Non-transgenic genome modification in plant cells

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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, non-transgenic 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 and petunia 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 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-Gonzalez, 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 non-homologous end joining (NHEJ) and not HR (Ray and Langer, 2002; Britt and May, 2003).

Various methods have been developed to indentify 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 (reviewed by 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. (Puchta et al., 1993, 1996) showed that DSB induction by the naturally occurring rare-cutting restriction enzyme I-
SceI leads to enhanced HR-mediated gene targeting in plants. Expression of I-SceI and another rare-cutting restriction 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; Paques and Duchateau, 2007). However, their wide application is hindered by the tedious and next to impossible re-engineering of such enzymes for novel DNA-target specificities (Paques 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, 2009 #3894; Townsend, 2009 #3892) and as an efficient tool for the induction of site-specific mutagenesis (e.g. Zhang, 2010 #4086; Lloyd, 2005 #3087) in plant species. The latter is more efficient and simpler to implement in plants as it does not require co-delivery 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 Cys_2His_2 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 recent reviews 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 PEG, 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, non-direct 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., 2002). 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 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, Figure 1A), transformed the vector into Agrobacterium cells and used the agroinfiltration method to inoculate Nicotiana benthamiana, Nicotiana tabacum cv. Samsung and Petunia hybrida cv. Burgundy Dream plants. Strong and uniform expression of the DsRed2 reporter gene in cells of leaves that developed after the infection was clearly observed 7 days post-inoculation (Figure S1). Continued and strong expression (even several months post-inoculation) was also clearly observed in various newly developed plant tissues and organs, including roots (Figure 2). DsRed2 expression was not observed in non-infected, control plants (data not shown). It is important to note that newly developed virus-infected tissues which 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-Δ2b-sgP::DsRed2-infected plants to infect the stems and leaves of healthy *N. benthamiana* and *P. hybrida* plants. Here again, strong and uniform DsRed2 expression was observed in plant tissues and organs developed on infected plants (e.g. Figure 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. Co-delivery 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 co-infection with two pTRVs, each carrying a different reporter gene, would result in the co-expression 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-Δ2b-sgP::Rssu-EGFP and used this construct in co-inoculation experiments with pTRV2-Δ2b-sgP::DsRed2 (both vectors are based on the structure of the generic vector pTRV2-Δ2b-sgP::GOI, Figure 1A) . Figure S3 shows that both reporter proteins (i.e. DsRed2 and Rssu-tagged EGFP) were co-expressed in infected *N. tabacum* mesophyll cells developed from agroinfiltrated plants. Co-expression 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 non-infected, control plants (data not shown). Note, however, that co-expression 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 of, 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 more uniform and consistent expression in infected cells. The genes were either separated by the *Thosea asigna* virus (TaV) sequence T2A (Figure 1B) or driven by separate double subgenomic promoters (Figure 1C). Figure 3A shows uniform distribution of both EGFP and DsRed2 in cells of various infected plant species when the pTRV2-Δ2b-sgP::DsRed2-T2A-EGFP vector, in which the reporter genes were separated by the T2A sequence, was used for infection. Similarly, co-expression of EGFP and DsRed2 could also be observed in plant cells infected by pTRV2-Δ2b-[sgP::EGFP][sgP::DsRed2], in which the expression of each reporter gene is driven by its own subgenomic promoter, as shown in Figure 3B for infected *N. tabacum* plants. A similar expression pattern was observed in *N. benthamiana* and *P. hybrida* 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 non-infected, 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; Tovkach et al., 2010) in which ZFN activity is measured by restoration of a mutated GUS-encoding gene (mGUS) (Figure 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 *N. tabacum* and *P. hybrida* plants using a mGUS construct which was engineered to carry the QQR ZFN target site (Tovkach et al., 2009; Tovkach et al., 2010). We also produced pTRV2-Δ2b-sgP::QQR vector (based on the generic vector pTRV2-Δ2b-sgP::GOI, Figure 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 which developed post-inoculation. pTRV2-mediated ZFN expression led to site-specific mutagenesis in a variety of tissues and organs which developed after the inoculation, as determined by GUS expression (Figure 4). Thus, for example, GUS expression was clearly visible in newly developed leaves of *N. tabacum* (Figure 4B) and *P. hybrida* (Figure 4C) plants. More importantly, ZFN-mediated targeting could also be detected in newly developed buds (Figure 4D and E), developing primordia (e.g. Figure 4F) and even flowers and reproductive tissues of infected plants (e.g. Figure 4G and H). GUS expression was not observed in the tissue of non-infected plants (data not shown).

