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CRISPR/Cas-mediated *in planta* gene targeting: current advances and challenges

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Highlight

We review recent advances in CRISPR/Cas-mediated gene targeting in plants, discuss their potential applications, and suggest further improvements for achieving higher efficiency.

Abstract

We can use gene targeting (GT) to make modifications at a specific region in a plant's genome and create high-precision tools for plant biotechnology and breeding. However, its low efficiency is a major barrier to its use in plants. The discovery of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas-based site-specific nucleases capable of inducing double-strand breaks in desired loci resulted in the development of novel approaches for plant GT. Several studies have recently demonstrated improvements in GT efficiency through cell-type-specific expression of Cas nucleases, the use of self-amplified GT-vector DNA, or manipulation of RNA silencing and DNA repair pathways. In this review, we summarize recent advances in CRISPR/Cas-mediated GT in plants and discuss potential efficiency improvements. Increasing the efficiency of GT technology will help us pave the way for increased crop yields and food safety in environmentally friendly agriculture.

Keywords: CRISPR-Cas, DNA sequence knock-in, DNA sequence replacement, homologous recombination, plant gene targeting

Introduction

Generating targeted genetic changes in living cells and organisms has historically been a great challenge in many species. Precise editing of genomic information is essential for a better understanding of gene function, the production of new plant traits, and the development of better breeding strategies. During the past decade, several technological breakthroughs have made efficient plant genome editing conceivable. Especially with the discovery of CRISPR/Cas, a gene editing technology that allows us to knock out or knock in genes, a great advance has been made (Jinek *et al.*, 2012; Schiml and Puchta, 2016; Puchta, 2017; Huang and Puchta, 2019; Wada *et al.*, 2020). Knocking out a gene in plants involves expressing the Cas protein and directing it to a precise genomic locus using specific guide RNA. There, the Cas protein cuts the genomic locus by snipping through both strands of DNA, and the cell's DNA repair mechanism fixes the cut using non-homologous end joining (NHEJ). NHEJ is efficient but inaccurate, introducing errors in the form of small insertions or deletions that are usually enough to knock out the gene. The result usually is the insertion or deletion of random nucleotides (nt) at the snip site, leading to the disruption of the reading frame and target gene's function**.** This method is widely used to produce stable single and multiple mutants in numerous plant species (Shen and Li, 2022; Gehrke *et al.*, 2022). The lack of predictability presents a particular obstacle in cases where the aim is to controllably change the function or regulation of a gene rather than knock it out or prevent it. In the case of GT, the cuts produced using CRISPR/Cas nucleases must be repaired very precisely using a second DNA repair mechanism called homology-directed repair (HDR), which occurs less efficiently in plants and whose frequency is dwarfed by that of NHEJ (Steinert *et al.*, 2016; Dong and Ronald, 2021; Shen and Li, 2022). Thus, one of the big questions in plant genome editing is how we can achieve fast and very efficient GT, i.e., inserting or replacing a desired DNA fragment in the given genomic locus.

Plant gene targeting and its potential applications

Why do we need GT in plants? (Fig.1). GT is a tool of vital importance for fundamental research as well as plant biotechnology and breeding. More specifically, it can be used: 1) **To achieve stable expression and precision-based DNA integration into the plant genome.** Currently used methods of DNA delivery into plants, such as *Agrobacterium tumefaciens* (*A. tumefaciens*)-mediated transformation or particle bombardment, lead to a random integration of the DNA in the plant genome. This may cause alterations in gene function, variations in gene expression levels, as well as altered agronomic traits (Meyer, 1995; Day *et al.*, 2000; Schubert *et al.*, 2004; Tang *et al.*, 2006; Rajeevkumar

et al., 2015; Gelvin, 2017; Jupe *et al.*, 2019). Therefore, targeted insertion of desirable sequences at a precise genomic site is of the highest interest to the plant community. Stable expression of transgenes is a prerequisite for most transgenic plants. The number of integrations into a plant genome is dependent on specific elements of the final T-DNA vectors used for transformation into *Agrobacterium tumefaciens,* such as (T)-DNA origins of replication (Oltmanns *et al.*, 2010; Ye *et al.*, 2011; Tark-Dame *et al.*, 2018). Furthermore, a number of T-DNA vectors used for crop transformation frequently result in 20-80% of transgenic plants containing vector sequences outside of the T-DNA region, which could be caused by either LB (left border, one of the two imperfect direct repeats required *in cis* for T-DNA transfer into plant cells) readthrough or LB T-DNA initiation in different T-DNA vector/crop combinations (Kononov *et al.*, 1997; Afolabi *et al.*, 2004; Podevin *et al.*, 2006; Oltmanns *et al.*, 2010; Ye *et al.*, 2011). Because of the likelihood of gene silence and regulatory requirements for backbone-free plants, the presence of vector backbone sequences and multiple copies of the transgene is undesired for biotechnology applications. **2) To improve the methods of studying gene functions**. GT would allow us to look at fluorescent-tagged protein fusions in the native genomic context, replace or insert promoters or regulatory elements to improve the activity of desired proteins or block the action of unwanted pathways. At the same time, different transgenic plants with different mutations in the sequence of the same genes to be inserted in the same genomic locus, could be compared. **3) Using GT to avoid RNA silencing.** Expression of transgenes is prone to silencing mediated by small RNAs (sRNAs), due in part to the involvement of native genomic components required to shield genes from the silencing machinery (Molnar *et al.*, 2011; Guo *et al.*, 2016; El-Sappah *et al.*, 2021). Terminator sequences and well-defined poly(A) sites, for example, have been shown to have a significant impact on transgene-derived sRNA accumulation (de Felippes *et al.*, 2020; de Felippes and Waterhouse, 2022). The capacity to insert the required genomic elements into the native genomic context may reduce the likelihood of sRNA-mediated gene silencing. This aspect will be discussed further in this review; **4) For a precision-based development of new agronomic traits.** The development of a global agriculture that can survive the worsening effects of climate change will be greatly aided by GT. It will lead to the creation of highly valuable crops that can better withstand biotic and abiotic stress, have a higher nutrient content, yield, or biomass, and are precisely edited for domestication.

Mechanisms of double-strand break repair

The error-prone NHEJ-based double strand break (DSB) repair can occur via two competing sub-pathways: classical (cNHEJ) and alternative (aNHEJ) (Fig. 2A-B). In the case of cNHEJ, the break ends are swiftly bound by the abundant, ring-shaped heterodimer KU70/KU80, which recruits additional repair proteins and allows the break to be re-ligated by DNA LIGASE 4 (LIG4). In addition to precise ligations, a cNHEJ route may result in minor deletions or insertions. Repair through the aNHEJ route, on the other hand, results in bigger deletions since microhomologies available at the break sites are

employed for annealing. As a result, the intermediate sequence is lost. POLY(ADP-RIBOSE)-POLYMERASE 1 (PARP1), a polymerase that competes with the KU complex, can bind the break ends and cause 5′- to 3′-resection of the DSBs, resulting in short single-strand overhangs (Audebert *et al.*, 2004; Wang *et al.*, 2006; Robert *et al.*, 2009; Truong *et al.*, 2013; Wolter *et al.*, 2021; Shen and Li, 2022). The exposed microhomologies can anneal, with polymerase Q (PolQ) stabilizing the resultant repair intermediate (Wyatt *et al.*, 2016; Black *et al.*, 2016; Seol *et al.*, 2018; Mara *et al.*, 2019). After nucleases destroy the protruding 3′ ends (Bennardo *et al.*, 2008), PolQ-initiated fill-in synthesis can proceed (Ahmad et al., 2008; Hogg et al., 2012). Finally, an XRCC1/Ligase III complex or Ligase I can ligate the break (Liang *et al.*, 2008; Charbonnel *et al.*, 2010; Martínez-Macías *et al.*, 2013; Masani *et al.*, 2016).

