

Nontransgenic Genome Modification in Plant Cells^{1[W][OA]}

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Zinc finger nucleases (ZFNs) are a powerful tool for genome editing in eukaryotic cells. ZFNs have been used for targeted mutagenesis in model and crop species. In animal and human cells, transient ZFN expression is often achieved by direct gene transfer into the target cells. Stable transformation, however, is the preferred method for gene expression in plant species, and ZFN-expressing transgenic plants have been used for recovery of mutants that are likely to be classified as transgenic due to the use of direct gene-transfer methods into the target cells. Here we present an alternative, nontransgenic approach for ZFN delivery and production of mutant plants using a novel *Tobacco rattle virus* (TRV)-based expression system for indirect transient delivery of ZFNs into a variety of tissues and cells of intact plants. TRV systemically infected its hosts and virus ZFN-mediated targeted mutagenesis could be clearly observed in newly developed infected tissues as measured by activation of a mutated reporter transgene in tobacco (*Nicotiana tabacum*) and petunia (*Petunia hybrida*) plants. The ability of TRV to move to developing buds and regenerating tissues enabled recovery of mutated tobacco and petunia plants. Sequence analysis and transmission of the mutations to the next generation confirmed the stability of the ZFN-induced genetic changes. Because TRV is an RNA virus that can infect a wide range of plant species, it provides a viable alternative to the production of ZFN-mediated mutants while avoiding the use of direct plant-transformation methods.

Methods for genome editing in plant cells have fallen behind the remarkable progress made in whole-genome sequencing projects. The availability of reliable and efficient methods for genome editing would foster gene discovery and functional gene analyses in model plants and the introduction of novel traits in agriculturally important species (Puchta, 2002; Hanin and Paszkowski, 2003; Reiss, 2003; Porteus, 2009). Genome editing in various species is typically achieved by integrating foreign DNA molecules into the target genome by homologous recombination (HR). Genome editing by HR is routine in yeast (*Saccharomyces cerevisiae*) cells (Scherer and Davis, 1979) and has been adapted for other species, including *Drosophila*, human cell lines, various fungal species, and mouse embryonic stem cells (Baribault and Kemler, 1989; Venken and Bellen, 2005; Porteus, 2007; Hall et al., 2009; Laible and Alonso-González, 2009; Tenzen et al., 2009). In plants, however, foreign DNA molecules,

which are typically delivered by direct gene-transfer methods (e.g. *Agrobacterium* and microbombardment of plasmid DNA), often integrate into the target cell genome via nonhomologous end joining (NHEJ) and not HR (Ray and Langer, 2002; Britt and May, 2003).

Various methods have been developed to identify and select for rare site-specific foreign DNA integration events or to enhance the rate of HR-mediated DNA integration in plant cells. Novel T-DNA molecules designed to support strong positive- and negative-selection schemes (e.g. Thykjaer et al., 1997; Terada et al., 2002), altering the plant DNA-repair machinery by expressing yeast chromatin remodeling protein (Shaked et al., 2005), and PCR screening of large numbers of transgenic plants (Kempin et al., 1997; Hanin et al., 2001) are just a few of the experimental approaches used to achieve HR-mediated gene targeting in plant species. While successful, these approaches, and others, have resulted in only a limited number of reports describing the successful implementation of HR-mediated gene targeting of native and transgenic sequences in plant cells (for review, see Puchta, 2002; Hanin and Paszkowski, 2003; Reiss, 2003; Porteus, 2009; Weinthal et al., 2010).

HR-mediated gene targeting can potentially be enhanced by the induction of genomic double-strand breaks (DSBs). In their pioneering studies, Puchta et al. (1993, 1996) showed that DSB induction by the naturally occurring rare-cutting restriction enzyme *I-SceI* leads to enhanced HR-mediated DNA repair in plants. Expression of *I-SceI* and another rare-cutting restric-

¹ This work was supported by Danziger Innovations Ltd., Danziger "Dan" Flower Farm, and the Israel Science Foundation (grant no. 269/09).

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www.plantphysiol.org/cgi/doi/10.1104/pp.110.164806

tion enzyme (*I-CeuI*) also led to efficient NHEJ-mediated site-specific mutagenesis and integration of foreign DNA molecules in plants (Salomon and Puchta, 1998; Chilton and Que, 2003; Tzfira et al., 2003). Naturally occurring rare-cutting restriction enzymes thus hold great promise as a tool for genome editing in plant cells (Carroll, 2004; Pâques and Duchateau, 2007). However, their wide application is hindered by the tedious and next to impossible reengineering of such enzymes for novel DNA-target specificities (Pâques and Duchateau, 2007).

A viable alternative to the use of rare-cutting restriction enzymes is the zinc finger nucleases (ZFNs), which have been used for genome editing in a wide range of eukaryotic species, including plants (e.g. Bibikova et al., 2001; Porteus and Baltimore, 2003; Lloyd et al., 2005; Urnov et al., 2005; Wright et al., 2005; Beumer et al., 2006; Moehle et al., 2007; Santiago et al., 2008; Shukla et al., 2009; Tovkach et al., 2009; Townsend et al., 2009; Osakabe et al., 2010; Petolino et al., 2010; Zhang et al., 2010). Here too, ZFNs have been used to enhance DNA integration via HR (e.g. Shukla et al., 2009; Townsend et al., 2009) and as an efficient tool for the induction of site-specific mutagenesis (e.g. Lloyd et al., 2005; Zhang et al., 2010) in plant species. The latter is more efficient and simpler to implement in plants as it does not require codelivery of both ZFN-expressing and donor DNA molecules and it relies on NHEJ—the dominant DNA-repair machinery in most plant species (Ray and Langer, 2002; Britt and May, 2003).

