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## Review

## Toward establishing an efficient and versatile gene targeting system in higher plants



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## ABSTRACT

Precise modification of plant genomes provides powerful tools for understanding gene function. Furthermore, pinpoint modification of endogenous plant genes is also an attractive method in molecular plant breeding. One of the best methods currently available to induce specific DNA sequence changes into genomes is gene targeting (GT) via homologous recombination (HR). GT can induce a variety of mutations, including substitution of several nucleotides as well as insertions and deletions. Of the various approaches taken to improve GT efficiency, utilization of engineered sequence-specific endonucleases to create targeted DNA double-strand breaks (DSBs) that stimulate HR at breaking sites is one of the most effective. As a universal selection system for GT cells, a target-gene-independent selection system such as positive–negative selection with subsequent elimination of the positive selection marker using *piggyBac* transposon or HR combined with mega-nuclease allows retention of the desired mutation without any trace of additional exogenous nucleotides. Furthermore, a novel GT system named *in planta* GT seems to have effectively overcome problems due to low transformation efficiency of the HR template into plant nuclei, and can adjust the timing of DSB induction at the target locus. In the *in planta* system, an HR template flanked by a nuclease recognition site is stably integrated into the plant genome; subsequent expression of nuclease simultaneously releases the HR template from the genome and induces a DSB at the target site. Here, we review recent developments in GT, giving examples of new techniques improving the efficiency of GT in plants.

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## 1. Introduction

Gene targeting (GT) is defined as the alteration of a specific DNA sequence in an endogenous gene at its original locus in the genome by homologous recombination (HR). Since the first report of GT of an integrated antibiotic-tolerant gene in the tobacco genome (Paszkowski et al., 1988) various approaches aimed at HR-dependent GT have been attempted in plants (for reviews, see Tzfira and White, 2005; Iida and Terada, 2005; Voytas, 2013). A recalcitrant problem with GT is its low frequency; when we

Abbreviations: GT, gene targeting; HR, homologous recombination; DSBs, DNA double strand breaks; ALS, acetolactate synthase; ZFNs, zinc finger nucleases; TALENs, transcription activator-like effector nucleases; CRISPR, clustered regularly interspaced short palindromic repeats; T-DNA, transfer DNA

