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REVIEW

Homology-based double-strand break-induced genome engineering in plants

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Abstract
Key message **This review summarises the recent progress in DSB-induced gene targeting by homologous recombination in plants. We are getting closer to efficiently inserting genes or precisely exchanging single amino acids.**
Abstract Although the basic features of double-strand break (DSB)-induced genome engineering were established more than 20 years ago, only in recent years has the technique come into the focus of plant biologists. Today, most scientists apply the recently discovered CRISPR/Cas system for inducing site-specific DSBs in genes of interest to obtain mutations by non-homologous end joining (NHEJ), which is the prevailing and often imprecise mechanism of DSB repair in somatic plant cells. However, predefined changes like the site-specific insertion of foreign genes or an exchange of single amino acids can be achieved by DSB-induced homologous recombination (HR). Although DSB induction drastically enhances the efficiency of HR, the efficiency is still about two orders of magnitude lower than that of NHEJ. Therefore, significant effort have been put forth to improve DSB-induced HR based technologies. This review summarises the previous studies as well as discusses the most recent developments in using the CRISPR/Cas system to improve these processes for plants.

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Keywords Double-strand break repair · Homologous recombination · Non-homologous end joining · Synthetic nucleases · Targeted mutagenesis · Gene targeting

Introduction

To specifically alter the genetic sequence of a plant has been a primary goal in plant biology, as targeted gene inactivation is crucial to understanding how genotypes influence phenotypes. Underlying most genetic manipulations is the induction of a sequence-specific DSB induced in the target gene, which activates repair mechanisms. Naturally, DSBs occur due to a variety of reasons: they can result from exposure to exogenous factors such as gamma radiation or from agents that either induces DNA damage directly or indirectly by enhancing cellular processes that produce free radicals. During meiosis, DSBs are induced in a programmed way to facilitate meiotic recombination, and thus, increasing genetic variability.

The basic principle of genome engineering is to induce DSBs on purpose via the use of nucleases at specific sites to initiate repair reactions that result in a more or less specific genomic change. In principle, plants have two major pathways of DSB repair: NHEJ and HR. The predominant pathway in the somatic cells of higher plants is NHEJ, however, when homologous sequences are available either on the sister chromatids or close to the break site (e.g., in tandem duplications) DSB repair can occur via HR (Puchta 2005). Multiple reviews on the use of NHEJ as the means for genome engineering using synthetic nucleases have been published recently (e.g., Voytas 2013; Puchta and Fauser 2014; Schaeffer and Nakata 2015; Weeks et al. 2015; Schiml and Puchta 2016), and more reviews are included in this special issue. The purpose of this review is

to focus on HR-based DSB-induced genome engineering in plants. First, the basic mechanism of DSB-induced HR will be discussed, and second, we will highlight the different approaches that have been used primarily with synthetic nucleases for genome engineering in plants.

The basic mechanisms of homologous recombination

HR occurs in somatic cells to prevent genetic errors and in meiotic cells to enable the equal distribution of the parental chromosomes and genetic exchange between homologues. HR is subdivided into conservative and non-conservative pathways. Frequently, only conservative mechanisms are considered as 'classical' HR, because in non-conservative mechanisms of HR, genetic material is lost. A prominent non-conservative mechanism is the so-called single-strand annealing (SSA) pathway that depends on homologous repetitive sequences that are arranged in close proximity (Fig. 1). Tandemly arranged homologous sequences are often found in plant genomes due to gene duplications. SSA can be almost as efficient as NHEJ: up to one out of three DSBs is repaired via SSA within repeated sequence regions of plant genomes (Siebert and Puchta 2002). After DSB induction, single-strand resections on both break sites take place until homologous sequences can anneal.