Since sister chromatids may be employed as a repair template, HR-based strategy for DSB repair mainly occurs in the late S and G2 phases of the cell cycle (Fig. 2C). Conservative and non-conservative HR pathways can take place. Single-strand annealing (SSA) is a prominent non-conservative path that requires homologous repetitive sequences arranged in close proximity (Siebert and Puchta, 2002). Once DSBs have been induced, both break sites undergo single-strand resections until homologous sequences can anneal. Ligations follow the degradation of overhanging single strands or the filling of gaps. Because the sequence between the two homologies is usually deleted, deletions occur as a result of this process. In contrast, the conservative HR does not lead to sequence loss. Synthesis-dependent strand annealing (SDSA) and DSB repair (DSBR) are two well-known subpathways of conservative HR-based repair that share the initial steps. In contrast to the DSBR, which plays a central role in meiotic recombination in plants and can lead to crossover events, experimental data suggests that practically all DSBs are repaired by the SDSA mechanism in somatic cells. In SDSA, after a DSB is induced, a homologous sequence is copied into the break site such that no sequence information is lost. After a DSB, the 3′ ends of the DNA strand are exposed by resecting both ends of the break. The so-called displacement loop results when a 3′ end invades a homologous sequence, displacing one of the strands along the way. The invading strand is subsequently elongated by copying the donor DNA sequence. After being liberated from the D-loop, the elongated single-strand can reanneal with the homologous single-stranded DNA on the other side of the break point. Because of its positive effects on genome stability, the SDSA pathway appears to be the primary mechanism behind conservative HR in somatic cells (Fig. 2) (Steinert *et al.*, 2016; Wolter *et al.*, 2021; Shen and Li, 2022).

Several strategies have been tried to shift the balance of DNA repair in plants toward HR-based repair. Because the DNA repair mechanism is generally similar across plant species, altering it might make targeted modifications to the genome more efficient. For instance, a considerable improvement in the efficiency of HR-based GT events was achieved using zinc finger nucleases (ZFNs) in the absence of critical DNA repair proteins, KU70 and LIG4 (nonhomologous end-joining, NHEJ), and SMC6B (sister-chromatid-based homologous recombination, HR) (Nishizawa-Yokoi *et al.*, 2012; Qi *et al.*, 2013). Moreover, depleting key components of the chromatin assembly factor 1 (CAF-1) complex leads

to a significant increase in the frequency of somatic HR in plants by roughly 40-fold (Endo *et al.*, 2016). This could be explained by a better accessibility of genomic areas to repair enzymes and foreign DNA, as well as a delay in the loading of histones onto freshly duplicated or repaired DNA. An increase in DSB, a G2-phase retardation leading to a faster endoreduplication, and an increase in mRNAs coding for HR-related proteins might also contribute to the shift in GT efficiency when the CAF-1 complex is depleted. Overexpression of proteins involved in the HR pathway, such as RAD proteins, which are involved in both DSB repair and HR, could serve as another strategy for increasing GT efficiency. As demonstrated in *Arabidopsis thaliana*, the overexpression of Rad52-1A or the yeast RAD54 gene enhanced the frequency of HR (Shaked *et al.*, 2005). Although NHEJ pathway mutants showed an improvement in GT efficiency, these mutations frequently cause an accumulation of spontaneous mutations in the genome. At the same time, heterologous recombinase overexpression may result in morphological abnormalities (Barakate *et al.*, 2020). As a result, while all the above-mentioned manipulations have the potential to improve GT, advances must be made to avoid unintended effects on plant growth. It is important to highlight that, unlike in angiosperms, GT is the primary mechanism of genetic transformation in the moss *Physcomitrium patens*. It has a high efficiency (up to 90%) of homologous recombination, leading to a high ratio of targeted integration into the moss genome. This is in contrast to the low efficiency of homologous recombination seen in most plant species. As demonstrated by several studies, homologous recombination in *P.patens* relies on the action of the RAD51 protein to repair double-strand breaks (DSBs). It has been proposed that the efficiency of GT is connected to the dominance of the gametophytic haplophase in *P.patens*' life cycle. Furthermore, a number of genes in *P.patens* can act as enhancers or repressors in homologous recombination-mediated DSB repair (Schaefer, 2001; Zhu, 2021).

CRISPR/Cas-mediated double-strand break induction: a breakthrough for gene targeting

DSBs must be repaired for genomic stability. DNA repair patterns are genetically determined and contribute to the evolution of the genome. Over the years, a wealth of knowledge has been developed on how DSBs are repaired in diverse eukaryotic genomes. The recent surge in interest in DSB repair mechanisms can be attributed to the need for rapid development of gene editing technologies (Shen and Li, 2022). The most widely used Cas protein for generating DSBs in plants, Cas9, is an RNA-dependent DNA endonuclease that interacts with a CRISPR RNA (crRNA), which is a small RNA of 20 nt that is complementary to the target sequence in the genome. It is required for Cas9 protein recruitment to the target site. crRNA sequence is fused to the scaffold tracrRNA (trans-activating crRNA), which serves as a binding scaffold for the Cas nuclease (Fauser *et al.*, 2014). For CRISPR/Cas systems to generate DSBs, a protospacer adjacent motif (PAM) next to the target recognition site is necessary. In addition to the most widely used Cas9 system from *Streptococcus pyogenes* (SpCas9), several Cas9 orthologs have been discovered in other bacteria. As different Cas orthologs require distinct PAM sites, multiple

CRISPR/Cas tools have been developed and are continuously in development to target nearly any genomic locus. For instance, a smaller Cas9 found in *Staphylococcus aureus (SaCas9*) has been shown to have more DSB activity than SpCas9 being now more frequently used (Wolter *et al.*, 2018). Both nucleases generate DSBs with blunt ends, with SpCas9 using NGG (N = any nucleotide; G = guanine) and SaCas9 using NNGRRT (R = A, adenosine, or G, guanine; T = thymine) as their corresponding PAM sites. The CRISPR/Cas12a system is another extensively used system to generate DSBs. Unlike Cas9, Cas12a produces staggered DNA ends, with the cleavage site located distal to its TTTV (V = A, C, or G) PAM sequence (Zetsche *et al.*, 2015; Mao *et al.*, 2021). One of the most widely used Cas12a variants (LbCas12a) was isolated from the *Lachnospiraceae bacterium* (Xu *et al.*, 2017; Begemann *et al.*, 2017*a*; Zhong *et al.*, 2018; Bernabé-Orts *et al.*, 2019) (Table 1).