ZFNs are artificial restriction enzymes composed of a fusion between an artificial Cys₂His₂ zinc-finger protein DNA-binding domain and the cleavage domain of the *FokI* endonuclease. The DNA-binding domain of ZFNs can be engineered to recognize a variety of DNA sequences (for review, see Durai et al., 2005; Porteus and Carroll, 2005; Carroll et al., 2006). The *FokI* endonuclease domain functions as a dimer, and digestion of the target DNA requires proper alignment of two ZFN monomers at the target site (Durai et al., 2005; Porteus and Carroll, 2005; Carroll et al., 2006). Efficient and coordinated expression of both monomers is thus required for the production of DSBs in living cells. Transient ZFN expression, by direct gene delivery, is the method of choice for targeted mutagenesis in human and animal cells (e.g. Urnov et al., 2005; Beumer et al., 2006; Meng et al., 2008). Among the different methods used for high and efficient transient ZFN delivery in animal and human cell lines are plasmid injection (Morton et al., 2006; Foley et al., 2009), direct plasmid transfer (Urnov et al., 2005), the use of integrase-defective lentiviral vectors (Lombardo et al., 2007), and mRNA injection (Takasu et al., 2010).

In plant species, however, efficient and strong gene expression is often achieved by stable gene transformation. Both transient and stable ZFN expression have been used in gene-targeting experiments in plants (Lloyd et al., 2005; Wright et al., 2005; Maeder et al., 2008; Cai et al., 2009; de Pater et al., 2009; Shukla et al., 2009; Tovkach et al., 2009; Townsend et al., 2009;

Osakabe et al., 2010; Petolino et al., 2010; Zhang et al., 2010). In all cases, direct gene-transformation methods, using polyethylene glycol, silicon carbide whiskers, or *Agrobacterium*, were deployed. Thus, while mutant plants and tissues could be recovered, potentially without any detectable traces of foreign DNA, such plants were generated using a transgenic approach and are therefore still likely to be classified as transgenic. Furthermore, the recovery of mutants in many cases is also dependent on the ability to regenerate plants from protoplasts, a procedure that has only been successfully applied in a limited number of plant species. Therefore, while ZFN technology is a powerful tool for site-specific mutagenesis, its wider implementation for plant improvement may be somewhat limited, both by its restriction to certain plant species and by legislative restrictions imposed on transgenic plants.

Here we describe an alternative to direct gene transfer for ZFN delivery and for the production of mutated plants. Our approach is based on the use of a novel *Tobacco rattle virus* (*TRV*)-based expression system, which is capable of systemically infecting its host and spreading into a variety of tissues and cells of intact plants, including developing buds and regenerating tissues. We traced the indirect ZFN delivery in infected plants by activation of a mutated reporter gene and we demonstrate that this approach can be used to recover mutated plants.

RESULTS

TRV Can Be Used for Indirect Foreign Gene Expression in Plants

The efficient use of viral-based vectors for transient expression of foreign genes in plant cells (Gleba et al., 2007) prompted us to explore whether viral vectors can be used as an alternative, nondirect gene-transfer method for the expression of ZFNs in plant cells. We selected the positive-strand RNA virus *TRV* because of its use in a wide range of plant species (Ratcliff et al., 2001; Liu et al., 2002a). Viral vectors are more often used for induced gene silencing (Carrillo-Tripp et al., 2006) than for overexpression of foreign genes (Lico et al., 2008). We thus first tested whether *TRV* can mediate the overexpression of a reporter gene in various target plant tissues, and in particular in the growing and newly developing tissues that are often used for regeneration and development of new plants. To this end, we cloned the red fluorescent reporter gene *DsRed2* under the control of the double subgenomic promoter (*sg-P*) to produce *pTRV2-Δ2b-sgP::DsRed2* (based on the generic structure of *pTRV2-Δ2b-sgP::GOI*; Fig. 1A), transformed the vector into *Agrobacterium* cells, and used the agroinfiltration method to inoculate *Nicotiana benthamiana*, tobacco (*Nicotiana tabacum* 'Samsung'), and petunia (*Petunia hybrida* 'Burgundy Dream') plants. Strong and uniform expression of the *DsRed2* reporter gene in cells of leaves that

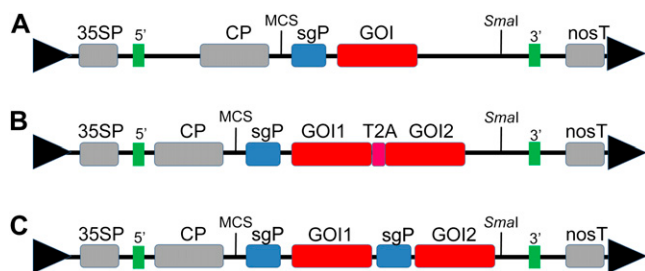


Figure 1. Structure and key features of pTRV-based expression vectors. A, pTRV- Δ 2b-sgP::GOI designed to drive the expression a single gene of interest (GOI) under the control of the sgP constitutive promoter. B, pTRV- Δ 2b-sgP::GOI1-T2A-GOI2 designed to drive the coexpression of two genes as a single transcript in which the coding sequences of the two genes (GOI1 and GOI2) are separated by a T2A sequence. C, pTRV- Δ 2b-[sgP::GOI1][sgP::GOI2] designed to drive the coexpression of two genes from two independent sg-Ps. The T-DNA region of each vector is presented. Also shown are (1) the constitutive 35S promoter (35sP) and the nopaline synthase terminator (nosT), needed for the production of primary viral transcript following agroinfiltration, and (2) the 5' and 3' untranslated regions of the TRV2 needed for viral replication and transcription. The multiple cloning site (MCS) includes *EcoRI*, *XbaI*, *KpnI*, *SacI*, and *XhoI*. CP, Coat protein.