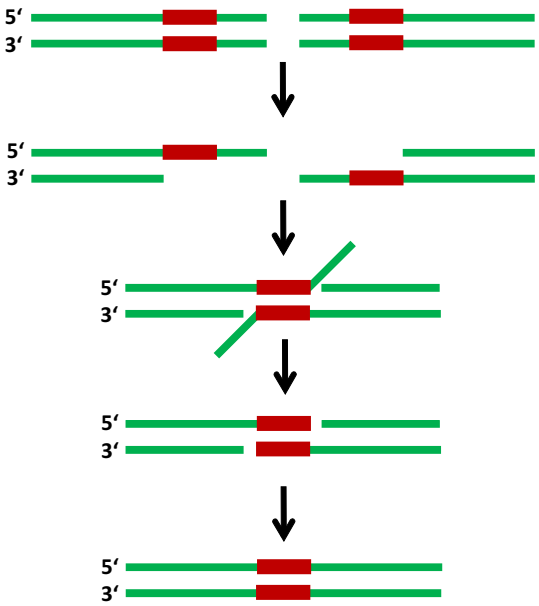

Fig. 1 Single-strand annealing (SSA) pathway of homologous recombination (HR). After DSB induction, the 5′ ends of the break side are resected to produce single-stranded 3′ overhangs. Due to homologies within the ssDNA regions, the two strands can directly anneal. The overhanging ends are either trimmed or single-stranded gaps are filled via DNA synthesis

Overhanging single-strands are degraded or gaps are filled, which is followed by ligations. This mechanism results in deletions, as the sequence stretch between both homologies is always lost.

Unlike the non-conservative SSA pathway, the conservative mechanisms of HR do not result in sequence loss. The two most prominent subpathways, which have common first steps in their reactions, are the synthesis-dependent strand annealing (SDSA) and the double-strand break repair (DSBR) pathways (Nassif et al. 1994; Szostak et al. 1983). Whereas, the DSBR is a prominent mechanism in meiotic recombination in plants (see Osman et al. 2011) that can result in crossover (CO) events between homologues, experimental evidence indicates that in somatic plant cells almost all DSBs are repaired via the SDSA mechanism (Puchta 1998). As shown in Fig. 2, this mechanism does not result in crossovers. The basic principle of this process is that after induction of a DSB a homologous sequence is copied into the break site, leading to no loss of sequence information. After a DSB occurs, both break ends are resected to expose the 3′ ends of the single-stranded DNA. One 3′ end invades a homologous sequence by displacing one of the strands along the way, producing the so-called displacement loop (D-loop). The invading strand is then elongated by copying the sequence information from the intact donor DNA. Later, the elongated single-strand is released from the D-loop, and then, can reanneal with the homologous single-stranded DNA on the opposite side of the break site. Thus, only non-crossover events occur. The SDSA pathway seems to be the predominant pathway responsible for conservative HR in somatic plant cells, as it is beneficial for genome stability. COs between the multiple repetitive sequences in plant genomes can lead to di- or acentric chromosomes.

Strategies developed to perform HR-based DSB-induced genome engineering in plants should, therefore, be developed along the lines of the SSA or SDSA pathway models.

Genome engineering in plants

Loss of function mutants can be created by using sequence-specific nucleases to induce DSBs at the target site in the coding sequences and their imprecise repair via NHEJ. Precise repair due to the induction of HR events, however, can alter plant genomes in a completely predictable way. In general, there are two different types of HR reactions that can be induced by a single DSB that are of interest for genome engineering. If the break is induced between directly repeated sequence motives, a controlled deletion can be achieved (Siebert and Puchta 2002). However, if a DSB is induced in a genomic locus in the presence of an extrachromosomal homologous repair template, the