We further investigated the molecular outcome of virus-mediated ZFN expression in infected plants by randomly analyzing several TRV-infected *P. hybrida* and *N. tabacum* 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 *N. tabacum* and *P. hybrida* 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 *N. tabacum* and *P. hybrida* 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. Figure 6A) revealed strong uniform expression. We next investigated the stability and heredity of the changes in those plants by harvesting seeds from flowering N. tabacum and P. hybrida plants (e.g. Figure 6B) and analyzing uidA-transgenic plantlets for GUS activity. As expected, GUS expression could clearly be detected in both P. hybrida (Figure 6C) N. tabacum (Figure 6D) seedlings, demonstrating that the virally expressed ZFN-induced mutations were stably inherited. Sequencing analyses of several P. hybrida seedlings (e.g. Figure 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 N. tabacum seedlings further confirmed the nature of the ZFN-induced mutation in this species (Figure 7A). RT-PCR analysis also revealed that, as expected from a non-seed-transmissible virus, the newly developed seedlings were free of viral particles (Figure 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* 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 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 expected 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 implementation 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 which can lead to high levels of ZFN expression in a wide variety of plant species, as well as organs and tissues, and which will allow regeneration of mutated and ZFN-free plants is thus needed.

The strong and uniform expression pattern observed in pTRV-infected plants (Figures 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 (Figures 4 and 5) and that mutant plants can be recovered from the
infected tissues (Figure 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 which 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 (Figure 5). Sequencing analysis of several N. tabacum and P. hybrida seedlings (Figure 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 N. tabacum 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 which 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 *P. hybrida*, 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-Δ2b-sgP generic plasmid was constructed by removing nt 1342 to 1647 from pTRV2 (accession no. AF406991) and adding the Pea early browning virus coat protein (CP) subgenomic promoter (sgP, nucleotides 323 to 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 *Hpal/Smal* 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 1086-1259, accession no. X00806) and cloning this into the *Hpal/Smal* sites of pTRV2-Δ2b-sgP. pTRV2-Δ2b-sgP::EGFP was generated by PCR-amplifying the EGFP coding sequence and cloning it into the *Hpal/Smal* sites of pTRV2-Δ2b-sgP. pTRV2-Δ2b-[sgP::EGFP] was generated by PCR-amplifying the EGFP coding sequence and cloning it into the *Hpal/Smal* 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::EGFP] and cloning the product into the *SmaI* site of pTRV2-Δ2b-sgP::DsRed. 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-Sacl 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/Smal 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, N. tabacum cv. Samsung and Petunia hybrida 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., 2002). Agrobacterium cultures were grown overnight at 28°C in LB 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 MS 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 MS with or without 1.5 µg ml⁻¹ cytokinin (BA). 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, Jena, Germany) was used for tissue and subcellular imaging. EGFP and DsRed2 were excited at 488 and 545 nm, and images were collected at 505-530 nm and 585-615 nm, respectively. Chlorophyll autofluorescence was excited at 488 nm and imaging was collected at 650-670 nm. For whole-plant and organ imaging, stereoscopic fluorescent microscope MZFLIII equipped with a DC300FX camera was used (Leica Microsystems Ltd. Wetzlar, Germany).

**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 (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).

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**References**


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Figure legends

**Figure 1** Structure and key features of pTRV-based expression vectors. (A) pTRV-Δ2b-sgP::GOI designed to drive the expression a single gene of interest (GOI) under the control of the sgP constitutive promoter. (B) pTRV-Δ2b-sgP::GOI1-T2A-GOI2 designed to drive the co-expression 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-Δ2b-[sgP::GOI1][sgP::GOI2] designed to drive the co-expression of two genes from two independent subgenomic promoters. The T-DNA region of each vector is presented. Also shown are (i) the constitutive 35S promoter (35sP) and the nopaline synthase terminator (nosT), needed for the production of primary viral transcript following agroinfiltration, and (ii) 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.

**Figure 2** pTRV-mediated expression of a single reporter gene (DsRed2) in newly developed tissues and organs. Plants were infected by pTRV-Δ2b-sgP::DsRed2. Images were taken by fluorescence stereoscope. DsRed2 expression is shown in red.

