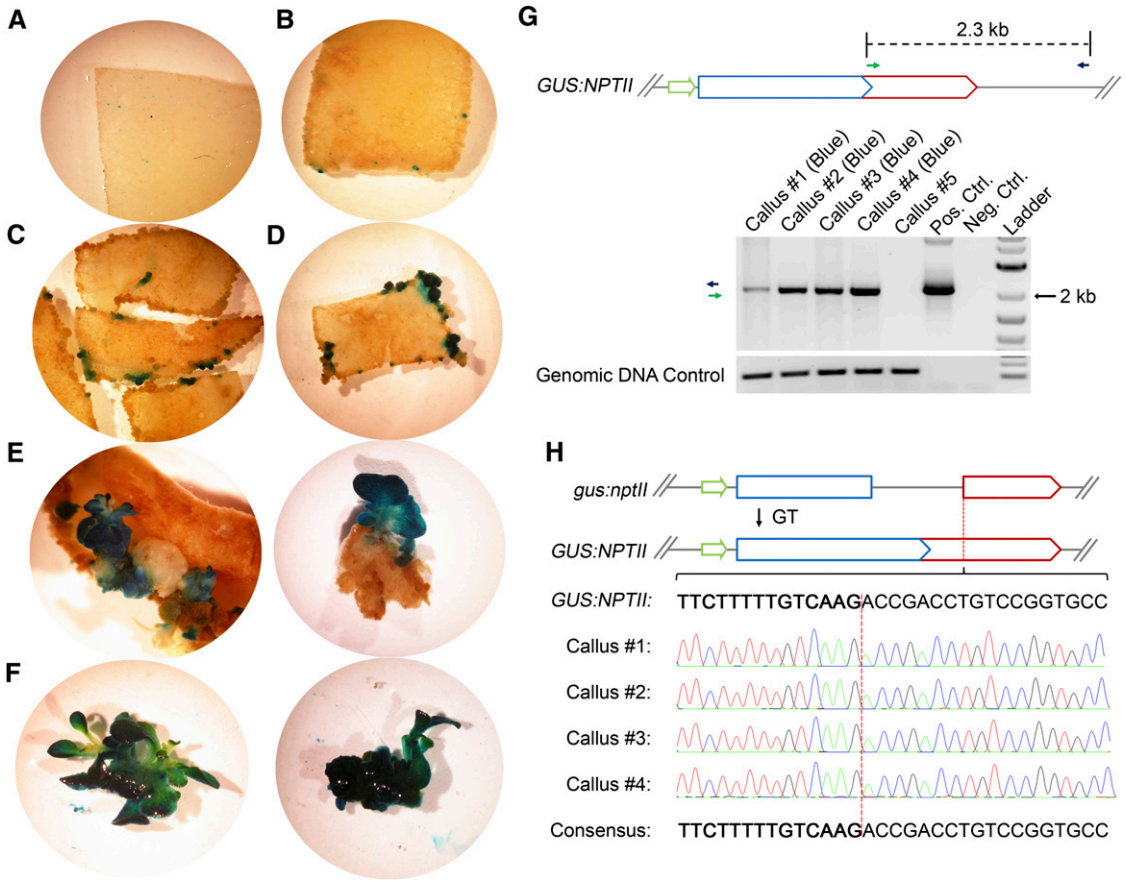

Figure 5. Regeneration of Cells with the Repaired *GUS:NPTII* Transgene.

(A) Image of a leaf disc stained in X-Gluc 0 d after plating.
(B) Image of a leaf disc stained in X-Gluc 7 d after plating.
(C) Image of leaf discs stained in X-Gluc 14 d after plating.
(D) Image of a leaf disc stained in X-Gluc 21 d after plating.
(E) Shoots with GUS activity 42 d after plating.
(F) Shoots with GUS activity 49 d after plating.
(G) PCR-based detection of the repaired *GUS:NPTII* transgene. Total DNA was extracted from four calli that stained blue. As a negative control, genomic DNA was extracted from callus that did not stain blue (Callus #5). Primers were designed to be homologous to sequence within the 600-bp modification sequence and ~1 kb downstream of the homologous DNA region carried by the repair template. The genomic DNA control used primers designed to amplify sequence within the endogenous *F-box* gene.
(H) DNA sequences of amplicons generated from calli #1 to #4.

support this hypothesis. Gene targeting frequencies of pZLSLD.R were similar to that of the two-component system (pLSLZ.D and pREP; Figure 6C). Together, these results illustrate the usefulness of single-component vectors for the delivery of GVRs for recombination-based genome editing.

DISCUSSION

The potential of geminiviruses for gene targeting has long been recognized, principally because their DNA genomes could serve as repair templates, and they replicate to high copy number. We found the key to realizing this potential was the use of sequence-specific nucleases to stimulate recombination between the virus and the chromosomal target. In addition, replication of repair templates and pleiotropic activities of Rep and RepA further enhanced gene targeting; however, these factors were insufficient to achieve gene targeting in the absence of a targeted chromosome break. Together, these data stress the importance of using geminivirus technology in conjunction with sequence-specific nucleases.