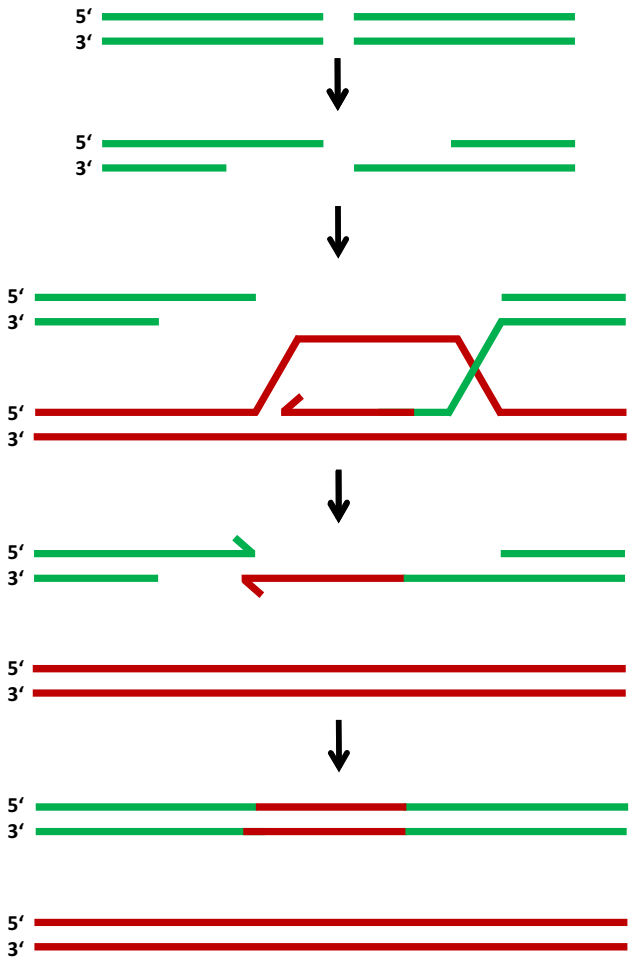

Fig. 2 Synthesis-dependent strand annealing (SDSA) pathway of homologous recombination (HR). After DSB induction, free 3′ overhangs are produced that can invade a second homologous dsDNA molecule. The invading strand forms a D-loop structure and repair synthesis is initiated using the homologous dsDNA as a template. The elongated 3′ end can reanneal with the second free 3′ end at the break site. The resulting dsDNA still contains two gaps on each side that are filled by DNA synthesis. Thus, genetic information is only copied from the homologous sequence into the DSB site, and the donor sequences stays unaltered. This leads only to non-crossover events

technique is referred to as gene targeting (GT) (Puchta et al. 1996). GT is of great interest because, on the one hand, new genes can be integrated into a specific target locus, and on the other hand, it is also possible to introduce subtle changes of single amino acids into a specific ORF. DSB-induced gene targeting has been the focus of interest in recent decades. A series of different approaches have been developed, and over time, different types of increasingly sophisticated synthetic nucleases have been applied. In the following paragraphs, we will discuss different approaches that have been taken over the years.

GT in plants: the early days

In bacteria or yeast cells, DNA integration takes place mostly via HR, and therefore, GT can easily be achieved using homologous sequences. As HR is not the main repair mechanism in higher organisms, such as plants, spontaneous "classical" GT events are quite hard to obtain, as they are so rare.

The first approaches to achieve "classical" GT were via simply applying a homologous sequence and screening for spontaneous GT recombination events (Fig. 3). The initial studies on GT events in higher eukaryotes were performed in mouse embryonic stem cells in 1987 (Doetschman et al. 1987; Thomas and Capecchi 1987), but it took longer to demonstrate that genes can be targeted in plant cells. In 1988, Paszkowski et al. (1988) demonstrated for the first time that GT is possible in plant cells, namely, in tobacco protoplasts. In these experiments, they transfected tobacco cells carrying copies of a partial, non-functional kanamycin-resistance gene with plasmids containing the missing part of the gene. Gene correction due to GT events was measured based on kanamycin-resistance. Two years later, Offringa et al. (1990) showed that GT is also possible using a T-DNA instead of plasmids. A third possibility is to apply GT donor sequences using chimeric RNA/DNA oligonucleotides. The DNA component of these chimeric oligos contains a "mutator" region of five nucleotides homologous to the target sequence that can contain one or two mismatches to the target, and therefore, can be introduced into the target site via repair mechanisms. The use of chimeric oligos to alter target genes has been reported in maize, tobacco, rice and wheat (Beetham et al. 1999; Dong et al. 2006; Okuzaki and Toriyama 2004; Zhu et al. 1999, 2000). In these studies and in some subsequent ones, the measured GT frequencies were rather low, with only one event per 10^4 to 10^5 targeting attempts, because the donor and the target sequences interact more or less randomly (for reviews see Puchta 2002; Puchta and Fauser 2013).

Using site-specific synthetic nucleases, a tool was in hand to induce DSBs at the target locus, thus increasing GT frequencies. The first experiments in plants were performed using the meganuclease I-SceI (Puchta et al. 1993). HR-mediated GT was shown to work in tobacco cells using an artificial target site. A particular DSB was induced in a stably transformed transgene with a transiently expressed I-SceI cassette and the break was subsequently repaired via HR using a T-DNA harbouring homologous sequences to the target sequence. Therefore, it was shown for the first time in plants that HR frequency can be increased by up to two orders of magnitude (Puchta et al. 1996).