developed after the infection was clearly observed 7 d post inoculation (Supplemental Fig. S1). Continued and strong expression (even several months post inoculation) was also clearly observed in various newly developed plant tissues and organs, including roots (Fig. 2). DsRed2 expression was not observed in noninfected, control plants (data not shown). It is important to note that newly developed virus-infected tissues that have not been exposed to *Agrobacterium* cells, and plants regenerating from such tissues, are not considered transgenics as they do not carry foreign DNA sequences. We nevertheless tested whether plants can also be mechanically inoculated by recombinant pTRV. We used sap from pTRV2- Δ 2b-sgP::DsRed2-infected plants to infect the stems and leaves of healthy *N. benthamiana* and petunia plants. Here again, strong and uniform DsRed2 expression was observed in plant tissues and organs developed on infected plants (e.g. Supplemental Fig. S2). Thus, while

agroinfiltration is simpler to perform than mechanical inoculation, it is possible to avoid using *Agrobacterium* altogether during infection of the target plants.

TRV Can Be Used for Dual Foreign Gene Expression in Plants

ZFN-mediated gene targeting typically requires the expression of two different ZFN monomers in a single cell. Codelivery of two ZFNs into a single cell often poses an obstacle to the use of this technology for genome editing in eukaryotic cells. This technical challenge has been addressed, for example, by using a lentiviral vector system (Lombardo et al., 2007) or dual-expression cassettes (Cai et al., 2009; de Pater et al., 2009). We thus tested whether TRV can drive the simultaneous expression of two foreign genes in infected plant tissues. We first tested whether coinfection with two pTRVs, each carrying a different reporter gene, would result in the coexpression of two genes in a single cell. We cloned the *Rssu-EGFP* (chloroplast-targeted EGFP fused to Rubisco transit peptide) reporter gene under the control of the sg-P promoter to produce pTRV2- Δ 2b-sgP::Rssu-EGFP and used this construct in coinoculation experiments with pTRV2- Δ 2b-sgP::DsRed2 (both vectors are based on the structure of the generic vector pTRV2- Δ 2b-sgP::GOI; Fig. 1A). Supplemental Figure S3 shows that both reporter proteins (i.e. DsRed2 and Rssu-tagged EGFP) were coexpressed in infected tobacco mesophyll cells developed from agroinfiltrated plants. Coexpression of both DsRed2 and EGFP in infected leaves of *N. benthamiana* plants was also observed following their mechanical inoculation (data not shown), whereas expression of GFP and DsRed2 was not observed in noninfected, control plants (data not shown). Note, however, that coexpression of the reporter genes was much less efficient than single-gene expression; in many instances, mosaic-like expression was observed in infected tissues and organs. This can potentially be attributed to uneven spread and/or competition between the two similar viruses in the infected cells.

We next tested whether the cloning of two reporter genes on a single pTRV2 construct can lead to their

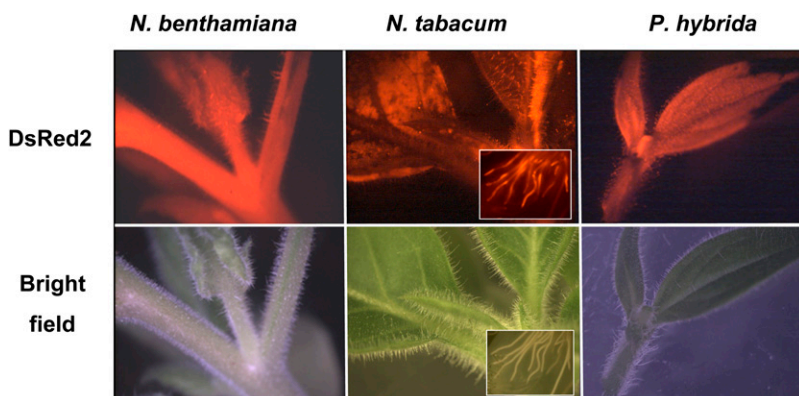


Figure 2. pTRV-mediated expression of a single reporter gene (DsRed2) in newly developed tissues and organs. Plants were infected by pTRV- Δ 2b-sgP::DsRed2. Images were taken by fluorescence stereoscope. DsRed2 expression is shown in red.

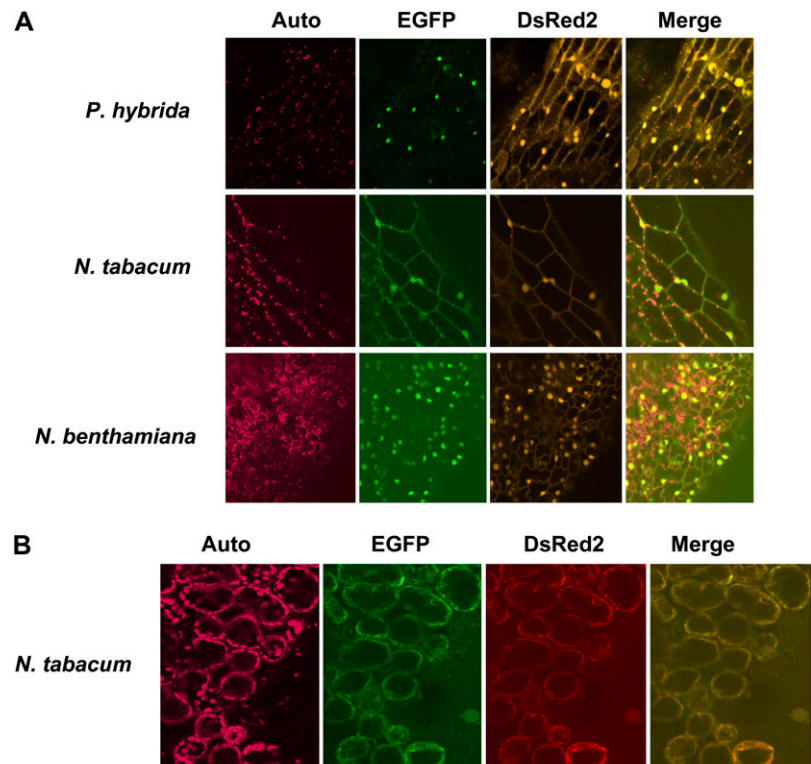
more uniform and consistent expression in infected cells. The genes were either separated by the *Thosaea asigna* virus sequence T2A (Fig. 1B) or driven by separate double sg-Ps (Fig. 1C). Figure 3A shows uniform distribution of both EGFP and DsRed2 in cells of various infected plant species when the pTRV2- Δ 2b-sgP::DsRed2-T2A-EGFP vector, in which the reporter genes were separated by the T2A sequence, was used for infection. Similarly, coexpression of EGFP and DsRed2 could also be observed in plant cells infected by pTRV2- Δ 2b-[sgP::EGFP][sgP::DsRed2], in which the expression of each reporter gene is driven by its own sg-P, as shown in Figure 3B for infected tobacco plants. A similar expression pattern was observed in *N. benthamiana* and petunia plants, infected by either agroinfiltration or direct infection methods, with continuous and strong expression in various plant tissues and organs of the infected plants, throughout their growth and development (data not shown). Neither GFP nor DsRed2 expression was observed in noninfected, control plants (data not shown).