Genome size constraints hindered the use of full viruses for genome engineering. One strategy to increase the cargo capacity of begomoviruses is to replace the coat protein gene with a desired heterologous sequence of up to 800 bp. This strategy permits the delivery of some genome engineering tools, including repair templates, meganucleases, ZFN monomers, and possibly sgRNA for the CRISPR/Cas system. However, the cargo capacity is insufficient for expression of large heterologous sequences,

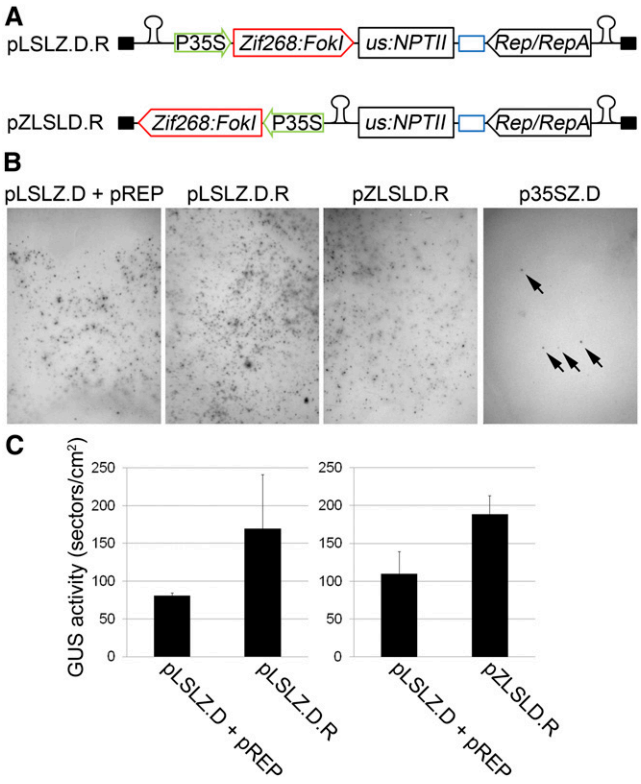

Figure 6. Single-Component Geminivirus Vectors Enable Efficient Genome Editing.

(A) Illustration of single-component GVR vectors. The blue box represents SIR sequence.

(B) Selected images of leaves transformed with pLSLZ.D and pREP, pLSLZ.D.R, pZLSLD.R, or p35SZ.D. Green and blue channels were removed from the images to better visualize GUS staining.

(C) Quantification of GUS activity in leaf tissue delivered GVR constructs. The left graph represents data from three leaves transformed with pLSLZ.D and pREP on one side and pLSLZ.D.R on the other side. The right graph represents data from two leaves transformed with pLSLZ.D and pREP on one side and pZLSLD.R on the other. Error bars represent SE. P values for both graphs were >0.05.

including TALENs and Cas9. For large cargo, there is a high likelihood that larger than wild-type genomes will recombine to smaller sizes. Virus genome size constraints are imposed by cell-to-cell movement through plasmodesmata and not by replication (Gilbertson et al., 2003). Therefore, to deliver large heterologous sequences, we deleted virus genes involved in cell-to-cell movement and retained the elements required for rolling-circle replication. This effectively eliminated constraints on cargo capacity and made it possible to simultaneously deliver sequence-specific nucleases and repair templates for gene targeting. In addition, this approach allowed us to maintain the ability for replicons to amplify to high-copy number by rolling-circle replication.

Compared with meganucleases and ZFNs, TALENs and CRISPR/Cas reagents are, in general, easier to construct and have increased efficacy. Furthermore, TALENs appear to have low frequencies of off-target cleavage (Mussolino et al., 2011), and CRISPR/Cas technology enables efficient multiplexed genome engineering (Cong et al., 2013; Wang et al., 2013). Therefore, it would be advantageous for new vectors for genome engineering to be compatible with TALEN and CRISPR/Cas technology. Our results indicate that GVRs accomplish this. By placing TALENs and Cas9/sgRNAs within GVRs, we achieved targeted mutagenesis of the endogenous *ALS* genes. For gene targeting, when the repair template is on the GVR, sequence-specific nucleases can be expressed using either GVRs or conventional T-DNA, both of which resulted in comparable levels of gene targeting. Taken together, these results suggest that not only are GVRs amenable for expression of TALENs and the CRISPR/Cas system, but they can be combined with other methods for delivery of sequence-specific nucleases, such as T-DNA.