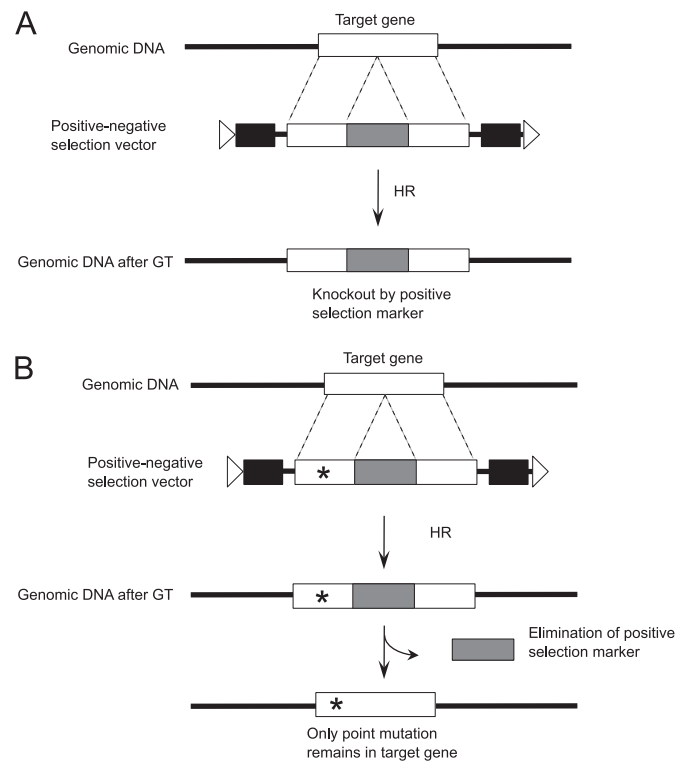
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transform a plant with a GT vector with homology to a target gene, almost all T-DNAs integrate randomly into the plant genome, and HR between the target gene and the GT vector occurs only rarely. The frequency of HR-dependent GT has been shown to be in the order of  $10^{-3}$  to  $10^{-6}$  relative to random integration of the GT vector (Iida and Terada, 2005; Vergunst and Hooikaas, 1999; Iida and Terada, 2004; Lee et al., 1990; Ray and Langer, 2002; Britt and May, 2003; Gong and Rong, 2003; Hanin and Paszkowski, 2003; Hohn and Puchta, 2003; Reiss, 2003). Following the discovery that induction of DSBs increases the frequency of HR by several orders of magnitude, engineered nucleases have emerged as the method of choice for improving GT efficiency, and reports of successful GT in mammals have increased drastically (e.g., Jung and Sander, 2013). There is no doubt that induction of DSBs at specific genomic locations is effective in improving GT efficiency in plants (for a review, see Voytas, 2013). However, GT comprises several processes; transformation of a GT vector with homology to the target locus, the occurrence of a DSB at the target site, and HR repair at the DSB site using the GT vector as a template for HR. In addition, a step to select GT cells is also important because, in most cases, GT-mediated modification of endogenous genes does not confer a selectable phenotype. In this review, we focus on the following four topics as key strategies for establishing an efficient and versatile GT system in higher plants: (1) positive–negative selection system – a target gene independent selection system for GT cells using selection markers located on the template DNA, (2) elimination of the positive selection marker to leave only the desired point mutations after positive–negative selection, (3) use of engineered nucleases to induce a DSB at the target locus for enhanced homologous recombination at target locus and (4) *in planta* GT, which addresses the problems of low transformation efficiency and can synchronize DSB induction and supply of the HR template because stably integrated HR template can be excised at the same time as DSB induction at the target gene.

### 1.1. A universal positive–negative selection system for GT

A GT system using a positive–negative selection marker originally developed to enrich rare knockout mutants in mice (Mansour et al., 1988) is capable of generating both knock-out and knock-in mutants (Fig. 1A). While HR-mediated sequence-specific integration of a transgene in mouse embryonic stem cells has been reported to be 1% or higher of the total integration events, most of which are random integration of the transgene by non-homologous end joining (Jasin et al., 1996). On the other hand, the targeted integration of a transgene into the endogenous homologous sequence in higher plants has been regarded to be in the order of 0.01% to 0.1% compared with random integration (Iida and Terada, 2005, 2004; Hanin and Paszkowski, 2003; Reiss 2003; Puchta, 2002). To detect GT event efficiently among the overwhelming random integration events, an approach using a strong positive–negative selection marker was developed. This approach was widely used for creating rice knockout mutant of following genes: Waxy (Iida and Terada, 2005, 2004; Terada et al., 2002; Ozawa et al., 2012), Alcohol dehydrogenase2 (ADH2) (Terada et al., 2007); Methyltransferase1a (Met1a) (Yamauchi et al., 2009); Repressor of silencing 1 (ROS1) (Ono et al., 2012); Domains rearranged methylase 2 (DRM2) (Moritoh et al., 2012);  $\beta$ 1,2-xylosyltransferase (Ozawa et al., 2012). In positive–negative selection vectors, positive selection markers are located within segments homologous to the target genes, and negative selection markers flank the targeted homologous sequence, acting to counter-select for random or non-targeted integration events. In higher plants, the *NPTII* gene conferring resistance to kanamycin or geneticin (G418) and the *HPT* gene for hygromycin resistance, have been used as positive selection markers. The *codA* gene encoding cytosine



**Fig. 1.** Positive–negative selection system of GT. The dashed lines indicate the homologous region between genomic DNA and the vector sequence. The grey rectangle indicates a positive selection marker, and the black rectangles indicate negative selection markers. The white arrowheads indicate the right and left border sequence of the T-DNA. (A) Knockout using positive–negative selection marker dependent GT. By counter-selection using positive and negative markers, knockout mutants of the target gene can be obtained. (B) Induction of point mutation using positive–negative selection. After elimination of the positive selection marker, only the point mutation shown by the asterisk will remain in the target gene.