pTRV-Mediated ZFN Expression Leads to Site-Specific Mutagenesis

Our data indicate that pTRV2 vectors can be used for the simultaneous efficient delivery of two foreign genes into cells and growing and developing organs of plants. We next tested whether pTRV2-mediated expression of a ZFN protein can lead to genomic modifications in infected plant cells. We used a visual

transgenic repair assay (Tovkach et al., 2009, 2010) in which ZFN activity is measured by restoration of a mutated GUS-encoding gene (mGUS; Fig. 4A). In this assay, a *uidA* gene is engineered to carry a stop codon within the 6-bp spacer of the ZFN target site, leading to premature termination of *uidA* translation in plant cells. Digestion of the *uidA* sequence and misrepair of the DSB site may lead to activation of GUS expression. We produced transgenic tobacco and petunia plants using a mGUS construct that was engineered to carry the QQR ZFN target site (Tovkach et al., 2009, 2010). We also constructed pTRV2- Δ 2b-sgP::QQR vector (based on the generic vector pTRV2- Δ 2b-sgP::GOI; Fig. 1A), which expresses the QQR ZFN under the control of sgP. Transgenic plants were then infected by agroinfiltration (or by direct delivery of viral virions) and targeting of the mGUS-coding sequence was detected by histochemical GUS staining of infected tissues that developed post inoculation. pTRV2-mediated ZFN expression led to site-specific mutagenesis in a variety of tissues and organs that developed after the inoculation, as determined by GUS expression (Fig. 4). Thus, for example, GUS expression was clearly visible in newly developed leaves of tobacco (Fig. 4B) and petunia (Fig. 4C) plants. More importantly, ZFN-mediated targeting could also be detected in newly developed buds (Fig. 4, D and E), developing primordia (e.g. Fig. 4F), and even flowers and reproductive tissues of infected plants (e.g. Fig. 4, G and H). GUS expression was not observed in the tissue of noninfected plants (data not shown).

Figure 3. pTRV-mediated coexpression of two reporter genes in newly developed plant cells. A, Coexpression of fluorescent reporter genes DsRed2 and EGFP in newly developed leaves of pTRV- Δ 2b-sgP::DsRed2-T2A-EGFP-infected plants. DsRed2 and EGFP fluorescence are in orange and green, respectively, and plastid autofluorescence is in dark red. B, Coexpression of DsRed2 and EGFP in newly developed leaves of pTRV- Δ 2b-[sgP::DsRed2][sgP::EGFP]-infected tobacco plant. DsRed2 and EGFP fluorescence are in red and green, respectively, and plastid autofluorescence is in dark red. Images in A and B are single confocal sections.