We observed that gene targeting was enhanced by pleiotropic activity of Rep and RepA. One hypothesis to explain these results derives from our understanding of how geminiviruses establish successful infection within host plants. Geminiviruses only infect nondividing cells and encode proteins that promote entry into S-phase (Horns and Jeske, 1991; Lucy et al., 1996; Kong et al., 2000; Ascencio-Ibáñez et al., 2008). Progression into S-phase is thought to provide geminiviruses with necessary host factors required for replication (e.g., nucleotides and DNA polymerase). In monocots, S-phase entry is mediated by the mastrevirus RepA protein: RepA interacts with the plant's RBR and sequesters RBR from E2F, thereby promoting cell cycle progression into S-phase. The BeYDV RepA has been shown to interact with RBR from maize (*Zea mays*), and our results suggest that RepA stimulates tobacco cells to enter S-phase (Liu et al., 1999). The basis for this latter conclusion is that in higher-eukaryotic cells, the frequency of homologous recombination increases as cells move out of a resting state and into S and G2 (Rothkamm et al., 2003). We believe promotion into S-phase by Rep/RepA underlies the stimulatory effect these proteins have in gene targeting.

Geminiviruses infect a wide range of plants, including both monocots and dicots. We modified the BeYDV, which belongs to the genus *Mastrevirus*. Most mastreviruses infect monocots; however, the BeYDV infects dicots, including tobacco species, *Datura stramonium*, tomato (*Solanum lycopersicum*), French bean (*Phaseolus vulgaris*), and *Arabidopsis* (Liu et al., 1997). Whereas BeYDV replicons should function within this list of species, they are unlikely to replicate in plants that are not hosts to the wild-type virus, including monocots. It may be possible to develop GVR systems for monocots using other mastreviruses (e.g., wheat dwarf virus and maize streak virus). Both wheat dwarf virus and maize streak virus have long been used for protein expression in monocots and replicate to similar copy numbers as our BeYDV replicon system (Timmermans et al., 1992; Palmer et al., 1999). Thus, there is promise for GVRs to enable efficient genome engineering across many plant species.

METHODS

Vector Construction

LSL T-DNA plasmids (pLSL, pLSLGUS, pLSLGFP, pLSLZ, pLSLT, pLSLC, and pLSLZ.D) were constructed using the *Agrobacterium tumefaciens* binary vector pCAMBIA1300. LIR and SIR sequences were derived from the mild BeYDV strain (Halley-Stott et al., 2007) (GenBank accession number DQ458791). The boundaries of the LIR and SIR sequences were defined following previously described methods (Mor et al., 2003). The cauliflower mosaic virus promoter (P35S) was positioned within the replicon and

upstream of the LIR. To enable translation of coding sequence that is located downstream of the LIR, splice donor and acceptor sequences were positioned upstream and downstream, respectively, of the LIR sequence that is formed upon circularization. To enable facile cloning, pLSL was modified to harbor Gateway-compatible attR1 and attR2 sequences between the upstream LIR and SIR elements. Conventional T-DNA expression plasmids (pREP, pREPK219H, p35SZ, and p35SZ.D) were constructed using the Gateway-compatible *Agrobacterium* binary vector pMDC32. Coding sequence for Rep and RepA was derived from the mild BeYDV strain (Halley-Stott et al., 2007).

Two entry vectors were constructed for cloning sequence-specific nuclease (or reporter protein) and repair template sequences into the pLSL and pMDC32 destination plasmids. The first entry vector (pNJB091) is a derivative of pZHY013 (Zhang et al., 2013) with attL2 sequence replaced with Nos-T and attL5 sequence. The second entry vector (pNJB080) is also a derivative of pZHY013, but here the *Fok*I and attL1 sequences were replaced with the chloramphenicol resistance gene, ccdB gene, and attL5 sequence.

To engineer pLSL vectors for the repair of the *gus:nptII* transgene, pNJB80 was modified to harbor repair template sequence (*us:NPTII*), and pNJB91 was modified to harbor the Zif268:*Fok*I ZFN coding sequence. To generate the *us:NPTII* sequence for cloning into pNJB80, pDW1269 (Wright et al., 2005) was used as a template in a PCR with the oligonucleotides 5′-CGCGGA**GCGGCCGC**AGCAGTCTTACTTCCATGATTTC-3′ and 5′-GATCGATCT**CTGCAG**CCCCGCGCGTTGGCCGATTCA-3′. Bold letters indicate *Not*I and *Pst*I recognition sites for cloning into pNJB80. The resulting repair template contained 600 bp of corrective sequence flanked by 1 kb of homologous sequence to the *gus:nptII* reporter. To generate Zif268:*Fok*I sequence for cloning into pNJB91, pDW1345 (Wright et al., 2005) was used as a template in a PCR with the oligonucleotides 5′-GCCCTTCA**CCATGG**CTTCCTCCCCTCCAAAGAAAAAG-3′ and 5′-GAACGATCG**GACGTC**CTATTAAAAGTTTATCTCACCGTTA-3′. Bold letters indicate the *Nco*I and *Aat*II restriction enzyme sites used for cloning into pNJB91. The resulting plasmids were used in a MultiSite Gateway (Life Technologies) recombination reaction with pLSL or pMDC32 to generate pLSLZ.D or p35SZ.D, respectively. pLSLZ and p35SZ were generated as described above; however, instead of using pNJB80 with the *us:nptII* sequence, we used a different pNJB80 vector which harbors ~3.0 kb of filler DNA sequence.