deaminase, and the DT-A gene encoding diphtheria toxin A fragment are used as negative selection markers because these genes confer lethal or conditional lethal phenotypes (for a review, see Iida and Terada, 2005). As an aside, positive–negative selection is useful not only for generating knock-out mutants of specific genes, but also for introducing point mutations that eliminate the positive selection marker following GT (Fig. 1B). Because it does not rely on gene-specific selection, the positive–negative selection system represents a universal strategy that can be used for any target gene. Several strategies to eliminate selection marker genes, such as site-specific recombination (Dale and Ow, 1991; Gleave et al., 1999), transposition systems (Goldsbrough et al., 1993) and HR (Puchta, 2000; Zubko et al., 2000), have been developed and are described below. The combination of positive–negative selection dependent GT and subsequent elimination of the positive selection marker gene can be applied to the induction of mutations in any gene of interest if the induced mutation itself does not provide a selectable phenotype (Terada et al., 2010).

### 2. Elimination of positive selection marker leaving only desired mutations

Accurate and effective excision of the positive marker gene is indispensable for exclusive introduction of only the desired mutations into the plant genome via GT using a positive–negative selection system. A large number of studies has demonstrated that site-specific recombination methods such as the *Cre/loxP* system from bacteriophage P1 and the *FLP/FRT* system from *Saccharomyces cerevisiae* allow the elimination of marker genes from randomly

integrated transgenes at a high frequency in model plants and crop species (reviewed by Wang et al., 2011). Furthermore, Terada et al. (2010) succeeded in Cre/loxP-mediated marker elimination from a GT locus. However, marker excision using site-specific recombination leaves dispensable sequences such as the recognition sequences of site-specific recombinase at the excised site. To overcome this problem, the use of *piggyBac* transposon – derived originally from the lepidopteran cabbage looper moth *Trichoplusia ni* – is currently attracting much interest. The *piggyBac* transposon integrates into the host genome at TTAA elements and excises without leaving a footprint at the excised site (Cary et al., 1989). More recently, Yusa et al. (2009) have shown in mammalian cells that the *piggyBac* transposon system enables excision of a selectable marker from a GT locus in the host genome without residual ectopic sequences. In plants, we have demonstrated that the animal-derived *piggyBac* transposon is capable of accurate and effective transposase-mediated transposition in rice (Yokoi et al., unpublished result), suggesting that a high frequency marker excision system for plant genomes could be established using *piggyBac*. When the positive selection marker is eliminated using the *piggyBac* transposon, the positive selection marker located on the *piggyBac* transposon must be exactly adjacent to the TTAA sequence because the *piggyBac* transposon excises without leaving a footprint precisely at the excised site TTAA elements (Cary et al., 1989). In cases where a TTAA sequence does not exist near the target site, *piggyBac*-dependent marker elimination is thus not preferred. Another method of precise excision of a positive selection marker uses mega-nuclease and HR repair by overlap sequence adjacent to a positive selection marker.

### 3. Use of engineered nucleases to induce DSBs at target site

Zinc finger nucleases (ZFNs) are synthetic restriction enzymes with zinc finger domains that recognize a specific DNA sequence, fused to the nuclease domain of the restriction enzyme *FokI* (Kim et al., 1996). Because the zinc-finger domain can be engineered to target novel DNA sequences, ZFNs have been exploited widely in eukaryotic systems for engineering of endogenous genome loci (for review, see Carroll 2008). In higher plants, Shukla et al. (2009) and Townsend et al. (2009) describe ZFN-mediated GT modification of the maize gene encoding inositol-1,3,4,5,6-pentakisphosphate 2-kinase and the tobacco acetolactate synthase (ALS) gene, respectively.

Transcription activator-like effector nucleases (TALENs) are fusions of the *FokI* cleavage domain and DNA-binding domains derived from TALE proteins. TALEs are naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*. Zhang et al. (2013) describe methods for the targeted modification of plant genomes using TALENs. Methods were optimized using tobacco (*Nicotiana tabacum*) protoplasts and TALENs targeting the ALS gene.