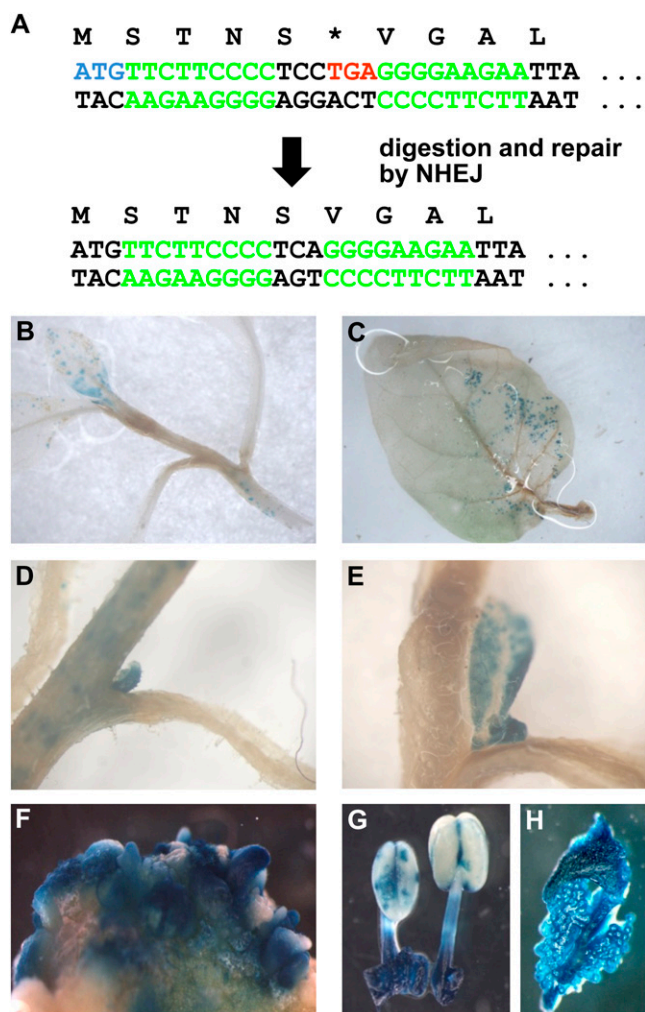


Figure 4. pTRV-mediated ZFN expression leads to site-specific mutagenesis in newly developed tissues of infected plants. A, Outline of the mGUS reporter gene-repair assay designed to monitor ZFN-mediated mutagenesis in transgenic plants. The mGUS-encoding gene is disrupted by a stop codon (in red) within the 6-bp spacer of the ZFN target site. Reconstruction of active GUS gene occurs by putative deletion of a CTG sequence. The ZFN binding sequences are shown in green and the GUS initiation codon in blue. B to H, Detection of site-specific mutagenesis events in newly developed tissues of pTRV- $\Delta 2b$ -sgP::QQR infected plants by 5-bromo-4-chloro-3-indolyl- β -glucuronic acid staining. GUS expression was detected in newly developed tobacco (B) and petunia (C) leaves, 13 and 22 d post inoculation, respectively, and in newly developed petunia (D) and tobacco (E) buds, 11 and 50 d post inoculation, respectively. GUS expression was also detected in petunia developing primordia (F) and petunia flower and reproductive tissues (G and H) of the GUS-positive mature plant.

We further investigated the molecular outcome of virus-mediated ZFN expression in infected plants by randomly analyzing several TRV-infected petunia and tobacco lines/tissues by PCR amplification and DNA sequencing. We observed a wide variety of changes in the mGUS-targeted region. Figure 5 shows the sequencing data of some of the detected mutation events, which revealed the presence of small deletions and/or

insertions at the target site, some of which could explain the reconstruction of a functional *uidA* gene.

Recovery of Mutated Tobacco and Petunia Plants

To demonstrate the feasibility of our approach for the generation of fully developed mutant plants, we sampled several petioles and lower leaf parts from infected tobacco and petunia plants: Tissues were placed into regeneration medium, primordia were subcultured, and buds were set to root and further develop into mature healthy plants. Analyses of GUS in primordia, buds, and mature plants (e.g. Fig. 6A) revealed strong uniform expression. We next investigated the stability and heredity of the changes in those plants by harvesting seeds from flowering tobacco and petunia plants (e.g. Fig. 6B) and analyzing *uidA*-transgenic plantlets for GUS activity. As expected, GUS expression could clearly be detected in both petunia (Fig. 6C) and tobacco (Fig. 6D) seedlings, demonstrating that the virally expressed ZFN-induced mutations were stably inherited. Sequencing analyses of several petunia seedlings (e.g. Fig. 7A) confirmed the nature of the ZFN-induced mutation and showed, as expected, a (single-type) mutation that could explain the reconstruction of a functional *uidA* gene in all siblings derived from a given plant. Similarly, sequencing analysis of tobacco seedlings further confirmed the nature of the ZFN-induced mutation in this species (Fig. 7A). Reverse transcription (RT)-PCR analysis also revealed that, as expected from a non-seed-transmissible virus, the newly developed seedlings were free of viral particles (Fig. 7B). Our findings thus show that viral vectors can be successfully used to induce permanent and heritable mutations in plants by indirect transfer of ZFNs into the target tissues, and that mutated plants can be recovered from existing organs, producing virus- and ZFN-free mutated offspring.

DISCUSSION

ZFN-mediated site-specific mutagenesis relies on inaccurate DSB repair by NHEJ and has been used to target various transgene and native sequences in *Arabidopsis thaliana* and tobacco plants (e.g. Lloyd et al., 2005; Maeder et al., 2008; de Pater et al., 2009; Tovkach et al., 2009; Osakabe et al., 2010; Zhang et al., 2010). ZFNs have also been used for transgene removal in tobacco plants (Petolino et al., 2010), leading to NHEJ-mediated truncated repair of the targeted sites. In addition, ZFNs (when delivered with donor DNA molecules) have been used to stimulate site-specific HR-mediated integration of donor DNA molecules into the genomes of tobacco and corn (*Zea mays*) plants (Shukla et al., 2009; Townsend et al., 2009). However, their expression in tobacco and corn target cells also led to site-specific mutagenesis. Thus, ZFNs hold great potential as site-specific mutagens and further development of this technology is ex-

Figure 5. Molecular analysis of ZFN-mediated mutagenesis events in petunia (P) and tobacco (T) plants. The initiation codon and the ZFN-binding sites on the top strand of the mGUS sequences are in blue and purple, respectively. The stop codon sequence is in red. The predicted outcome of positive (+) or negative (-) GUS expression is indicated on the right.