To generate pLSLT, pNJB91 was modified to harbor the T30 TALEN pair sequence (Zhang et al., 2013). This sequence contains the two T30 TALEN open reading frames linked by a T2A translational skipping sequence. The N- and C-terminal truncation for both TALENs was NΔ152/C63 (Miller et al., 2011). The T30 TALEN sequence was cloned from pZHY528 into pNJB91 using standard cloning techniques. The resulting plasmid was used in a MultiSite Gateway recombination reaction with pLSL and pNJB80 containing the *us:NPTII* repair template to generate pLSLT.

To generate pLSLC, pNJB91 was modified to harbor a codon-optimized Cas9 gene from *Streptococcus pyogenes*, and pNJB80 was modified to harbor the *Arabidopsis thaliana* U6 RNA Pol III promoter followed by sgRNA sequence. The Cas9 gene was codon optimized for dicotyledonous plants and synthesized (GenScript) with C- and N-terminal SV40 NLS signals and an N-terminal 3x FLAG-tag sequence. The U6:gRNA sequence was synthesized on gBlocks (Integrated DNA Technologies).

Single-component LSL vectors (pLSLGFP.R, pLSLZ.D.R, and pZLSLD.R), where Rep/RepA coding sequence was positioned within the replicon, were constructed in T-DNA plasmids with the pCAMBIA backbone. Sequence of features within pLSLZ.D.R can be found in Supplemental Figure 14. Boundaries for the LIR and SIR were determined using the annotated sequence for the mild BeYDV strain (GenBank accession number DQ458791).

Plant Material

Tobacco (*Nicotiana tabacum* var Xanthi) plants, both wild type and transgenic (Wright et al., 2005), were grown at 21°C with 60% humidity under a 16-h-light and 8-h-dark cycle. Leaves (fully expanded upper leaves) from 4- to 6-week-old tobacco plants were used for *Agrobacterium* transformation. When comparing gene targeting or targeted mutagenesis frequencies between different T-DNA molecules, one leaf per plant was infiltrated.

Agrobacterium Transformation

T-DNA vectors were introduced into *Agrobacterium* (GV3101/pMP90) using the freeze-thaw method. Single transformed colonies were grown overnight at 28°C in 3 mL of Luria-Bertani starter culture containing kanamycin (50 μg/mL) and gentamycin (50 μg/mL). The next day, 1 mL of starter culture was used to inoculate 50 mL of Luria-Bertani culture containing kanamycin (50 μg/mL), gentamicin (50 μg/mL), 10 mM MES, and 20 μM acetosyringone. After reaching an OD_{600} of ~1 (around 16 h), cells were pelleted and resuspended to an OD_{600} of 0.2 using infiltration buffer (10 mM MES, 150 μM acetosyringone, and 10 mM $MgCl_2$, pH 5.6). Resuspended cultures were incubated at room temperature for 2 to 4 h. For experiments requiring the coinfiltration of two different *Agrobacterium* strains, cultures were mixed together in a 1:1 ratio, or, for three different strains, in a 1:1:1 ratio. When comparing two different *Agrobacterium* samples, the ODs of the solutions were adjusted accordingly to ensure equal concentrations of individual *Agrobacterium* strains. Leaves from tobacco plants were infiltrated with *Agrobacterium* using a 1-mL syringe. Immediately following infiltration, plants were watered and covered with a plastic dome to maintain high humidity. Plastic domes were removed ~24 h after infiltration.

GUS Assay

To visualize cells expressing GUS, leaf tissue was vacuum infiltrated with X-Gluc solution (10 mM phosphate buffer, 10 mM EDTA, 1 mM ferricyanide, 1 mM ferrocyanide, 0.1% Triton X, and 1 mM X-Gluc). Leaf tissue was then incubated in X-Gluc solution at 37°C for ~24 h. Chlorophyll was removed by incubating stained leaf tissue in 80% ethanol for ~1 week.

Tobacco Regeneration

Leaves from 4- to 5-week-old tobacco plants were infiltrated with pLSLZ.D and pREP. As a control for selection and regeneration of kanamycin resistant cells, additional leaves were infiltrated with pCAMBIA2301 (T-DNA that contains a kanamycin resistance and also the *GUS* gene). Two separate experiments were performed; for each experiment, three to five leaves from different plants were infiltrated with each T-DNA or T-DNA pair. Five days after infiltration, leafs were removed from the plant and surface sterilized by submersion in 10% bleach for ~20 min. Leaves were then washed three times in sterile water. Sterilized leaf tissue was cut into ~1 × 1-cm discs using a surgical blade. Leaf discs were plated onto regeneration media (4.4 g Murashige and Skoog medium with vitamins, 1 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, 30 g Suc, and 8 g agar, pH 5.7) containing kanamycin (100 μg/mL) and Timentin (200 μg/mL). As a negative control for tobacco selection, nontransformed leaf tissue was sterilized and plated. Discs were transferred to new plates weekly.