Recently, it was shown that several modifications in the Cas gene sequence can significantly improve its activity in generating DSBs and GT. For instance, a mutated version of LbCas12a, temperature-tolerant LbCas12a (ttLbCas12a), was recently reported to be more efficient compared to the wild-type version in *A.thaliana* and *Nicotiana tabacum* for both mutagenesis and GT (Schindele and Puchta, 2020; Merker *et al.*, 2020; Huang *et al.*, 2021). Moreover, the number of NLSs (nuclear localization signals) used in association with the Cas9 gene has a significant impact on its activity. In addition, a major boost in editing efficiency was obtained by inserting 13 introns into the Cas9 coding sequence. The intronized Cas9 gene was found to be effective in various plant species, including *A.thaliana*, *Nicotiana benthamiana*, and *Catharanthus roseus* (Grützner *et al.*, 2021; Ursache *et al.*, 2021). In a similar fashion, an intron containing version of LbCas12, ttLbCas12a-i, was generated. Short introns from *A.thaliana* genes with canonical donor and acceptor sites (AGGT) were placed into the coding sequence of LbCas12a. ttLbCas12a-i version, when driven under the ubiquitous PcUBi4-2 promoter, significantly (2.7 fold) improved the GT targeting efficiency in *A.thaliana* (Schindele *et al.*, 2022). Introns have been known for many years to have a positive effect on gene expression (Callis *et al.*, 1987; Marillonnet *et al.*, 2005; Christie *et al.*, 2011; Grützner *et al.*, 2021) and this effect is attributed to a decrease in RNA-mediated gene silencing. Transgenes become comparable to transposons, as both are intronless genes. In fact, a study where transgenes with or without introns were compared found that sRNA densities in exons of intronless genes were comparable to exons of transposable elements (Christie *et al.*, 2011). Therefore, the higher efficiency of intronized-Cas9 and ttLbCas12a-i could be attributed to a drop in transgene-silencing, allowing a more sustained expression of the nuclease during plant development (Grützner *et al.*, 2021; Schindele *et al.*, 2022) and higher efficiencies in gene editing and GT.

The discovery of compact CRISPR-Cas systems may allow for approaches where the size of Cas protein is a limiting factor. As a result, several research lines are currently focused on developing small and adaptable CRISPR-Cas systems that will enable the next generation of GT techniques. Recently, CasΦ (700 to 800 amino acids compared to 1368 of SpCas9), a compact Cas that requires a T-rich minimal PAM and a wide range of working temperatures, was discovered, making it an excellent

addition to the existing plant genome editing tools (Al-Shayeb *et al.*, 2020; Pausch *et al.*, 2020). Large bacteriophages encoding CasΦ are found in diverse ecosystems, which may provide a broad range of optimal temperatures for CasΦ activity (Al-Shayeb *et al.*, 2020). CasΦ and its variants can generate stably inherited gene edits with high specificity and low off-target effects, as demonstrated in *A.thaliana* and maize (Pausch *et al.*, 2020; Li *et al.*, 2023). Other, most recently reported, yet not tested in plants and very compact Cas proteins, such as Cas12f, CasMINI, and *Acidibacillus sulfuroxidans* Cas12f1 *(As*Cas12f1) consisting of 529 aa, 526 aa, and 422 aa, respectively, have shown to be robust and efficient in gene editing activity in mammalian cells (Xu *et al.*, 2021; Wu *et al.*, 2021; Xin *et al.*, 2022). Although the compact Cas systems might open up new avenues for GT, their efficiency must be further tested in plants.

Recent studies indicated that timing the occurrence of DSBs using developmentally regulated promoters, such as an egg-specific promoter (Wang *et al.*, 2015) or a reproductive tissue-associated promoter (Yan *et al.*, 2015), has been found to considerably improve gene editing and GT efficiency in *A.thaliana* (Wolter *et al.*, 2018). It will be fascinating to explore if the advantages of egg cell-specific expression of different Cas genes can be transferred and lead to GT improvements in crop species. The temperature is another factor that influences GT efficiency. For instance, when Cas9-based GT was performed on tomatoes at temperatures ranging from 18 to 31 degrees Celsius, it was found that the frequency of the GT rose proportionally with temperature, going from around 1 percent at the lower temperature to about 5 percent at the higher (Vu *et al.*, 2019). This might be explained by recent data showing that exposure to 37°C boosts both SpCas9 cleavage activity and sgRNA levels *in vivo*. This is most likely because the optimal temperature for SpCas9 activity is far higher than the temperature at which plants normally grow. As demonstrated by LeBlanc and colleagues, heat stress does not raise the amounts of SpCas9 in *A.thaliana* or Citrus plants, but it does significantly increase the levels of sgRNA. At the same time, SpCas9 is more effective in producing double-stranded DNA breaks at 37°C than at 22°C, as shown by *in vitro* experiments, suggesting a possible contributing mechanism for the influence of temperature *in vivo* on CRISPR/Cas9 (LeBlanc *et al.*, 2018). Such temperature adjustments might prove useful, but they would require careful monitoring to prevent negative impacts on plant growth. Furthermore, adding transcriptional and translational enhancer sequences, such as the omega translational enhancer, the dMac3 translational enhancer, or the UBQ10 *A.thaliana* 1st intron, have been reported to improve Cas9 activity and, in turn, the efficiency of CRISPR/Cas9-mediated targeted mutagenesis in crop species like barley and potato (Gasparis *et al.*, 2018; Kusano *et al.*, 2018; Peng *et al.*, 2020).

Manipulation of RNA-silencing machinery and its potential use for improving the gene targeting

sRNAs are essential regulators of gene expression all across eukaryotic organisms. In plants, sRNAs are involved in modulating development and reproduction, genome reprogramming and stability,

defense against pathogens, and providing a broad adaptive response to stress (Borges and Martienssen, 2015; Singh *et al.*, 2018). Plant sRNAs can be divided into two main categories: microRNAs (miRNAs) and small interfering RNAs (siRNAs). To fulfill their biological function, after being processed from long double-strand RNA (dsRNA), all sRNAs are loaded into ARGONAUTE (AGO) proteins. AGO proteins, the main component of the RNA-induced silencing complex (RISC), regulate endogenous target genes by transcriptional gene silencing (TGS) or posttranscriptional gene silencing (PTGS), as well as invading plant viruses (Vaucheret, 2008; Bologna and Voinnet, 2014; Borges and Martienssen, 2015). As a way to fight back against antiviral silencing, many plant viruses have evolved viral suppressors of RNA silencing (VSRs) (Burgyán and Havelda, 2011). These VSRs suppress RNA silencing through different mechanisms, including inhibition of the processing of dsRNAs to siRNAs, the sRNA/AGO assembly, AGO activities, and/or dsRNA/siRNA-sequestration.