mGUS	GACGGTACCATGTTCTTCCCCTCC	TG--AGGGGAAGAATTACGTCCTGTAGAAACCCCA	-
T7	GACGGTACCATGTTCTTCCCCTCC	TG--GAGGGGAAGAATTACGTCCTGTAGAAACCCCA	-
P47	GACGGTACCATGTTCTTCCCCTCC	TG--AAGGGGAAGAATTACGTCCTGTAGAAACCCCA	-
P41	GACGGTACCATGTTCTTCCCCTCC	TGTGAGGGGAAGAATTACGTCCTGTAGAAACCCCA	-
P30	GACGGTACCATGTTCTTCCCACCCG	--AGGGGAAGAATTACGTCCTGTAGAAACCCCA	+
P29	GACGGTACCATGTTCTTCCCCTCT	TG--AGGGGAAGAATTACGTCCTGTAGAAACCCCA	-
T115	GACGGTACCATGTTCTTCCCCTC	--G--AGGGGAAGAATTACGTCCTGTAGAAACCCCA	-
T11	GACGGTACCATGTTCTTCCCCT	--TG--AGGGGAAGAATTACGTCCTGTAGAAACCCCA	-
T6	GACGGTACCATGTTCTTCCCCT	-----AGGGGAAGAATTACGTCCTGTAGAAACCCCA	+
P62	GACGGTACCATGTTCTTCCCCT	---G--AGGGGAAGAATTACGTCCTGTAGAAACCCCA	-
T4	GACGGTACCATGTTCTTCCCCTC	-----GGGAAGAATTACGTCCTGTAGAAACCCCA	-
T114	GACGGTACCATGTTCTTCCCCT	-----GGGAAGAATTACGTCCTGTAGAAACCCCA	-
T101	GACGGTACCATGTTCTTCCC	-----GGGAAGAATTACGTCCTGTAGAAACCCCA	-
P26	GACGGTACCATGTTCTTCC	-----GGGAAGAATTACGTCCTGTAGAAACCCCA	-
P49	GACGGTACCATGTTCTTCCC	-----TCGTCCTGTAGAAACCCCA	-
P48	GACGGTA--AG	-----GGGAAGAATTACGTCCTGTAGAAACCCCA	-
T104	GACGGTACCATGT	-----TTACGTCCTGTAGAAACCCCA	-
P51	GACGGTA	-----GAATTACGTCCTGTAGAAACCCCA	-
T111	GACGGTACCATG	-----TTCTGTAGAAACCCCA	+
P34	GACGGTACCATG	-----TTCTT-----CCCCA	-
P25	GAC	-----CCCCA	-

pected to accelerate gene discovery and lead to the development of novel crop plants.

Key to the implementation of ZFNs for site-specific mutagenesis is their efficient expression in regenerating cells or tissues, from which mutated plants can potentially arise. Transgenic approaches have been used in *Arabidopsis* for efficient expression of ZFNs in L2 cells of the shoot apical meristem from which mutated seeds will eventually develop. Both induction (e.g. heat shock- or estrogen-inducible stable expression systems; Lloyd et al., 2005; Tovkach et al., 2009; Zhang et al., 2010) and overexpression (i.e. a constitutively expressed ZFN; de Pater et al., 2009) have been used to drive the expression of ZFNs in transgenic *Arabidopsis* plants. In a similar approach, ZFN-overexpressing tobacco lines have been crossed with target tobacco lines, allowing the ZFNs to function and remove their target sequence from the target tobacco plant genomes (Petolino et al., 2010). While in *Arabidopsis*, tobacco, and other species that can be propagated by seeds, the ZFN transgene can potentially be eliminated in successive generations, the mutated offspring are likely to be classified as transgenic, due to stable incorporation of the ZFN expression cassette in the parental lines.

Transient ZFN expression can potentially be used as an alternative to ZFN-expressing transgenic plants. Indeed, direct plasmid transfer and *Agrobacterium*-mediated gene-transfer methods have been the methods of choice for ZFN delivery into tobacco and corn target cells, respectively (Shukla et al., 2009; Townsend et al., 2009). Nevertheless, while proven useful for generating ZFN-free mutated plants (as determined by molecular analysis), the use of direct, albeit transient DNA-transfer methods for the delivery of ZFN-expression constructs into target cells may still lead to unwanted and hard to detect traces of foreign DNA in the mutated lines. Thus, even when using transient ZFN expression, crop plants

can potentially be classified as transgenic or be subjected to extensive investigation to confirm that they do not possess any traces of foreign DNA within their genome.

The recovery of mutants from transient ZFN expression experiments depends on the ability to regenerate plants from single cells without direct selection, a procedure that has only been successfully applied to a limited number of plant species (e.g. tobacco protoplasts). Therefore, while ZFN technology is a powerful tool for site-specific mutagenesis, its wider implemen-

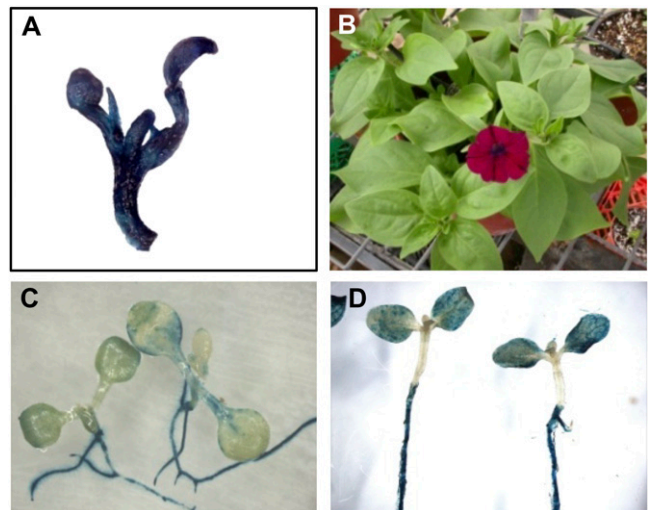


Figure 6. Mutant plants can develop directly, without a regeneration step, from virus-infected plants and can stably pass the mutation on to their offspring. Uniformly GUS-stained plantlets, exemplified here with infected petunia (A), which were regenerated from infected plants, and allowed to root, mature (B), and set seed. Also shown are GUS-stained petunia (C) and tobacco (D) seedlings obtained from mature, virus-infected plants.