PCR-Based Detection of Circularized GVRs and the *GUS:NPTII* Transgene

To detect the presence of circularized GVRs, total DNA was extracted from plant tissue and used as a template for PCR. Oligonucleotides were designed to be homologous to DNA sequence located upstream and downstream of LIR sequence in the circular replicon (5′-GTTTCACTTCACACATTATTACTG-3′ and 5′-TGTTGAGAACTCTCGACGTCCTGC-3′). To minimize strand transfers during amplification, PCR was performed using the Expand Long Template PCR system (Roche).

To detect the presence of a repaired *GUS:NPTII* transgene, total DNA was extracted from leaf or callus tissue and used as a template for PCR. Oligonucleotides were designed to be homologous to DNA sequence located within the corrective sequence on the repair template and sequence ~1 kb downstream of the region of homology harbored on the repair template (5′-GTCGGTGAACAGGTATGGAAT-3′ and 5′-CTACATACCTCGCTCTGCTAATC-3′).

Detection of ZFN-, TALEN-, and Cas9-Induced Mutations

Experiments that used GVRs to mediated expression of ZFNs, TALENs, and CRISPR/Cas elements for targeted mutagenesis were performed similarly: One side of a leaf was delivered pLSL, and the other side was infiltrated with pLSL and pREP. Five to seven days after infiltration, total DNA was extracted and assessed for mutations at the appropriate sequence-specific nuclease target site. To detect Zif268:*Fok*I-induced mutations, the *gus:nptII* target locus was amplified using primers that are homologous to sequences 270 bp upstream and 214 bp downstream of the *Mse*I site (present within the spacer region of the Zif268 binding sequences; 5′-AAGGTGCACGGGAATATTTCGCGC-3′ and 5′-GCCATGATGGATACTTTCTCG-3′). The resulting amplicons were digested for 16 h at 37°C with *Mse*I and resolved by gel electrophoresis. Cleavage-resistant amplicons were gel purified, cloned into pJET1.2, and sequenced. To detect TALEN-induced mutations at the *SurA* and *SurB* loci, total DNA was first predigested with *Alu*I for 16 h at 37°C. Digested DNA was used as a template for a PCR with primers designed to amplify a 573-bp product encompassing the T30 binding site (5′-GACGGTGCAGAAAGTGAAGTA-3′ and 5′- TATGGCCCAGGAGTGTCTAA-3′). The resulting amplicons were digested for 16 h at 37°C with *Alu*I and resolved by gel electrophoresis. Cleavage-resistant amplicons were gel purified, cloned into pJET1.2, and sequenced. To detect Cas9-induced mutations, the target DNA sequence present within *SurA* and *SurB* was amplified by PCR (forward, 5′-TGACATCTGGTGGATTAGGAGC-3′; reverse, 5′-CAATCACATCCAACAAGTATGGC-3′). The resulting 408-bp amplicons were digested with *Alw*I for 16 h at 37°C. Cleavage-resistant products were gel purified and cloned into pJET1.2 and sequenced.

Data Collection and Statistics

Following staining in X-Gluc, the density of blue sectors was quantified in leaf tissue. Three nonoverlapping images were taken for each infiltrated region (leaf halves) using a camera attached to a stereoscope (Nikon). Each image captured 1.8 cm^2 of leaf tissue, totaling 5.4 cm^2 for each leaf half. Image locations were directed to capture the entire number of blue sectors for each leaf half. For samples where the total number of blue sectors exceeded 5.4 cm^2 of area, regions with the highest density of blue sectors were imaged. Leaves without detectable GUS activity were excluded from the data analysis. ImageJ software was used to calculate the number of blue sectors per image. P values in Figures 4 to 6 were generated using a paired, two-tailed Student's *t* test. P values in Figures 2 and 3 were generated using a two-tailed Student's *t* test.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ADH1*, AT1G77120.1; mild strain of BeYDV, DQ458791; *SurA*, X07644.1, *SurB*, X07645.1; pCPCbLCVA.007, AY279345; and pCPCbLCVB.002, AY279344.

Supplemental Data

The following materials are available in the online version of this article.

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

Supplemental Figure 2. Phenotype of *Arabidopsis* Plants Infected with Cabbage Leaf Curl Virus.

Supplemental Figure 3. Temporal Control of Targeted Double-Strand Breaks in Whole Plants.

Supplemental Figure 4. PCR-Based Detection of Geminivirus-Mediated Gene Targeting.

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

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

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

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

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

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

Supplemental Figure 11. Exploring the Role of Repair Templates for Geminivirus Replicon-Mediated Gene Targeting.