**Figure 3** pTRV-mediated co-expression of two reporter genes in newly developed plant cells. (A) Co-expression of fluorescent reporter genes DsRed2 and EGFP in newly developed leaves of pTRV-Δ2b-sgP::DsRed2-T2A-EGFP-infected plants. DsRed2 and EGFP fluorescence are in orange and green, respectively, and plastid autofluorescence is in dark red. (B) Co-expression of DsRed2 and EGFP in newly developed leaves of pTRV-Δ2b-[sgP::DsRed2][sgP::EGFP]-infected *N. tabacum* plant. DsRed2 and EGFP fluorescence are in red and green, respectively,
and plastid autofluorescence is in dark red. Images in panels 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 mutated GUS (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-H) Detection of site-specific mutagenesis events in newly developed tissues of pTRV-Δ2b-sgP::QQR infected plants by X-Gluc staining. GUS expression was detected in newly developed *N. tabacum* cv. Samsung (B) and *P. hybrida* (C) leaves, 13 and 22 days post-inoculation, respectively, and in newly developed *P. hybrida* (D) and *N. tabacum* cv. Samsung (E) buds, 11 and 50 days post-inoculation, respectively. GUS expression was also detected in *P. hybrida* developing primordia (F) and *P. hybrida* flower and reproductive tissues (G and H) of the GUS-positive mature plant.

**Figure 5** Molecular analysis of ZFN-mediated mutagenesis events in *P. hybrida* (P) and *N. tabacum* (T) plants. The initiation codon and the ZFN-binding sites on the upper 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.

**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 *P. hybrida* (A), which were regenerated from infected plants, and allowed to root, mature (B) and set seed. Also shown are GUS-stained *P. hybrida* (C) and *N. tabacum* (D) seedlings obtained from mature, virus-infected plants.
Figure 7 Molecular analysis of randomly selected GUS-positive *P. hybrida* and *N. tabacum* seedlings. (A) Mutagenesis events in *P. hybrida* (P) and *N. tabacum* (T) offspring. The initiation codon and the ZFN-binding sites on the upper strand of the mGUS sequences are in blue and purple, respectively. Stop codon is in red. (B) Upper panel: RT-PCR analysis of pTRV genomes in infected, healthy and mutated seedlings of *P. hybrida* and *N. tabacum*. Lower panel: RT-PCR analysis of housekeeping gene (the plastid 23S RNA gene). pTRV genomes (identified by RT-PCR amplification of TRV2 sequence coat protein[CP]) were detected in infected, but not healthy or mutated seedlings (M, DNA marker ladder).
Supplemental figure legends

**Figure S1** Expression of fluorescent reporter gene DsRed2 in cells of newly developed leaves of plants which were agroinfiltrated with pTRV-Δ2b-sgP::DsRed2. DsRed2 and plastid autofluorescence are shown in orange and dark red, respectively.

**Figure S2** Expression of fluorescent reporter gene DsRed2 in tissues and organs of plants which were mechanically infected with pTRV-Δ2b-sgP::DsRed2. DsRed2 expression is shown in red.

**Figure S3** Expression of chloroplast-targeted Rssu-EGFP and DsRed2 fluorescent reporter genes in newly developed tissues and organs of plants which were co-infected with pTRV2-Δ2b-sgP::Rssu-EGFP and pTRV2-Δ2b-sgP::DsRed2. DsRed2 expression is shown in orange, EGFP expression in green and plastid autofluorescence in purple.
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mGUS GACGGTACCATGTTCTTCCCTCCCTCTCTG--AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
T7 GACGGTACCATGTTCTTCCCTCCCTCTCTG--GAGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P47 GACGGTACCATGTTCTTCCCTCCCTCTCTCTG--AAGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P41 GACGGTACCATGTTCTTCCCTCCCTCTCTCTGAGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P30 GACGGTACCATGTTCTTCCCTCCCTCCCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P29 GACGGTACCATGTTCTTCCCTCCCTCTCTCTG---AAGGGGAAGAATTACGTCCTGTAGAAACCCCA -
T115 GACGGTACCATGTTCTTCCCTCCCTCTCTCTG---G---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
T11 GACGGTACCATGTTCTTCCCTCCCTCTCTCTG---TG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
T6 GACGGTACCATGTTCTTCCCTCCCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P62 GACGGTACCATGTTCTTCCCTCTCTCTCTCTG---G---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
T4 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P114 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
T101 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P26 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P49 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P48 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P104 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P51 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P111 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P34 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -
P25 GACGGTACCATGTTCTTCCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTG---AGGGGAAGAATTACGTCCTGTAGAAACCCCA -

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