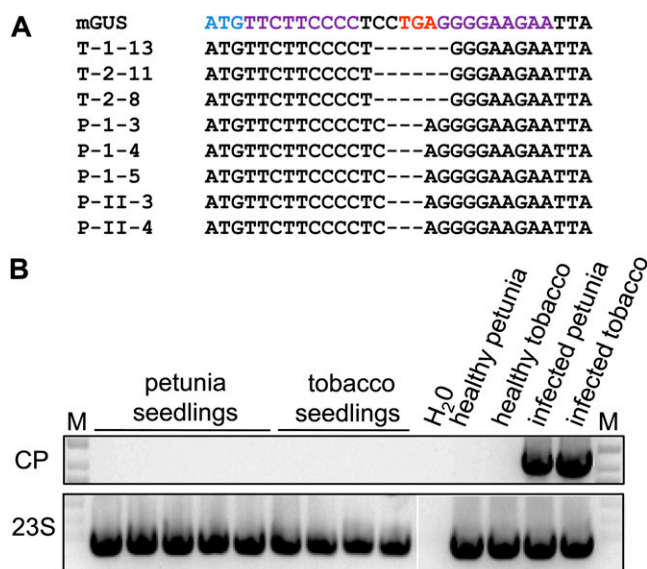


Figure 7. Molecular analysis of randomly selected GUS-positive petunia and tobacco seedlings. A, Mutagenesis events in petunia (P) and tobacco (T) offspring. The initiation codon and the ZFN-binding sites on the top strand of the mGUS sequences are in blue and purple, respectively. Stop codon is in red. B, Top section, RT-PCR analysis of pTRV genomes in infected, healthy, and mutated seedlings of petunia and tobacco. Bottom section, RT-PCR analysis of housekeeping gene (the plastid 23S RNA gene). pTRV genomes (identified by RT-PCR amplification of TRV2 sequence coat protein) were detected in infected, but not healthy or mutated seedlings (M, DNA marker ladder).

tation for plant improvement may be somewhat limited, by both its restriction to certain plant species and legislative restrictions imposed on transgenic plants. An infection system that can lead to high levels of ZFN expression in a wide variety of plant species, as well as organs and tissues, and that will allow regeneration of mutated and ZFN-free plants is thus needed.

The strong and uniform expression pattern observed in pTRV-infected plants (Figs. 2 and 3), which was attributed to the virus's systemic movement through the infected plant's cells and organs, led us to suggest TRV as an efficient vector for the delivery of ZFNs into growing and developing plant tissues. Furthermore, since expression of ZFNs can lead to site-specific mutagenesis, we suggested that the mutated plants could potentially be regenerated or even directly developed from the infected tissues without the need for direct DNA transfer into them. Indeed, we demonstrate that virus-mediated ZFNs can lead to site-specific mutagenesis in newly developed tissues (Figs. 4 and 5) and that mutant plants can be recovered from the infected tissues (Fig. 6). The ability of pTRV to move from cell to cell and to target different tissues could potentially allow the recovery of mutated plants from various other cells and tissues. We should emphasize that newly developed virus-infected tissues that have not been directly transformed, and plants regenerating from such tissues, are not transgenic.

Furthermore, we demonstrated that plants can also be mechanically inoculated by recombinant pTRV from the sap of infected plants. It is thus possible to avoid using direct gene-transfer methods during infection of the target plants.

ZFN-induced genomic DSBs can be repaired by NHEJ. This error-prone DNA-repair mechanism often leads to small deletions, insertions, and/or substitutions at the ZFN-cleavage site (Le Provost et al., 2010; Weinthal et al., 2010). As expected, and in line with previous studies demonstrating that ZFN-mediated site-specific mutagenesis can lead to a variety of molecular changes at the break sites (Lloyd et al., 2005; Wright et al., 2005; Maeder et al., 2008; Cai et al., 2009; de Pater et al., 2009; Shukla et al., 2009; Tovkach et al., 2009; Townsend et al., 2009; Osakabe et al., 2010; Petolino et al., 2010; Zhang et al., 2010), we observed a wide variety of changes in the mGUS-targeted region (Fig. 5). Sequencing analysis of several tobacco and petunia seedlings (Fig. 7) confirmed the heritable nature of the ZFN-induced mutation in these species and RT-PCR analysis revealed that, as expected from a non-seed-transmissible virus, the offspring of tobacco and petunia seedlings were virus free.

There are thus several advantages to using RNA viral vectors over direct transformation methods: Viruses do not integrate into the genome, they often lead to high gene expression in a variety of target tissues (Marillonnet et al., 2005; Gleba et al., 2007; Lindbo, 2007; Lico et al., 2008), they move from cell to cell (Ratcliff et al., 2001), and they are not transmissible through seeds (Ratcliff et al., 2001). The ability of TRV, for example, to target more than 400 species, including a wide variety of commercially important plant species (<http://www.dpvweb.net/dpv/showdpv.php?dpvno=398>), suggests that it may be useful for targeting experiments and transgene removal in model and economically important plants. Furthermore, our strategy could potentially be adopted for use with other plant viruses capable of infecting plant species that may not be susceptible to TRV.

To conclude, our data show that ZFN-expressing viral particles can travel to various plant tissues and organs and that ZFN expression can lead to genomic changes in newly developed plant tissues and organs. We also show that fully developed and healthy mutated plants can be recovered from the virus-infected tissues. Moreover, our report extends the use of ZFN technology to petunia, a commercially important plant species, and suggests that the pTRV vectors we developed can be used to extend the use of ZFN-targeting technology beyond model plants.