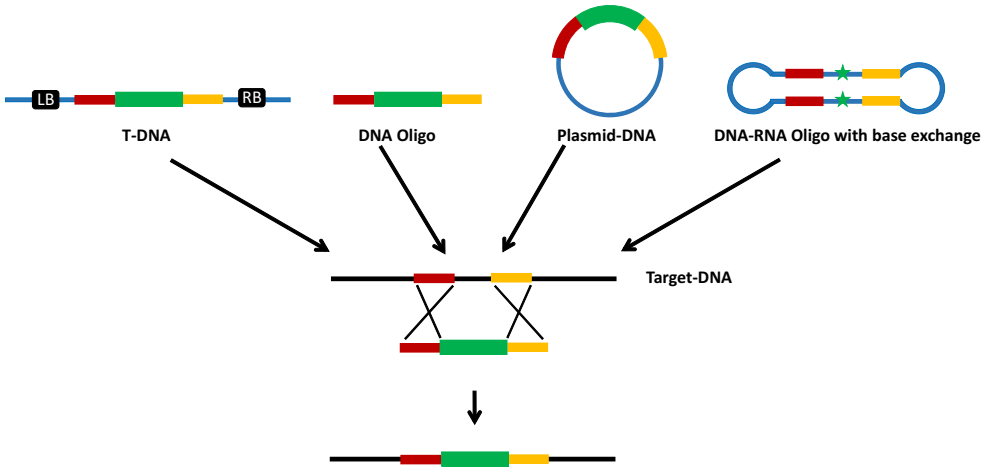

Fig. 3 Different templates to initiate "classical" gene targeting in plants. T-DNAs, DNA oligonucleotides, DNA-plasmids or DNA–RNA oligonucleotides can be used for GT. HR within the target site using the flanking homologous regions (*red*, *yellow*) of the respective donor sequence leads to the desired gene insertion or modification (colour figure online)

GT in plants using synthetic nucleases

Over the years, new types of site-specific nucleases became available and replaced meganucleases for use in genome engineering. Meganucleases are of limited applicability, as they have only one particular recognition sequence, making the knockout of natural genes nearly impossible. Manipulation of the enzymes' binding site has to be performed without harming its endonuclease activity, and this is difficult given that both activities are combined in one domain. New sequence-specific nucleases were developed that combined a DNA-binding domain with a separate nuclease domain derived from the type IIS enzyme FokI. One of these is the so-called zinc-finger nuclease (ZFN) that operates as a dimer. In 2005, GT was demonstrated with high efficiency in tobacco protoplasts by inducing a DSB with a ZFN in an artificial locus (Wright et al. 2005). Here, two disrupted marker genes with recognition sites for the ZFN were restored by GT. After transformation with both the nuclease and the donor template with the correct marker sequences, GT frequencies of up to 10 % in selected lines were measured. This experiment showed that by using customizable synthetic nucleases, GT with higher frequencies and at desired loci was feasible in plants. Improved GT frequencies were also achieved in maize using ZFNs. Here, a heterologous donor molecule was used, expressing an herbicide resistance gene in the target locus when integrated by GT. The GT events were demonstrated in 20 % of the selected lines (Shukla et al. 2009). Another approach using ZFNs for GT was performed in tobacco cells, which also inserted resistance cassettes into the target genes, leading to GT efficiencies of up to 4 % (Townsend et al. 2009). Additionally, GT was found to be possible in Arabidopsis. Plants expressing a ZFN targeting the protoporphyrinogen oxidase (PPO) gene were transformed with a T-DNA harbouring an incomplete PPO gene, making the enzyme butafenacil-herbicide insensitive. Based on this analysis, GT frequencies of up to 3.1×10^{-3} per transformation event could be detected (Pater et al. 2013).

As ZFNs are relatively difficult to customise for a specific sequence, multiple studies using the, at that time, newly developed transcription activator-like effector nucleases (TALENs) were initiated a few years later. This class of sequence-specific nucleases is, like the ZFNs, composed of a DNA-binding domain and a FokI nuclease domain that act as dimers (Miller et al. 2011). With TALENs, it was possible to modify the endogenous genes for many different plant species, such as *Brachypodium*, rice, maize and tobacco, with even higher frequencies than was possible with ZFNs (Mahfouz et al. 2011; Shan et al. 2013; Zhang et al. 2013). GT experiments using TALENs in tobacco protoplasts showed that it is possible to alter the *ALS* gene with a donor sequence differing by 6 bp to the original gene sequence with frequencies of up to 4 % (Zhang et al. 2013).