As we mentioned above, the integration of transgenes is frequently prone to gene silencing. Recent studies showed that RNA-silencing pathways can be manipulated to improve the gene editing efficiency of CRISPR/Cas9 systems (Mao *et al.*, 2018, 2021; Chiong *et al.*, 2021). In 2018, Mao and collaborators found out that silencing AGO1 (the main effector of PTGS in *A.thaliana*) by introducing an AGO1-RNAi cassette into the CRISPR/Cas9 vector significantly increased the gene editing efficiency. They showed that this gene editing efficiency coincided with a significant increase in Cas9 transcript levels in RNAi-AGO1-CDS compared to control samples (Mao *et al.*, 2018). Similarly, co-expression of the RNA silencing suppressor P19, *Tomato bushy stunt virus* (TBSV) P19 protein which is one of the best-studied VSRs (Csorba *et al.*, 2015), showed a strong correlation between the severity of the P19-caused effects and gene editing efficiency (Mao *et al.*, 2018). P19 functions as a suppressor of RNA interference by forming homodimers that bind the siRNAs produced. The sequestering of siRNAs by P19 prevents the RISC from being programmed with these molecules. This inhibits the endonuclease activity of RISC and thus interferes with the degradation of any RNA corresponding to siRNA, enhancing the transient expression of transgenes in many plant species (Saxena *et al.*, 2011). Similarly, another study also reported an increased frequency of mutations when co-expressing Cas9 protein with VRSs (such as P19 or P126), associating the gene editing increase with higher levels of Cas9 expression (Chiong *et al.*, 2021). It will be fascinating to see how the P19 co-expression strategy affects GT efficiency in plants.

Moreover, it was recently described in mammals that endogenous miRNAs could target the sgRNA backbone sequence, and the use of miRNA sponges to block miRNA activity led to a significantly increased rate of DNA cleavage by the CRISPR/Cas9 system (Huang *et al.*, 2020). Therefore, before using the CRISPR/Cas9 system in plants, the targeting of the guide RNA by endogenous miRNAs could be explored for the specific plant species to be edited. In the event that any putative interaction between endogenous miRNA and the sgRNA backbone is found, the miRNA activity could be prevented by adding specific mimicry (Franco-Zorrilla *et al.*, 2007), a well-established technology in plants to inhibit miRNA, to the CRISPR/Cas9 vector. The inhibition of RNA silencing by AGO1-RNAi cassette or VRS

could also avoid sgRNA targeting. Some studies have suggested that CRISPR/Cas9 systems cause serious off-target problems (Grünewald *et al.*, 2019). In mammalian cells, artificial miRNAs (amiRNAs) were used to reduce off-target events and increase the efficiency of DNA cleavage by the CRISPR/Cas9 system (Huang *et al.*, 2020). Aside from natural miRNAs, amiRNAs use natural miRNA scaffolds to target unusual RNAs (Yamada *et al.*, 1986). amiRNA-based approaches do not disrupt native cellular processes and may provide safer RNAi expression vectors compared with short hairpin RNAs (shRNAs). In this way, adding artificial miRNAs (amiRNAs) targeting the sgRNA spacer significantly abolished off-target editing without significantly inhibiting the cleavage at the on-target site, taking advantage of the difference in binding efficiency of the sgRNA between on- and off-targets (Huang *et al.*, 2020).

Donor template delivery for homologous double-strand break repair

Floral dip transformation is a simple and effective method for delivering transgenes and creating CRISPR knock-out and knock-in mutants in *A.thaliana* and its close relatives, but for most other plant species, tissue culture is required to regenerate whole plants from somatic cells (Clough and Bent, 1998; Bent, 2000). In tissue culture, phytohormones are added to the growth medium to promote cell division and maintain the resulting callus in the same developmental state as meristematic cells. As the number and activity of meristematic cells vary greatly between plant species, it is sometimes necessary to tailor procedures to each unique plant. Transiently expressing developmental regulators such as *WUSCHEL* (*WUS*), *SHOOT MERISTEMLESS* (*STM*), *ISOPENTENYL TRANSFERASE* (*IPT), GDNF FAMILY RECEPTOR ALPHA 4 (GFR4)*, and *GRF-INTERACTING FACTOR 1* (*GIF1*) can greatly improve transformation success (Debernardi *et al.*, 2020). This method has the potential to be important in the future development of GT strategies across a broad range of plant species resistant to transformation. Whether it's through a floral dip or a callus-based transformation, *A. tumefaciens* is the most often utilized plant pathogen strategy for creating transgenic plants or knock-in or knock-out mutants. This pathogen is capable of transporting its T-DNA to the nucleus of the plant host cell, where it is randomly integrated into the genome. The *A. tumefaciens*-based approach has been widely used for the delivery of CRISPR-Cas components due to its simplicity and low cost. Another technique used for genetic transformation, particularly in monocots, is particle bombardment. It requires coating metallic microprojectiles (typically gold, silver, or tungsten) with DNA constructs, which are subsequently shot into plant cells under high pressure. Using gold microparticles carrying CRISPR-Cas components that stably integrate into the host genome, targeted mutations have been successfully produced in a variety of plant species (Li *et al.*, 2015).

In *P.patens*, successful transformation (about 2% of the regenerated plants) of moss protoplasts with a plasmid containing the sgRNAs and SpCas9 for targeting the PpAPT target locus confering resistance to 2-fluoroadenine, was achieved. Mainly, deletions were observed, most of them resulting from

alternative end-joining driven DNA repair pathway. Collonnier and colleagues further demonstrated that, in the presence of a donor DNA template sharing sequence homology with the PpAPT gene, most transgene integration events in *P.patens* occur by homology-driven repair (HDR). At the same time, Cas9-induced DSBs are repaired with nearly equal frequency by mutagenic illegitimate recombination (Collonnier *et al.*, 2017).

At the same time, DNA-free editing in plants may be possible through the use of ribonucleoproteins (RNPs) to deliver preassembled Cas9-sgRNA into plant protoplasts (Woo *et al.*, 2015; Andersson *et al.*, 2018; González *et al.*, 2020). Protoplasts are uncovered plant cells generated using enzymatic degradation of the polysaccharide-rich cell walls. They can be transfected via electroporation or polyethylene glycol (PEG) treatment. The RNP's gene-editing complex can cleave target DNA sequence soon after delivery, bypassing the cellular transcription and translational machinery, and is then rapidly eliminated. Therefore, in comparison to CRISPR-Cas expression using DNA, off-target cleavage can be significantly reduced. Recently, examples of introducing LbCas12a- and AsCas12a-crRNA RNPs into soybean and tobacco protoplasts have been demonstrated (Kim *et al.*, 2017). In addition to the above-mentioned delivery techniques, viral vectors have been shown to be employed as short-term delivery vehicles for CRISPR-Cas components in plants (Platt *et al.*, 2014; Lau and Suh, 2017). As an advantage, due to the fast viral replication, CRISPR-Cas components accumulate to high levels in the host cell, potentially allowing for effective genome editing. Additionally, the transient viral expression approach is a great screening tool for estimating the efficiency and specificity of sgRNAs.