Supplemental Figure 12. Detection of Rep and RepA Transcripts in Tobacco Protoplasts.

Supplemental Figure 13. Expression of Green Fluorescent Protein with Single-Component Geminivirus Replicon Vectors.

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

Supplemental Methods.

ACKNOWLEDGMENTS

We thank members of the Voytas lab for the helpful discussion and insights with this work. We thank Dominique Robertson for the kind donation of the CaLCuV plasmids. This research was supported by a grant to D.F.V. from the National Science Foundation (DBI 0923827) and by a grant to J.G.-H. from Fundación Alfonso Martín Escudero.

AUTHOR CONTRIBUTIONS

N.J.B. and D.F.V. designed the research. N.J.B., J.G.-H., and T.C. performed the experiments. N.J.B. analyzed the data. N.J.B., T.C., and P.A.A. contributed reagents/materials/analysis tools. N.J.B. and D.F.V. wrote the article.

Received October 21, 2013; revised December 8, 2013; accepted December 18, 2013; published January 17, 2014.

REFERENCES

Ascencio-Ibáñez, J.T., Sozzani, R., Lee, T.J., Chu, T.M., Wolfinger, R.D., Cella, R., and Hanley-Bowdoin, L. (2008). Global analysis of Arabidopsis gene expression uncovers a complex array of changes

impacting pathogen response and cell cycle during geminivirus infection. Plant Physiol. **148:** 436–454.

Ayar, A., Wehrkamp-Richter, S., Laffaire, J.B., Le Goff, S., Levy, J., Chaignon, S., Salmi, H., Lepicard, A., Sallaud, C., Gallego, M.E., White, C.I., and Paul, W. (2013). Gene targeting in maize by somatic ectopic recombination. Plant Biotechnol. J. **11:** 305–314.

Bibikova, M., Beumer, K., Trautman, J.K., and Carroll, D. (2003). Enhancing gene targeting with designed zinc finger nucleases. Science **300:** 764.

Cai, C.Q., et al. (2009). Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Mol. Biol. **69:** 699–709.

Christian, M., Cermak, T., Doyle, E.L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A.J., and Voytas, D.F. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. Genetics **186:** 757–761.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science **339:** 819–823.

Datla, R.S.S., Hammerlindl, J.K., Pelcher, L.E., Crosby, W.L., and Selvaraj, G. (1991). A bifunctional fusion between beta-glucuronidase and neomycin phosphotransferase: A broad-spectrum marker enzyme for plants. Gene **101:** 239–246.

Desbiez, C., David, C., Mettouchi, A., Laufs, J., and Gronenborn, B. (1995). Rep protein of tomato yellow leaf curl geminivirus has an ATPase activity required for viral DNA replication. Proc. Natl. Acad. Sci. USA **92:** 5640–5644.

Ellis, J., and Bernstein, A. (1989). Gene targeting with retroviral vectors: Recombination by gene conversion into regions of nonhomology. Mol. Cell. Biol. **9:** 1621–1627.

Elmer, S., and Rogers, S.G. (1990). Selection for wild type size derivatives of tomato golden mosaic virus during systemic infection. Nucleic Acids Res. **18:** 2001–2006.

Etessami, P., Watts, J., and Stanley, J. (1989). Size reversion of African cassava mosaic virus coat protein gene deletion mutants during infection of *Nicotiana benthamiana*. J. Gen. Virol. **70:** 277–289.

Fauser, F., Roth, N., Pacher, M., Ilg, G., Sánchez-Fernández, R., Biesgen, C., and Puchta, H. (2012). In planta gene targeting. Proc. Natl. Acad. Sci. USA **109:** 7535–7540.

Gardiner, W.E., Sunter, G., Brand, L., Elmer, J.S., Rogers, S.G., and Bisaro, D.M. (1988). Genetic analysis of tomato golden mosaic virus: The coat protein is not required for systemic spread or symptom development. EMBO J. **7:** 899–904.

Gilbertson, R.L., Sudarshana, M., Jiang, H., Rojas, M.R., and Lucas, W.J. (2003). Limitations on geminivirus genome size imposed by plasmodesmata and virus-encoded movement protein: Insights into DNA trafficking. Plant Cell **15:** 2578–2591.

Gleba, Y., Marillonnet, S., and Klimyuk, V. (2004). Engineering viral expression vectors for plants: The 'full virus' and the 'deconstructed virus' strategies. Curr. Opin. Plant Biol. **7:** 182–188.

Halley-Stott, R.P., Tanzer, F., Martin, D.P., and Rybicki, E.P. (2007). The complete nucleotide sequence of a mild strain of bean yellow dwarf virus. Arch. Virol. **152:** 1237–1240.

Hayes, R., Petty, I., Coutts, R., and Buck, K. (1988). Gene amplification and expression in plants by a replicating geminivirus vector. Nature **334:** 179–182.