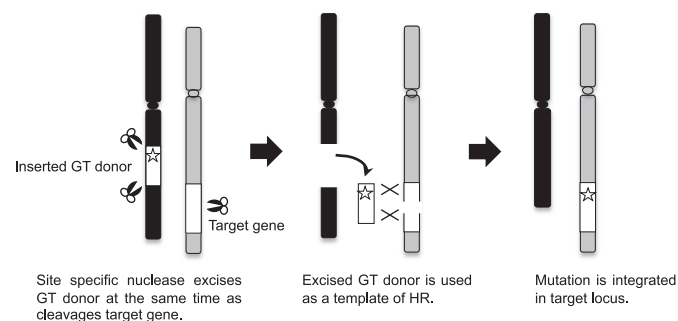
ZFNs and TALENs differ in the number of base pairs recognized by one DNA-binding module. In the case of ZFNs, one DNA-binding module consisting of approximately 30 amino acids recognizes three nucleotides; combining DNA-binding modules enables recognition of DNA sequences of 9–18 bp (Liu et al., 1997). TALENs contain DNA-binding domains composed of a series of 33–35 amino-acid repeat domains, each recognizing a single base pair. Thus a minimum of only four types of DNA-binding module are needed, recognizing A, T, C, and G. The single-base recognition of TALE-DNA binding repeats affords greater design flexibility than the triplet-restricted ZFNs. However, TALENs are physically larger than ZFNs recognizing the same number of nucleotides.

Distinct from the engineered nucleases described above, RNA-guided DNA endonucleases of the CRISPR-Cas9 system have recently emerged on the scene (Wiedenheft et al., 2012; Horvath

and Barrangou, 2010; Terns and Terns, 2011). The CRISPR (clustered regularly interspaced short palindromic repeats) system uses a short, non-coding RNA (crRNA) to target a Cas9 nuclease to complementary (protospacer) sequences in the host genome. The CRISPR-Cas9 system can serve as the basis of a simple and highly efficient method for performing genome editing in bacteria, yeast and human cells as well as *in vivo* in whole organisms such as fruitflies, zebrafish and mice (Wang et al., 2013; Shen et al., 2013; DiCarlo et al., 2013; Jiang et al., 2013; Jinek et al., 2013; Hwang et al., 2013; Cong et al., 2013; Mali et al., 2013; Cho et al., 2013; Gratz et al., 2013). A considerable advantage of the CRISPR-Cas9 system over other DNA binding protein methodologies is that it utilizes a small RNA molecule to target the nuclease of the system to a specific nucleic acid target. The same Cas9 nuclease can be used to cleave different target sites guided by different RNA molecules. Vector construction in the CRISPR-Cas9 system is facilitated by the small size of the Cas9 nuclease compared to ZFN and TALEN proteins, and because only the 20 bp guide-RNA, which assigns target locus, needs to be changed for each application. On the other hand, high off-target effects of the CRISPR-Cas9 system have been reported in human cells (Fu et al., 2013). During the preparation of this manuscript, three groups reported successful examples of CRISPR-Cas9 mediated target genome modification in plants (Shan et al., 2013; Li et al., 2013; Nekrasov et al., 2013). Shan et al. (2013) succeeded to disrupt endogenous genes in rice and wheat. The highest mutation frequency was 38% in rice protoplast. They also obtained gene knockout plants by bombardment of Cas9 plasmid and sgRNA expression plasmid together to rice calli; 9.4% of regenerated transgenic plants possessed mutation in target gene, PDS and biallelic mutations were identified in these plants. Li et al. (2013) reported disruption of *Arabidopsis* and *Nicotiana benthamiana* endogenous genes. They also showed that GT occurred in 10.7% of *N. benthamiana* protoplasts co-expressing Cas9 and sgRNA concurrently supplied a HR template. Nekrasov et al. (2013) succeeded to knockout *Nicotiana benthamiana* endogenous gene by transiently expressing Cas9 and sgRNA via *Agrobacterium*-mediated method. These reports clearly showed that CRISPR-Cas9 system can be used to induce DNA breaks at defined chromosomal locus in plants. In addition to ZFNs and TALENs, CRISPR-Cas9 system must be the powerful tool for inducing DSBs at desired locus.