One of the potentially major constraints on successful GT is the delivery and availability of the repair template sequence at the correct time and in sufficient quantity for it to be incorporated. A number of reported examples employ an all-in-one strategy, the *in planta* GT (ipGT) system, that consists of a T-DNA construct that includes a Cas gene driven by a strong constitutive promoter, a sgRNA driven by the AtU6-26 promoter, and a donor DNA sequence (Fauser *et al.*, 2012; Schiml *et al.*, 2014; Wolter *et al.*, 2018; Miki *et al.*, 2018; Merker *et al.*, 2020; Steinert *et al.*, 2015) (Fig. 3A). Miki et al. have reported a sequential transformation strategy for *A.thaliana* HR-mediated GT. A design containing an HR donor and a single-guide RNA (sgRNA) can be transformed into parental lines that already carry the Cas9 nuclease driven under egg cell or early embryo-specific promoters. Using this system, precise knock-in of the GFP reporter in multiple genes as well as successful gene replacements (5.3–9.1%) were achieved (Miki *et al.*, 2018). Because the sequential technique requires plants that already express Cas9, it is limited in its applicability to diverse plant species. A further advancement in the all-in-one strategy was implemented by flanking the repair template with homology arms with target sites for the Cas9 nuclease (Hahn *et al.*, 2018). Such a strategy allows for the excision of the homology template from the T-DNA backbone. In barley, a successful and heritable GT using the all-in-one strategy was achieved where the codon-optimized SpCas9 was driven under a ubiquitous promoter. Lawrenson and colleagues used this strategy to create a targeted deletion of around 150 bp of the barley coding sequence while simultaneously fusing in-frame mCherry in its place. Starting from 14 original transgenic plants, two

plants appeared to have the required GT event. From one of these T0 plants, three independent GT events were identified, two of which were heritable (Lawrenson *et al.*, 2021). By developing a high-frequency HR-mediated intra-genomic selectable marker-free intra-genomic GT strategy in maize (High-IGHR), Barone and colleagues reported a significant improvement in the all-in-one strategy. The donor template from pre-integrated T-DNA was mobilized at the same time as specific DSBs were generated using Cas9 driven under the heat shock-inducible Hsp26 promoter. The construct was built in such a way that the release of the donor template, followed by DNA repair, activated the expression of the selectable marker gene inside the donor locus. The donor template, which is flanked by homology arms and Cas9 cut sites, was placed between the selection marker and the upstream promoter, rendering the selectable marker inactive in this arrangement. In T0 plants, this technique resulted in up to 4.7% targeted insertion of the donor sequence into the target locus, with up to 86% identified donor template release and a 99% mutation rate at the donor locus and genomic target location, respectively. This method enables the screening of non-chimeric heritable GT events in a single generation (Barone *et al.*, 2020). Although several papers claim effective GT in plants utilizing this simple all-in-one technique, further work is needed to improve its efficiency in plants.

Since the early days of genetic engineering, plant viruses have been used as heterologous gene expression vectors. Since then, a number of *A. tumefaciens*-based viral vectors have been designed for straightforward inoculation into plant cells. Recent research suggests that viral vectors might be used as transitory delivery vehicles for CRISPR-Cas components, including donor template sequences for HR repair (Fig. 3B). To date, one of the most popular viral delivery systems for CRISPR components has been based on geminivirus replicons due to their ability to both push the plant cells into an S-phase-like state, where HR repair takes place more frequently, and replicate to high copy numbers, thereby delivering a high copy number of the repair template to the target site. The coat and movement proteins, which contain part of the viral genome, can be substituted by the repair template and delivered to the plant in linear form on a T-DNA construct using *A. tumefaciens*. The viral REP proteins are produced once within the cell, leading to rolling circle replication and the generation of multiple copies of the repair template. The Cas gene can also be carried on the same T-DNA, allowing for simultaneous DSB at the target site and large-scale generation of the repair template. This method was successfully applied to tomato (Čermák *et al.*, 2015; Dahan-Meir *et al.*, 2018; Vu *et al.*, 2020), wheat (Gil-Humanes *et al.*, 2017) rice (Wang *et al.*, 2017), and potato (Butler *et al.*, 2016). However, the attempts with *A.thaliana* and barley were not successful (Hahn *et al.*, 2018; Lawrenson *et al.*, 2021). It is possible that the benefits of replicons for producing heritable GT are limited to specific plant species or target sequences in the genome. At the same time, the choice of crRNAs, the efficiency of promoters driving Cas genes and crRNAs might also influence GT efficiency in different species.

Screening methods for CRISPR/Cas-based gene targeting

For identifying successful GT events, conventional approaches like polymerase chain reaction or enzyme digestion followed by sequencing have been applied. These approaches, however, are not optimal for large-scale screening. The use of a visible marking can greatly speed up the screening of GT events and the selection of nonchimeric plants. In *A.thaliana*, visual screening for seeds that do emit the RFP, mCherry, GFP, and mTURQ2 signals under a fluorescent stereo microscope can be used to screen for successful transformation and gene editing events in the T1 generation, followed by fluorescence-free seed screening in the T2 generation for a quick isolation of the Cas9-free mutants (Tsutsui and Higashiyama, 2016; Durr *et al.*, 2018; Wu *et al.*, 2018; Aliaga-Franco *et al.*, 2019; Ursache *et al.*, 2021; Bieluszewski *et al.*, 2022). The red fluorescent DsRED protein expressed in the endosperm or embryos allowed the selection of seeds carrying the gene editing cassette in maize, rice (Tsutsui and Higashiyama, 2016; Aliaga-Franco *et al.*, 2019; Qi *et al.*, 2020; Yan *et al.*, 2021) Although this strategy has been extensively used for producing knockout mutants, its application in producing GT events has not been thoroughly investigated. In rice and *A.thaliana*, *RUBY* has been found to be an effective selection marker for transformation events. It is based on betalain, which gives a bright red color and is naturally found in species such as beets, dragon fruit, and Swiss chard (He *et al.*, 2020). An EGFP (enhanced green fluorescent protein) and RUBY-based system was successfully employed to screen transformed grape cells carrying CRISPR/xCas9, CRISPR/LbCpf1 and cytidine base editor-based genome editing cassettes (Ren *et al.*, 2022). Although *RUBY* has yet to be fully explored in gene editing and GT, it has the potential to be an efficient and very convenient reporter when combined in the same cassette with CRISPR/Cas components and the repair template, which is visible to the naked eye without the use of any special equipment.

Hahn and colleagues used restoring trichome formation in a glabrous *A.thaliana* mutant by repairing a defective *glabrous1* gene as a visual marker. *GLABROUS1* (*GL1*) is an R2R3-MYB transcriptional master regulator of trichome production that was previously also identified as a non-invasive, endogenous visual marker for Cas9 activity in *A.thaliana* (Hahn *et al.*, 2018). On the other hand, in *A.thaliana*, anthocyanin accumulation is regulated by the *PRODUCTION OF ANTHOCYANIN PIGMENTS 1* (*PAP1*) gene. Incorporating PAP1 into a CRISPR/Cas9 vector causes the leaves of transgenic plants to take on a characteristic purple color. This phenotype may be used to track the activity and presence of the T-DNA cassette in plants. A phenotypic marker like this might be used to identify genome-edited and transgene-free plants (Liu *et al.*, 2019). Lao and colleagues recently demonstrated that ectopic expression of *FLOWERING LOCUS T* (*FT*), a mobile flower-promoting signal, in conjunction with a Cas9 cassette can efficiently reduce the time investment for screening gene editing events by promoting T1 transformant flowering (Lao *et al.*, 2022). Even though this method hasn't been used to screen for GT events yet, it could be used with GT procedures to speed up the creation of stable, heritable, transgene-free GT events harboring plants.