MATERIALS AND METHODS

DNA Constructs

pTRV2- Δ 2b-sgP generic plasmid was constructed by removing nucleotides 1,342 to 1,647 from pTRV2 (accession no. AF406991) and adding the pea (*Pisum sativum*) early browning virus coat protein sg-P (nucleotides 323–509,

accession no. X78455) as a *XhoI/SmaI* fragment. pTRV2-Δ2b-sgP::DsRed2 was constructed by PCR amplifying the DsRed2 coding sequence and cloning it into the *HpaI/SmaI* sites of pTRV2-Δ2b-sgP. pTRV2-Δ2b-sgP::Rssu-EGFP was constructed by fusing the EGFP coding sequence with the transit peptide of pea ribulose-1,5-bisphosphate carboxylase small subunit (Rssu; nucleotides 1,086–1,259, accession no. X00806) and cloning this into the *HpaI/SmaI* sites of pTRV2-Δ2b-sgP. pTRV2-Δ2b-[sgP::EGFP] was generated by PCR amplifying the EGFP coding sequence and cloning it into the *HpaI/SmaI* sites of pTRV2-Δ2b-sgP. pTRV2-Δ2b-[sgP::EGFP][sgP::DsRed2] was constructed by PCR amplification of the sgP-DsRed2 sequence from pTRV2-Δ2b-sgP::DsRed and cloning the product into the *SmaI* site of pTRV2-Δ2b-[sgP::EGFP]. To generate pTRV2-Δ2b-sgP::DsRed2-T2A-EGFP, the T2A sequence was first cloned into pBluescript SK, to which the EGFP and DsRed2 sequences were added, and the resultant DsRed-T2A-EGFP fragment was cloned as a *KpnI-SacI* fragment into pTRV2-Δ2b. pTRV2-Δ2b-sgP::QEQ-ZFN was constructed by PCR amplification of the QEQ-ZFN coding sequence from pSAT4.hspP.QQR (Tovkach et al., 2009) and cloning as a *XhoI/SmaI* fragment into pTRV2-Δ2b-sgP-NLS.

Transgenic Plants

The pRCS2.[KAN][QQR-TS*mGUS] binary vector (Tovkach et al., 2009), carrying a mutated GUS reporter gene with the QQR ZFN (Lloyd et al., 2005) recognition site and a functional plant kanamycin resistance gene, was used for the transformation of *Nicotiana benthamiana*, tobacco (*Nicotiana tabacum* ‘Samsung’) and petunia (*Petunia hybrida* ‘Burgundy Dream’) using the standard leaf disc transformation method (Guterman et al., 2006).

Viral Infection and Production of Target Plantlets

For agroinoculation, pTRV1 and recombinant pTRV2 vectors were mobilized into *Agrobacterium* strain EHA105 as previously described (Liu et al., 2002b). *Agrobacterium* cultures were grown overnight at 28°C in Luria-Bertani medium supplemented with 50 mg L⁻¹ kanamycin and 100 μM acetosyringone. Cells were harvested by centrifugation and resuspended to an OD₆₀₀ of 5 in Murashige and Skoog buffer supplemented with 10 mM MgSO₄ and 100 μM acetosyringone. Following an additional 3 h of incubation at 28°C, pTRV1 bacterial suspension was mixed with pTRV2 suspension at a 1:1 ratio. The mixed culture was then diluted 10-fold with inoculation buffer and infiltrated into the abaxial side of a leaf using a 2-mL syringe. For mechanical infection, sap from 2- to 3-week-old infected plants was extracted by grinding infected plant tissues with a mortar and pestle in 20 mM phosphate buffer pH 6.8. The extract was centrifuged to remove cells debris, mixed with carborundum, and rubbed on young leaves of healthy plants. To generate mutant plants from primordia, petioles, or basal parts of leaves of infected plants, they were cultured on Murashige and Skoog with or without 1.5 μg mL⁻¹ benzyl adenine. The plant organs were transferred to fresh medium once every 2 weeks. The cytokinin concentration was reduced according to the level of regeneration success.

Imaging

A confocal laser-scanning microscope (CLSM510, Zeiss) was used for tissue and subcellular imaging. EGFP and DsRed2 were excited at 488 and 545 nm, and images were collected at 505 to 530 nm and 585 to 615 nm, respectively. Chlorophyll autofluorescence was excited at 488 nm and imaging was collected at 650 to 670 nm. For whole-plant and organ imaging, stereoscopic fluorescent microscope MZFLIII equipped with a DC300FX camera was used (Leica Microsystems Ltd.).

Analysis of Gene-Targeting Events

Gene-targeting events were detected by GUS staining of virus-infected plants and their offspring as previously described (Tovkach et al., 2009). For molecular analysis of targeting events, total DNA was isolated from virus-infected plant tissues and seedlings of putatively targeted plants according to Bernatzky and Tanksley (1986), and was subjected to PCR and sequence analysis as described previously (Tovkach et al., 2009). For detection of viral sequences, total RNA was purified from plant tissues using the plant RNA/DNA purification kit (Norgen Biotek) and viral sequences were amplified using access RT-PCR (Promega).

Supplemental Data

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

Supplemental Figure S1. Expression of fluorescent reporter gene DsRed2 in cells of newly developed leaves of plants that were agroinfiltrated with pTRV-Δ2b-sgP::DsRed2.

Supplemental Figure S2. Expression of fluorescent reporter gene DsRed2 in tissues and organs of plants that were mechanically infected with pTRV-Δ2b-sgP::DsRed2.

Supplemental Figure S3. Expression of chloroplast-targeted Rssu-EGFP and DsRed2 fluorescent reporter genes in newly developed tissues and organs of plants that were coinfecting with pTRV2-Δ2b-sgP::Rssu-EGFP and pTRV2-Δ2b-sgP::DsRed2.

ACKNOWLEDGMENTS

We thank Danziger “Dan” Flower Farm for providing the plant material. A.V. is an incumbent of the Wolfson Chair in Floriculture.

Received August 26, 2010; accepted September 23, 2010; published September 27, 2010.

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