The most recent sequence-specific nuclease available for targeted gene manipulation is the RNA-guided Cas9 nuclease derived from the bacterial CRISPR/Cas system (clustered regularly interspaced short palindromic repeats/CRISPR associated). A common factor with meganucleases, ZFNs and TALENs is that the nuclease itself has to be altered to target the desired sequence. Cas9 on the other hand uses a guide RNA to direct the enzyme to the target site. Altering the RNA to facilitate target recognition is simple, as only 20 nts have to be exchanged whilst the nuclease itself remains unmodified. Its simple structure makes the RNA-guided Cas9 nuclease an extremely easily applicable tool for genome manipulations. Targeted gene insertion was shown to be effective in maize protoplasts via

biolistic transformation using the Cas9-sgRNA system (Svitashev et al. 2015). The donor DNA was located on the same plasmid as the Cas9-sgRNA cassette. Three different donor DNAs (one double-stranded vector DNA and two single-stranded oligos) were tested in the alteration of the *ALS2* gene, which can be edited to result in sulfonylurea-resistant plants. All experiments led to altered *ALS2* genes, showing that small single-stranded oligos are sufficient for gene editing. Integration frequencies of up to 4.1 % were achieved using the Cas9-sgRNA system, whilst parallel approaches using the same donor template with a meganuclease led to approximately 5 times fewer integration events. That single-stranded oligos in combination with the CRISPR/Cas system are also able to precisely edit crop plants was shown for flax (*Linum usitatissimum*) plants (Sauer et al. 2016). The authors were able to create herbicide resistance plants by introducing point mutations in the *EPSPS* genes by applying the DNA-oligos in combination with DSB induction via Cas9.

The general use of the Cas9-mediated GT approach was also shown to work in soybean, where a resistance cassette was integrated into the *DD43* target site (Li et al. 2015).

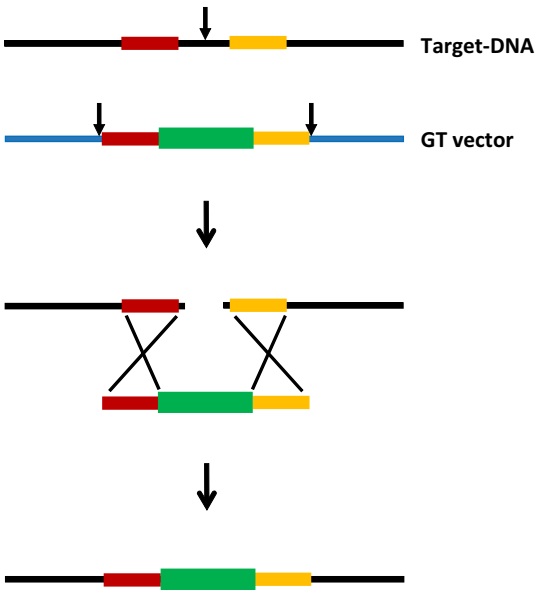

Fig. 4 In planta gene targeting. By simultaneous induction of a DSB in the target locus as well as at both ends of the GT donor vector, which is integrated in the plant genome, both the target and the donor vector get activated for the GT reaction

In planta gene targeting

A sophisticated method to enhance GT events was established in 2012 using the meganuclease I-SceI. Here, the enzyme not only cuts within the target site but also within the transgenic donor sequence. The donor in this case is stably integrated into the genome, so that this system can achieve GT events independent of transformation efficiencies (Fig. 4). Therefore, this strategy seems to be especially attractive for crop plants that are hardly transformable. After transformation of the target vector and the nuclease, the targeting vector gets excised, and then, can trigger GT with up to 1 % efficiency (Fauser et al. 2012). More recently, this in planta GT approach has been adopted for the Cas9 enzyme in Arabidopsis plants. Using this strategy, the number of T-DNAs needed was reduced to only one harbouring the donor sequence as well as the Cas9-sgRNA expression cassettes. Here, DSBs were induced simultaneously in a targeting vector and also in the endogenous *ADH1* target locus by a Cas9 nuclease. Via HR repair, it was possible to integrate a resistance cassette into the *ADH1* locus at the break site (Schiml et al. 2014).