### 4. A novel *in planta* GT system for plant species with low transformation efficiency

Although engineered nucleases provide a means of introducing diverse custom alterations at specific genomic locations, application



**Fig. 2.** *In planta* GT system. A GT donor vector flanked by the recognition sequence of engineered nuclease is first integrated into plant genome. By expressing engineered nuclease, excision of the GT donor vector and cleavage of the target gene occur simultaneously. Induction of a DSB at target locus stimulates HR and the well-timed supply of HR template enables efficient GT.

of this technology to plant GT is still limited by methods for delivering the nucleases and HR templates to the relevant cell types. In the case of mammals, typically, nuclease-encoding mRNAs and HR template DNAs can be delivered into cells temporarily and promptly. Thus, nuclease-coding mRNAs are transcribed immediately to provide functional nuclease to create DSBs at the target site. However, in the case of higher plants, an efficient RNA delivery system has not yet been established. Instead, *Agrobacterium*-mediated transformation of DNA or direct injection of DNA using a particle gun are commonly used to express proteins of interest. In *Agrobacterium*-mediated transformation, a single-stranded transfer DNA (T-DNA) protected by coat proteins is delivered from *Agrobacterium* to the plant cell nucleus (Tinland et al., 1994), where naked single-stranded molecules are converted to double strands (Rodenburg, 1989). These double-stranded T-DNAs are used as templates for HR. Because transcription and translation of engineered nucleases needs some time, we cannot harmonize the timing of expression of engineered nuclease tightly with the existence of the HR template. In addition, *Agrobacterium* mediated GT is only applicable to plant species, in which an efficient transformation system is established.

A novel GT method called *in planta* GT (Fig. 2; Fauser et al., 2012; Ayar et al., 2013) promises to solve both problems. In this system, a GT donor vector flanked by two engineered nuclease recognition sites is first stably integrated into the plant genome. Expression of a site-specific endonuclease cuts within the target and also excises the chromosomal transgenic donor if the recognition sequence of a engineered nuclease present at both ends of the GT donor is the same as that in the target gene. HR between the excised GT donor vector and target locus results in *in planta* GT. Conventional GT approaches rely on the generation of a very large number of transformation events, while the *in planta* GT system requires only a minimum transformation efficiency because, once plant materials in which the HR template and an inducible expression cassette for the engineered nuclease are integrated into the plant genome are obtained, these transgenic cells can be clonally propagated for use in inducing GT. Thus, this *in planta* GT system is suitable for plant species with low transformation efficiency.

## 5. Concluding remarks

Comparative genomics and structural- and computational-based protein engineering provide useful information for plant molecular breeding. Furthermore, many agronomically valuable traits and natural variations are now known to be due to a small number of point mutations. We previously showed an example where site-directed mutagenesis of an endogenous gene via GT gave superior results to ectopic expression of the mutated gene, i.e., the herbicide tolerance level of plants homozygous for a modified ALS gene exceeded that of rice plants over-expressing the same mutated ALS gene (Endo et al., 2007). Furthermore, site-directed mutagenesis via GT seems to run a lower risk of gene silencing due to the use of intrinsic cis-acting elements to regulate transcription of the mutated target gene at the original locus, and due to the lack of additional copies of the target gene as would be present in conventional transgenic approaches. However, undesirable random insertions of genes for sequence-specific endonucleases and GT donors are not avoidable. Thus, selecting elite plants, which is proven not to possess these undesirable insertions is important for molecular breeding. Deep sequencing using next generation sequencer may help to select appropriate plants. We believe that combining information on mutations conferring interesting traits with highly efficient GT system and accurate analysis technology has enormous potential to produce specifically designed plants for both research and agronomy.

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