Hayes, R.J., Coutts, R.H.A., and Buck, K.W. (1989). Stability and expression of bacterial genes in replicating geminivirus vectors in plants. Nucleic Acids Res. **17:** 2391–2403.

Hefferon, K.L., Moon, Y.-S., and Fan, Y. (2006). Multi-tasking of nonstructural gene products is required for bean yellow dwarf geminivirus transcriptional regulation. FEBS J. **273:** 4482–4494.

Horns, T., and Jeske, H. (1991). Localization of abutilon mosaic virus (AbMV) DNA within leaf tissue by in situ hybridization. Virology **181:** 580–588.

Horváth, G.V., Pettkó-Szandtner, A., Nikovics, K., Bilgin, M., Boulton, M., Davies, J.W., Gutiérrez, C., and Dudits, D. (1998). Prediction of functional regions of the maize streak virus replication-associated proteins by protein-protein interaction analysis. Plant Mol. Biol. **38:** 699–712.

Huang, Z., Chen, Q., Hjelm, B., Arntzen, C., and Mason, H. (2009). A DNA replicon system for rapid high-level production of virus-like particles in plants. Biotechnol. Bioeng. **103:** 706–714.

Huang, Z., Phoolcharoen, W., Lai, H., Piensook, K., Cardineau, G., Zeitlin, L., Whaley, K.J., Arntzen, C.J., Mason, H.S., and Chen, Q. (2010). High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. Biotechnol. Bioeng. **106:** 9–17.

Kim, Y.G., Cha, J., and Chandrasegaran, S. (1996). Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. Proc. Natl. Acad. Sci. USA **93:** 1156–1160.

Kong, L.J., Orozco, B.M., Roe, J.L., Nagar, S., Ou, S., Feiler, H.S., Durfee, T., Miller, A.B., Gruissem, W., Robertson, D., and Hanley-Bowdoin, L. (2000). A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. EMBO J. **19:** 3485–3495.

Laufs, J., Wirtz, U., Kammann, M., Matzeit, V., Schaefer, S., Schell, J., Czernilofsky, A.P., Baker, B., and Gronenborn, B. (1990). Wheat dwarf virus Ac/Ds vectors: Expression and excision of transposable elements introduced into various cereals by a viral replicon. Proc. Natl. Acad. Sci. USA **87:** 7752–7756.

Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M., and Sheen, J. (2013). Multiplex and homologous recombination-mediated genome editing in Arabidopsis and *Nicotiana benthamiana* using guide RNA and Cas9. Nat. Biotechnol. **31:** 688–691.

Liu, L., Saunders, K., Thomas, C.L., Davies, J.W., and Stanley, J. (1999). Bean yellow dwarf virus RepA, but not rep, binds to maize retinoblastoma protein, and the virus tolerates mutations in the consensus binding motif. Virology **256:** 270–279.

Liu, L., van Tonder, T., Pietersen, G., Davies, J.W., and Stanley, J. (1997). Molecular characterization of a subgroup I geminivirus from a legume in South Africa. J. Gen. Virol. **78:** 2113–2117.

Lloyd, A., Plaisier, C.L., Carroll, D., and Drews, G.N. (2005). Targeted mutagenesis using zinc-finger nucleases in Arabidopsis. Proc. Natl. Acad. Sci. USA **102:** 2232–2237.

Lombardo, A., Genovese, P., Beausejour, C.M., Colleoni, S., Lee, Y.L., Kim, K.A., Ando, D., Urnov, F.D., Galli, C., Gregory, P.D., Holmes, M.C., and Naldini, L. (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat. Biotechnol. **25:** 1298–1306.

Lucy, A.P., Boulton, M.I., Davies, J.W., and Maule, A.J. (1996). Tissue specificity of *Zea mays* infection by maize streak virus. Mol. Plant Microbe Interact. **9:** 22–31.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science **339:** 823–826.

Marton, I., Zuker, A., Shklarman, E., Zeevi, V., Tovkach, A., Roffe, S., Ovadis, M., Tzfira, T., and Vainstein, A. (2010). Nontransgenic genome modification in plant cells. Plant Physiol. **154:** 1079–1087.

Matzeit, V., Schaefer, S., Kammann, M., Schalk, H.J., Schell, J., and Gronenborn, B. (1991). Wheat dwarf virus vectors replicate and express foreign genes in cells of monocotyledonous plants. Plant Cell **3:** 247–258.

Miller, J.C., et al. (2011). A TALE nuclease architecture for efficient genome editing. Nat. Biotechnol. **29:** 143–148.

Mor, T.S., Moon, Y.S., Palmer, K.E., and Mason, H.S. (2003). Geminivirus vectors for high-level expression of foreign proteins in plant cells. Biotechnol. Bioeng. **81:** 430–437.

Mussolino, C., Morbitzer, R., Lütge, F., Dannemann, N., Lahaye, T., and Cathomen, T. (2011). A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. Nucleic Acids Res. **39:** 9283–9293.