Replicon-induced GT

In 2014, Baltes et al. (2014) presented an innovative approach that demonstrated that the induction of GT events was also possible using geminivirus-based replicons. It was demonstrated that DNA carried by geminiviruses can be used as a template for homologous recombination (Fig. 5). Tobacco plants were transformed with T-DNA constructs harbouring the minimal parts necessary for geminivirus replication, a ZFN and a donor template. In this process, after transformation, the rolling circle replication of the replicon is initiated at the large intergenic regions (LIRs) that flank the T-DNA construct, leading to the circularisation of the construct. Thereafter, the ZFN is expressed and induces a DSB in a defective GUS target gene. GT events occur using the supplied correct donor template sequence, copying it via GT in the target gene and leading to gene restoration. With this geminivirus-based method, the donor template is replicated multiple times, and a GT enhancement of greater than two orders of magnitude has been observed. Recently, this technique was also shown to work with TALENs and Cas9 in the tomato (Čermák et al. 2015).

Manipulating the enzyme machinery during DSB-induced GT

One major obstacle for performing GT in plants is its low efficiency. This is because GT is based on HR, which is only a minor pathway in higher organisms for repairing breaks in somatic cells. One approach to enhance HR efficiency is to express the HR-related genes of bacteria or yeast in plants, because in lower organisms, HR occurs

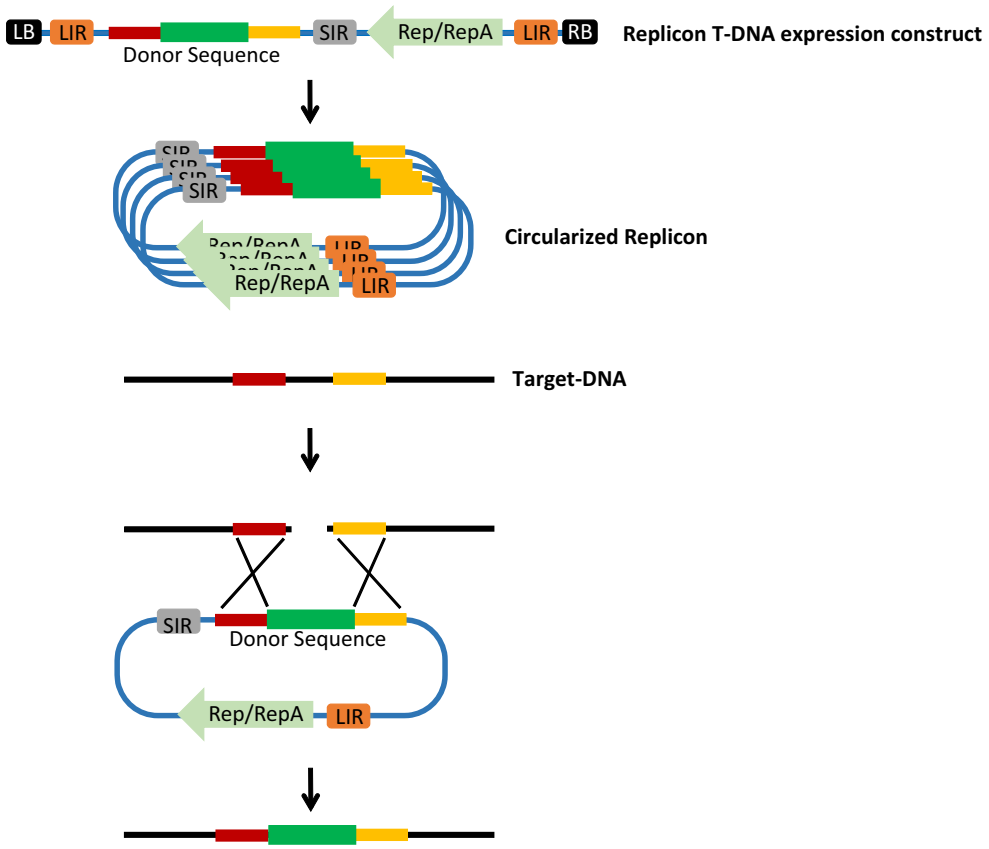

Fig. 5 Geminivirus-based replicon-mediated GT. After delivery of the T-DNA to the plant cell nucleus, the rolling circle replication of the replicon is initiated at the large intergenic regions (LIRs) that flank the T-DNA construct, leading to the circularisation of the targeting vector. GT can be triggered by inducing a DSB in the target locus, leading to integration of the donor sequence (*green*) (colour figure online)