Different strategies can be used in plants that can be transformed and regenerated by tissue culture. The most common method for getting transgene-free plants is to extract them by Mendelian segregation.

This method typically entails inserting CRISPR/Cas9 cassettes into DNA and screening transgenic plants based on antibiotic resistance. He and colleagues exploited OsC1 ectopic expression as a visual marker in rice to trigger anthocyanin production. They showed that expressing OsC1 under the control of the rice ACTIN promoter causes anthocyanin production in calli, seedlings, leaf vasculature, and grains, providing a visual marker for transgenic and transgene-free rice selection (He *et al.*, 2020). In soybean, Kumar and colleagues used the *Ochrobactrum haywardense* H1-8-mediated embryonic axis transformation method for the delivery of CRISPR-Cas9 components, including the GT repair template, to regenerate T0 soybean plants 6–8 weeks after transformation. Positive–negative selection (PNS) can serve as an approach for finding HR-mediated GT events. An example could be the use of the diphtheria toxin A-fragment, *NEOMYCIN PHOSPHOTRANSFERASEII* (*NPTII*) as a negative marker, which was used in the directed modification of several endogenous rice genes (Nishizawa-Yokoi *et al.*, 2012; Shimatani *et al.*, 2017). The programmed self-elimination strategy can be employed to reduce the cost and time of selecting progeny in favor of Cas9-free individuals. He et al. used suicide genes like *BARNASE* and *CMS* to enrich and isolate transgene-free rice. CMS is a male gametophyte-specific lethal protein, while BARNASE is a toxic nuclease. The *BARNASE* gene was driven by the early embryo specific REG2 promoter in the Cas9 expression system, and the *CMS* gene was driven by the 35S promoter, with the expectation that plants carrying the Cas9 gene and the two suicide genes would be eliminated in the same generation. Using this method, the authors were able to enrich and identify transgene-free individuals in the T1 generation (He *et al.*, 2018). A variety of different visual markers can be used. PDS (Phytoene desaturase) mutations in apples interfere with photosynthesis and result in bleached leaves. Yellow leaves can be observed when targeting a photosynthesis gene, *CAO* (*CHLOROPHYLLIDE-A OXYGENASE*), in rice and wheat. The absence of purple color in tomato hypocotyls can be induced by the inactivation of *DFR* (*DIHYDROFLAVONOL 4-REDUCTASE*), which is important in anthocyanin production (Danilo *et al.*, 2019). In *P.patens*, mutations in *APT* (*ADENINE PHOSPHORIBOSYLTRANSFERASE*) allow survival on 2-fluoroadenine media, providing another visual selective marker (Collonnier *et al.*, 2017). Targeting *ALS* (*ACETOLACTATE SYNTHASE*) providing resistance to the herbicide chlorsulfuron after can be employed to optimize HR-mediated gene editing (Svitashev *et al.*, 2015; Sun *et al.*, 2016; Oz *et al.*, 2021) Transgenes can be eliminated in asexually reproducing plants using nuclease-based editing, site-specific recombination methods, or transposition (He *et al.*, 2022). Haploid induction coupled with gene editing may be a possible solution for transforming species that are resistant to traditional approaches (He *et al.*, 2022).

Conclusions and perspectives

A lot of research is currently focused on increasing the effectiveness of GT in plants. The plants generated using GT are intended to have one copy number reflecting the target gene and to be in genomic areas that match the targets' natural positions. GT techniques could make use of the large

amounts of genomic data that are now freely available in databases and may be linked to the stability of the modifications applied at specific genomic regions. This would be ideal for enhancing crop agricultural characteristics, such as increasing the expression of a desired product or blocking a competing or undesirable pathway. Currently, as discussed in the review, a lot of research is focused on optimizing the potentially limiting steps determining the efficiency of CRISPR/Cas-mediated GT: a) spatial-temporal control of Cas protein activity; b) discovery and optimization of Cas proteins for the most efficient DSB formation; c) optimal repair template delivery; d) fast and efficient transformation and screening methods for GT events and transgene-free plants.

Base- and prime editing, which is not discussed in this review, is an alternative to HR-based precision genomic alteration and can also result in desired modifications with high accuracy. Unlike HR, base editors are not dependent on the production of DSBs and a repair template. A Cas9 nickase coupled with a reverse transcriptase unit is utilized in prime editing (Anzalone *et al.*, 2019). To circumvent the limitations of previous gene integration techniques, Yarnall and colleagues created the PASTE system in human cell lines by combining CRISPR-based primary editing with precise site-specific integrases. They achieved programmable integration of cargo sequences of up to 36 kb by employing Cas9, reverse transcriptase, and large serine integrases, with efficiencies of up to 50–60% in cell lines and 4-5% in human hepatocytes and T cells (Yarnall *et al.*, 2022). Although prime editing in plants has limitations in the size of targeted changes that can be induced, its combination with integrases still has to be explored. Another strategy for investigating plant gene function and crop enhancement is RNA editing. Cas13, the first-class II effector that operates entirely on RNA and is catalyzed by its two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains, is a new and very important addition to the CRISPR toolset. Furthermore, Cas13 proteins, like Cas12a, have the capacity to process pre-crRNA without the need of a tracrRNA. With the development of an effective and precise RNA targeting CRISPR system, a wide range of targeted RNA manipulations, such as site-specific RNA editing, are now possible. In mammalian cells, a deactivated Cas13b that binds to target RNA but lacks endonuclease activity has been combined with a particular adenosine deaminase or cytosine deaminase for editing of double-stranded RNA, targeted manipulation of the endogenous transcripts, and alterations in splicing and translation without making permanent changes at the genomic level (Cox *et al.*, 2017; Kavuri *et al.*, 2022).

Although all these strategies aim to improve the efficiency and overcome the limitations of the CRISPR/Cas system, there are some features that are difficult to solve. For instance, not all genes can be edited, as they need to harbor a PAM sequence. RNAi-related proteins, in particular prokaryotic ARGONAUTES proteins, are also pointed out as possible alternatives to CRISPR-effector complexes as genome-editing tools (Hegge *et al.*, 2018). Although pAgo-mediated DNA-guided DNA interference has not been tested in plants and additional information about the mechanism of action of the pathway and the characteristics of pAgos is needed in order to manipulate them biotechnologically, it represents a potential tool to generate mutants for crop improvement as an alternative or complement to the

CRISPR/Cas9 system. Another alternative strategy with a lot of potential is the development of transposon-based systems, such as piggyBac, originally isolated from an insect, which integrates into the host genome at TTAA elements and excises without leaving a footprint at the excised site. Recent advances in piggyBac transposase, an insect DNA transposase, imply that optimized versions of piggyBac might one day insert transgenes into plant genomes with high specificity (Endo *et al.*, 2021). In conclusion, new CRISPR/Cas systems as well as alternative GT systems are constantly being developed, implying that the toolbox for plant GT will extend further in the coming years, providing new approaches for precise genome editing in plants.