Palmer, K.E., Thomson, J.A., and Rybicki, E.P. (1999). Generation of maize cell lines containing autonomously replicating maize streak virus-based gene vectors. Arch. Virol. **144:** 1345–1360.

Puchta, H., Dujon, B., and Hohn, B. (1996). Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proc. Natl. Acad. Sci. USA **93:** 5055–5060.

Qi, Y., Li, X., Zhang, Y., Starker, C.G., Baltes, N.J., Zhang, F., Sander, J.D., Reyon, D., Joung, J.K., and Voytas, D.F. (2013). Targeted deletion and inversion of tandemly arrayed genes in *Arabidopsis thaliana* using zinc finger nucleases. G3 (Bethesda) **3:** 1707–1715.

Regnard, G.L., Halley-Stott, R.P., Tanzer, F.L., Hitzeroth, I.I., and Rybicki, E.P. (2010). High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. Plant Biotechnol. J. **8:** 38–46.

Reiss, B., Schubert, I., Köpchen, K., Wendeler, E., Schell, J., and Puchta, H. (2000). RecA stimulates sister chromatid exchange and the fidelity of double-strand break repair, but not gene targeting, in plants transformed by Agrobacterium. Proc. Natl. Acad. Sci. USA **97:** 3358–3363.

Rothkamm, K., Krüger, I., Thompson, L.H., and Löbrich, M. (2003). Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol. Cell. Biol. **23:** 5706–5715.

Russell, D.W., and Hirata, R.K. (1998). Human gene targeting by viral vectors. Nat. Genet. **18:** 325–330.

Shen, W.H., and Hohn, B. (1994). Amplification and expression of the β-glucuronidase gene in maize plants by vectors based on maize streak virus. Plant J. **5:** 227–236.

Shen, W.H., and Hohn, B. (1991). Mutational analysis of the small intergenic region of maize streak virus. Virology **183:** 721–730.

Shukla, V.K., et al. (2009). Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature **459:** 437–441.

Smith, J., et al. (2006). A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. Nucleic Acids Res. **34:** e149.

Stenger, D.C., Revington, G.N., Stevenson, M.C., and Bisaro, D.M. (1991). Replicational release of geminivirus genomes from tandemly repeated copies: Evidence for rolling-circle replication of a plant viral DNA. Proc. Natl. Acad. Sci. USA **88:** 8029–8033.

Suárez-López, P., and Gutiérrez, C. (1997). DNA replication of wheat dwarf geminivirus vectors: effects of origin structure and size. Virology **227:** 389–399.

Timmermans, M.C., Das, O.P., and Messing, J. (1992). Trans replication and high copy numbers of wheat dwarf virus vectors in maize cells. Nucleic Acids Res. **20:** 4047–4054.

Townsend, J.A., Wright, D.A., Winfrey, R.J., Fu, F., Maeder, M.L., Joung, J.K., and Voytas, D.F. (2009). High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature **459:** 442–445.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell **153:** 910–918.

Wang, Q., and Taylor, M.W. (1993). Correction of a deletion mutant by gene targeting with an adenovirus vector. Mol. Cell. Biol. **13:** 918–927.

Ward, A., Etessami, P., and Stanley, J. (1988). Expression of a bacterial gene in plants mediated by infectious geminivirus DNA. EMBO J. **7:** 1583–1587.

Wright, D.A., Townsend, J.A., Winfrey, R.J., Jr., Irwin, P.A., Rajagopal, J., Lonosky, P.M., Hall, B.D., Jondle, M.D., and Voytas, D.F. (2005). High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J. **44:** 693–705.

Wright, E.A., Heckel, T., Groenendijk, J., Davies, J.W., and Boulton, M.I. (1997). Splicing features in maize streak virus virion- and complementary-sense gene expression. Plant J. **12:** 1285–1297.

Xie, Q., Sanz-Burgos, A.P., Guo, H., García, J.A., and Gutiérrez, C. (1999). GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Mol. Biol. **39:** 647–656.

Zhang, F., Maeder, M.L., Unger-Wallace, E., Hoshaw, J.P., Reyon, D., Christian, M., Li, X., Pierick, C.J., Dobbs, D., Peterson, T., Joung, J.K., and Voytas, D.F. (2010). High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. Proc. Natl. Acad. Sci. USA **107:** 12028–12033.

Zhang, X., and Mason, H. (2006). Bean yellow dwarf virus replicons for high-level transgene expression in transgenic plants and cell cultures. Biotechnol. Bioeng. **93:** 271–279.

Zhang, Y., Zhang, F., Li, X., Baller, J.A., Qi, Y., Starker, C.G., Bogdanove, A.J., and Voytas, D.F. (2013). Transcription activator-like effector nucleases enable efficient plant genome engineering. Plant Physiol. **161:** 20–27.

Zuo, J., Niu, Q.W., and Chua, N.H. (2000). Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. **24:** 265–273.