with a high efficiency. Heterologous expression of the strand exchange protein RecA from *E. coli* in tobacco protoplasts led to the enhancement of intrachromosomal HR by one order of magnitude (Reiss et al. 2000). The overexpression of the HR strand invasion protein RAD54 from *S. cerevisiae* in Arabidopsis led to an enhanced GT frequency of the cruciferin gene, which codes for a seed storage protein (Shaked et al. 2005). By using egg cell-specific expression of RAD54, it was possible to control the protein expression both temporally and spatially. With this egg cell-specific expression of RAD54, GT efficiency was able to be increased by tenfold in Arabidopsis (Even-Faitelson et al. 2011). Instead of heterologous expression of HR-related proteins, GT efficiency could also be increased by manipulating the break repair pathways via the suppression of proteins involved in NHEJ. However, difficulties in suppressing NHEJ proteins arise, as there is more than one NHEJ-related pathway operating in plant cells. By blocking or knocking out proteins of the classical end joining pathway, proteins of an alternative NHEJ mechanism can still efficiently carry out the repair of DSBs (Charbonnel et al. 2011). On the other hand, mutating proteins that are involved in the suppression of HR, such as RECQ4A, FANCM or RTEL1, lead to a hyper-recombination phenotype in Arabidopsis (Endo et al. 2006; Hartung et al. 2007; Knoll and Puchta 2011; Recker et al. 2014). Modulating the expression of such proteins might also help to increase GT efficiencies. In 2012, Kwon et al. (2012) showed that by overexpressing OsRecQI4 and OsExo1, two proteins involved in the resection of the DSB side during HR, DSB-induced HR frequencies can be altered in rice calli. After DSB induction by I-SceI, the restoration of a disrupted GUS gene was detected by counting blue sectors after staining. An increase in HR events was also measured after DSB induction. When RecQ4 or Exo1 were overexpressed, the frequencies of HR events increased drastically and were even higher when both proteins were overexpressed simultaneously. Instead of overexpression, Qi et al. (2013) used a mutant of the SMC6B gene, which is involved in sister chromatid exchange during recombination, and showed that GT efficiency could be increased at three different loci in Arabidopsis. Following SMC6B knockout, sister chromatid exchange is impaired (Watanabe et al. 2009), therefore, an

extrachromosomal template might be used for more efficient repair, leading to higher GT rates.

By combining two methods, Endo et al. (2015) demonstrated increasing GT efficiencies in rice calli by simultaneously enhancing GT whilst suppressing NHEJ. In a first round of transformation, they used a Cas9 construct to knockout the *ligase 4* gene, which is an important factor during NHEJ repair. In the second transformation step, a GT vector harbouring the correct donor template as well as a Cas9 construct targeting a disrupted *acetolactate synthase* (ALS) gene were applied to the *lig4*-deficient calli cells. GT frequencies of up to 1 % have been achieved with this method. Without *lig4*-disruption, GT frequencies were two- to three-fold lower.

One obstacle with this approach is that the manipulation of the HR repair machinery can lead to a destabilisation of the genome, as higher HR efficiencies can also lead to undesirable recombination events between repetitive sequence motives that are abundantly present in larger plant genomes.