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IGB, NPA, NB and RU participated in the design and writing of the paper.

Conflict of interest

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Data availability

No data is associated with this manuscript.

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Tables

Table 1. Cas variants used for generating GT events in different plant species.

Cas variant	PAM	Plant species	Type of GT event
SpCas9	NGG	***Arabidopsis thaliana***	• 10 bp deletion repair in *glabrous1 (gl1)* (Hahn *et al.*, 2018) • Two amino acid substitutions in *PROTOPORPHYRINOGEN OXIDASE* (*PPO*) genes (de Pater *et al.*, 2018) • Insertion of a reporter gene (Shan *et al.*, 2018; Peng *et al.*, 2020) • Gene replacement (Zhao *et al.*, 2016)
		Glycine max	• Insertion of a resistance marker (Li *et al.*, 2015)
		Linum usitatisium	• Single nucleotide substitutions for herbicide resistance and to convert BFP to GFP (Sauer *et al.*, 2016)
		Lycopersicon esculentum	• Repair of 281-bp deletion (Dahan-Meir *et al.*, 2018) • Insertion of a promoter (Čermák *et al.*, 2015) • Amino acid exchange (Danilo *et al.*, 2019)
		Solanum tuberosom:	• Single nucleotide modifications for herbicide resistance (Butler *et al.*, 2016)
		Oryza sativa	• Insertion of two restriction enzyme target sites (Shan *et al.*, 2013) • Single nucleotide exchange for herbicide resistance (Ali *et al.*, 2020)
		Triticum aestivum	• Insertion of a reporter gene (Gil-Humanes *et al.*, 2017)
		Zea mays	• Insertion of a promoter (Shi *et al.*, 2017) • Single nucleotide mutation repair (using 800bp fragment for replacement) (Svitashev *et al.*, 2015) • Insertion of a resistance gene and multiple small GT events (Barone *et al.*, 2020)
		Physcomitrium patens	• Mutagenesis and reparation of an antibiotic resistance gene (Collonnier et al., 2017)
SpCas9 nickase	NGG	***Arabidopsis thaliana***	• Insertion of a resistance marker (Schiml *et al.*, 2014) • Point mutations in acetolactate synthase (ALS) gene (Wolter *et al.*, 2018)
SaCas9	NGRRT	***Arabidopsis thaliana***	• Point mutations in acetolactate synthase (ALS) gene (Wolter *et al.*, 2018)
		Nicotiana tabacum	• Multiple modifications in ALS gene (Huang *et al.*, 2021)
hSpCas9	NGG	***Arabidopsis thaliana***	• GFP insertion (Miki *et al.*, 2018)
		Zea mays	• Insertion of a resistance marker (Barone *et al.*, 2020)
LbCas12	TTTV	***Lycopersicon esculentum***	• Insertion of a promoter and a resistance marker (Vu *et al.*, 2020)

		Nicotiana benthamiana	• Nucleotide exchange to convert GFP to YFP (Eini *et al.*, 2022)
		Nicotiana tabacum	• Multiple modifications in ALS gene (Huang *et al.*, 2021)
		Oryza sativa	• Insertion of a resistance marker (Begemann *et al.*, 2017*b*) • Two amino acid exchange in *OsALS* gene (Li *et al.*, 2019, 2020)
ttLbCas12a	TTTV	***Arabidopsis thaliana***	• Amino acid exchange in *ALS* gene (Merker *et al.*, 2020; Schindele *et al.*, 2022)
		Nicotiana tabacum	• Multiple modifications in *ALS* gene (Huang *et al.*, 2021)
ttLbCas12a-I	TTTV	***Arabidopsis thaliana***	• Amino acid exchange in *ALS* gene (Schindele *et al.*, 2022)
FnCas12	NTTN	***Oryza sativa***	• Insertion of a resistance marker (Begemann *et al.*, 2017*b*)

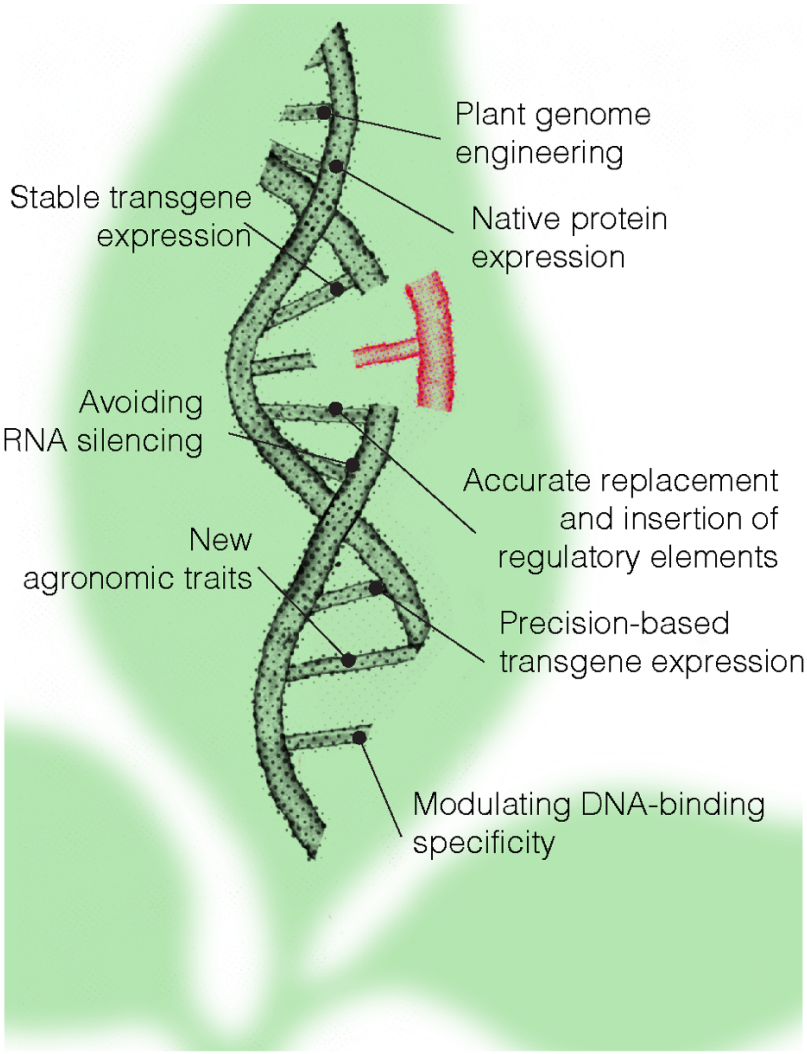
Figure legends

Fig. 1. Potential applications of plant gene targeting. The ability of efficiently inserting or replacing DNA fragment at a specific region in a plant's genome would greatly improve our way of studying gene functions, allow us to look at proteins under truly endogenous regulatory control and improve the current breeding strategies.

Fig. 2. The mechanisms of non-homologous end joining (NHEJ) and homology-directed repair (HDR). (A) The error-prone NHEJ-based DSB repair can take place via two competing sub-pathways: classical (cNHEJ) and alternative (aNHEJ). NHEJ is efficient but inaccurate, resulting in errors in the form of small insertions or deletions. (B) Homologous recombination (HR)-based strategy for DSB repair can occur via conservative and non-conservative pathways. The conservative HR does not lead to sequence loss. Synthesis-dependent strand annealing (SDSA) sub pathway of HR-based repair strategy is the primary mechanism behind conservative HR in plant somatic cells.

Fig. 3. Repair template delivery strategies for homology-directed DNA repair-based gene targeting. (A) Example of an all-in-one strategy that consists of a T-DNA construct carrying a Cas gene driven by a strong ubiquitous or germline-specific promoter, a sgRNA driven by the AtU6-26 promoter, a donor DNA repair sequence and selection marker for screening the GT events. (B) geminiviral replicon-based delivery of the repair template for HR-mediated GT. Once within the plant cell, the viral REP proteins are produced, leading to the rolling circle replication and the generation of multiple copies of the repair template. LB, left T-DNA border; RB, right T-DNA border; Term,

terminator sequence; HA, homology arm; LIR, long intergenic region; SIR, short intergenic region; Rep/RepA -replication proteins.



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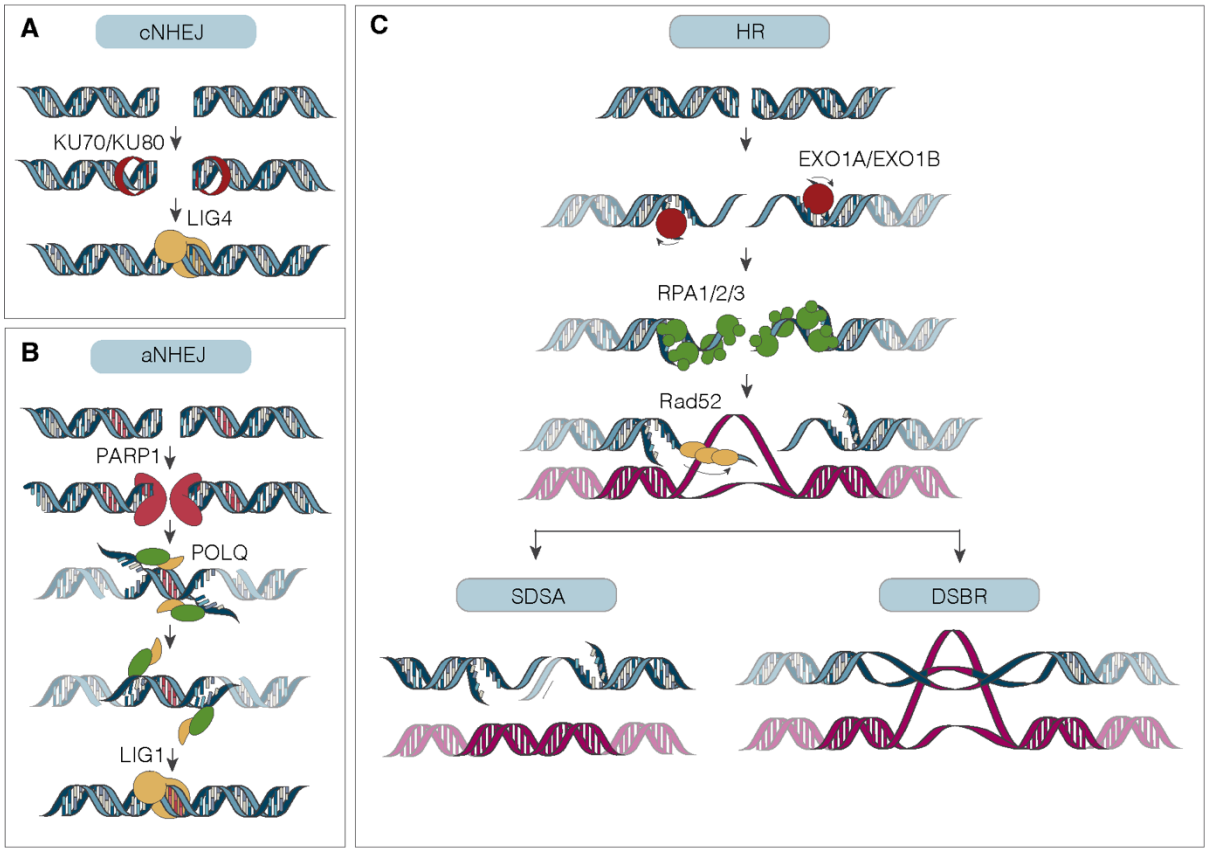

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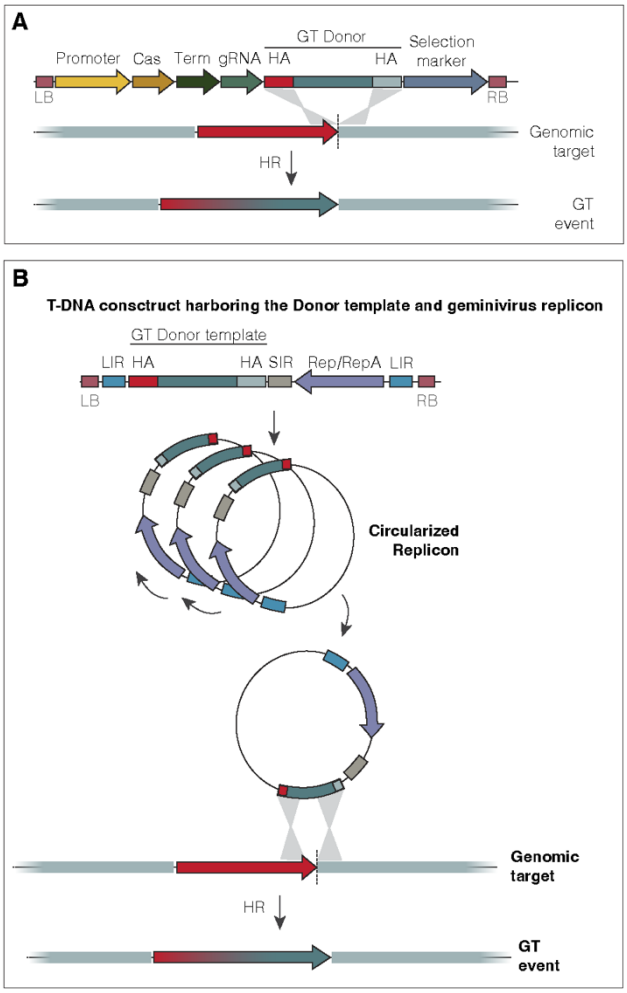

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