GT with positive–negative selection

A method to enrich transgenic cells that carry out GT events is positive–negative selection (PNS). The basis of PNS is the simultaneous use of positive- and negative-selectable markers. Notable, by using a negative-selectable marker, the active selection against random integration events is possible, as they are based on NHEJ and represent the huge majority of the transgenic cells that are obtained after transformation (for a review see Shimatani et al. 2014). The positive selection marker is located between two sequences that are homologous to the target sequence and can be used for the selection of HR-based integration events. The negative selection markers flank both ends of the homologous sequences and should be eliminated if HR-based GT takes place. If random integration of the vector occurs, the cells retain the negative selection marker in the genome and will not be able to survive under selection. PNS-mediated GT was first developed for plants by Terada et al. (2002) when they used this method to knockout the *waxy* locus in maize. GT events were detected in calli surviving PNS as shown by subsequent PCR analysis of specific targeted sequences. GT frequencies of 6.4×10^{-4} were achieved with this PNS screening method. Recently, this method was improved by combining it with an *nptII*-resistance gene and its antisense RNA (Nishizawa-Yokoi et al. 2015). When both the sense and antisense *nptII* constructs are transcribed, this leads to the suppression of the expression of the sense *nptII* gene, rendering the plants geneticin-sensitive. Therefore, with this approach only one resistance gene is needed, as the sense *nptII* gene is utilised as the positive marker and the antisense gene as the negative marker. When marker-free gene editing is desired, the positive marker can be removed after the integration event. The marker can be placed between two *loxP* sites, and therefore, can be removed after GT by Cre-*loxP*-mediated recombination. As the recognition sites for the recombinases are still present in the target gene after excision, the group of Seiichi Toki recently applied the *piggyBac* transposon for this purpose. This transposon integrates at "TTAA" sequences and is excised without leaving any footprint behind. This was demonstrated by removing the positive marker with the *piggyBac* transposon, whereupon the target gene was reactivated. In this case, a luciferase gene was reconstituted and the transposition of the *piggyBac* transposon was visualised via luminescence (Nishizawa-Yokoi et al. 2014). To date, PNS has only been applied for "classical GT" in plants; therefore, it will be interesting to see if PNS is also able to improve the efficiency of DSB-induced GT.

Outlook

In recent years, many new tools for genome engineering have become available, of which the Cas9-sgRNA enzyme complex plays the most prominent role, as it seems to work in almost every plant species tested. Using this system along with other synthetic nucleases, new approaches to enhance GT events may become available. To date, GT frequencies are still low and the detection of events is laborious and requires extensive screening. Combinations of the different approaches discussed above should open the door to make GT more efficient. Not only can the enzyme machinery be manipulated but also the PNS method could be applied.

Interestingly, it was shown that the Cas9 nickase derived from the CRISPR/Cas system is able to induce HR with a SSB (single-strand break) instead of a DSB in plants at frequencies comparable to that shown by the nuclease (Fauser et al. 2014). Whether the induction of SSBs is also able to enhance GT efficiency will be an interesting question for future research.

By mutating both nuclease domains of Cas9 (dCas9), the enzyme can act as a cargo protein, guiding proteins of interest to the desired genetic sequences, e.g., transcriptional activators or repressors. Targeted activation or repression in plants using dCas9 constructs has recently been demonstrated (Piatek et al. 2015). Such an approach might also be an attractive way to manipulate the efficiency of HR and/or NHEJ.

With the CRISPR/Cas system, a wide spectrum of gene modifications is possible. However, for more complex

genomic engineering approaches, it is desirable to induce different cellular functions at the same time in a single cell, e.g., DSB induction in the target gene and gene inactivation of proteins involved in NHEJ. A way to achieve this is the adaption of additional Cas9 nucleases for genome manipulations. The Cas9 nuclease commonly used is derived from *Streptococcus pyogenes*. It was shown that Cas9 orthologues originating from *Streptococcus thermophilus* and *Staphylococcus aureus* seem to work with comparable efficiencies to that of the *S. pyogenes* Cas9 (Esvelt et al. 2013; Jinek et al. 2012; Kleinstiver et al. 2015; Ma et al. 2015; Ran et al. 2015). In contrast to *S. pyogenes*, these orthologues have different PAM requirements for target recognition and are accompanied by specific sgRNAs. Recently, it has been demonstrated that these orthologues work efficiently in *A. thaliana* (Steinert et al. 2015). It was also shown that *S. aureus* Cas9 can induce intrachromosomal HR by inducing a DSB in an SSA-dependent GUS-reporter line. DSB induction occurred without interspecies interference, as only species-specific combinations of Cas9 and sgRNA led to DSB induction, demonstrating that the Cas9 enzymes tested act without cross-species interference, and therefore, can be used for more complex cellular approaches. With the Cas9 orthologues, the toolbox for genome and transcriptome engineering in plants is becoming larger and we are taking further steps towards developing synthetic plant genomes (for a review see Puchta 2016). Whereas, synthetic plant genomes are obviously a long way off, it does not seem to be out of the question that DSB-induced GT in plants will become increasingly efficient in the near future due to the application of the various recently developed innovative new strategies discussed in this review.

Author contribution statement All authors wrote, read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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