CHAPTER 3

Genome editing by CRISPR/Cas via homologous recombination

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Introduction

Throughout cell development, DNA can suffer damages that threaten genome integrity and cell survival. One of the most harmful lesions is the double-strand DNA break (DSB) because it may lead to loss of genomic information. DSBs may occur naturally during cellular metabolism or they may be triggered by external factors (Deriano; Roth, 2013). Either way, these damages are instantly repaired by the cell, mainly by two pathways: Non-Homologous End Joining (NHEJ) or Homology-Directed Repair (HDR). Unlike the repair via NHEJ, which simply joins the ends of the cleaved DNA (see Chapter 2), the HDR pathway requires the presence of an identical or very similar template, an intact sequence, to repair the lesioned DNA with accuracy (Heyer et al., 2010). The possibility to deliver a template to be used in HDR represents the key element for gene editing via the homologous recombination (HR) pathway, which may be exploited as one of the several new breeding techniques (NBTs).

In a natural system, the template used in the repair process via HR is the sister chromatid of the corresponding damaged region. This repair mechanism is less prone to error than NHEJ since it uses an identical unbroken DNA sequence as a template for the repair (Puchta, 2005). However, as long as a sequence is homologous to the regions that flank the DNA cleavage point, any sequence could be used as a template to resynthesize the DSB. Gene Targeting (GT) explores this characteristic using an exogenous sequence, instead of the sister chromatid, as a template, leading the cell to introduce the genomic modification of interest via HDR (Paszkowski et al., 1988; Voytas, 2013; Puchta; Fauser, 2013). Genome editing by HDR has been used to promote insertions into the genome and to exchange certain bases or regions in the target sequence (Huang; Puchta, 2019).

For this purpose, systems based on Zinc-Finger nucleases (ZFNs), Transcription Activator-like Effector nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) can induce DSBs. The DSB is created by an

exogenous nuclease, in the case of the CRISPR/Cas system by a Cas nuclease, which induces the cell to activate a DNA repair pathway, such as NHEJ or HR. The importance of DSB for gene-editing technologies was first demonstrated in maize (Athma; Peterson, 1991; Lowe et al., 1992), but at that time the authors did not even imagine its current application. Studying transposable elements, the group observed that DSB could activate the homologous recombination repair pathway. This study was later replicated with a tobacco endonuclease (I-SceI), in which the induction of DSBs increased the frequency of HDR in up to 100 folds (Puchta et., 1996). Thanks to these studies, now it is possible to intentionally explore the DNA repair mechanism via HDR, inducing double-strand DNA breaks by nucleases in a targeted way.

Despite the great potential of GT in biotechnology, its use is still limited due to the low frequency of HDR in somatic cells, around one event per 10,000 (Puchta, 2005). This low frequency can be attributed to the dominance of NHEJ-mediated DNA repair in somatic cells, as this mechanism takes place in all cell-cycle phases. In contrast, HDR occurs only in the S and G2 stages during interphase, when homologous DNA sequences (sister chromatids) are available to be used as templates for repair (Tamura et al., 2002). Thereby, new approaches have been developed to increase the frequency of HDR. Some techniques seek to increase the number of DNA templates using viral replicons, thus favoring GT (Baltes et al., 2014). On the other hand, other strategies aim to overexpress genes involved in HDR or to silence genes responsible for the NHEJ pathway, consequently increasing the frequency of genome editing by HDR (Shaked et al., 2005; Qi et al., 2013; Endo et al., 2016).

In this chapter, we will describe how to implement a gene-editing strategy via HR by CRISPR, covering from the general functioning of the mechanism, going through practical examples of genome editing and the steps to be considered before starting a project, until finally giving a practical direction on how to develop experiments to obtain an edited plant.

HDR mechanism

In eukaryotes, the occurrence of DSB activates the DNA repair system. Then, due to structural changes in the chromatin, a series of complexes are recruited to this region, culminating in cell cycle arrest (Kastan; Bartek, 2004). Depending on which phase the cell cycle stops, a different repair pathway will be prioritized. Certain proteins are vital in this process, and if the cell is in the S or G2 phases, HDR may occur (Vu et al., 2019). In animals, two protein complexes (KU70/80 and DNA-PK)

lead the cell cycle to halt in the S/G2 phases. However, in plant cells, only the KU complex is present, which may contribute to the low frequency of this type of repair in plants (West et al., 2002).

After the cell cycle has stopped, as soon as all the ideal conditions for HDR are established, including the presence of a DNA template, different HDR mechanisms may be triggered in eukaryotic cells, such as gene conversion or synthesis-dependent strand annealing (SDSA), single-stranded annealing (SSA), or crossing-over/non-crossing-over via double Holliday junction (dHj). However, only homologous recombination via SDSA – the main pathway of HDR in somatic plant cells – can generate products with precise sequences, which is the main goal of GT (Szostak et al., 1983; Puchta et al., 1996).

The HDR mechanism can be conceptually divided into three phases: pre-synapse, synapse, and post-synapse (Heyer et al., 2010). During the pre-synapse, a heterotrimeric complex (MRN; reviewed in Manova; Gruska, 2015) bounds to the free ends of the DNA (Figure 1A). Then, the cleaved double-strand ends undergo resection by nucleases, which digest the DNA in the 5'-3' direction, leaving the 3' ends in the single-stranded sequences (ssDNA). The damaged DNA is then restructured to form the ssDNA-RPA complex, by attaching the replication protein A (RPA) to the ssDNA (Figure 1B). Next, the ssDNA-RPA complex recruits the RAD51 protein, forming nucleoprotein filaments or presynaptic filaments (Figure 1C). In the synapse phase, these RAD51 filaments search for the homologous sequence (sister chromatid or exogenous donor template) and, after finding it, the ssDNA-RPA complex invades the template strand, assembling on it and forming a displacement loop (D-loop) (Figure 1D). Finally, in the post-synaptic phase, the broken DNA is resynthesized in a template-dependent manner (Figure 1E) (Puchta, 1998; Puchta, 2005; Heyer et al., 2010; Renkawitz et al., 2014). After the formation of the D-loop, the repair by SDSA occurs: the 3' end of the ssDNA is used as a primer, initiating DNA polymerization and the replication of the sequence present in the target-strand template (Puchta, 1998).

Although SDSA repair represents the main HDR route, DNA from somatic cells can also be repaired via SSA. When the resection process (a key step that generates single-stranded DNA) is extended, RAD52 recombinase decouples RPA, directing the repair system to the SSA pathway (Renkawitz et al., 2014). The CRISPR gene-editing strategies via HR described in this chapter consider DNA repair by the SDSA mechanism since it is the only one that will accurately edit the region of interest, as previously mentioned (Puchta, 2005).

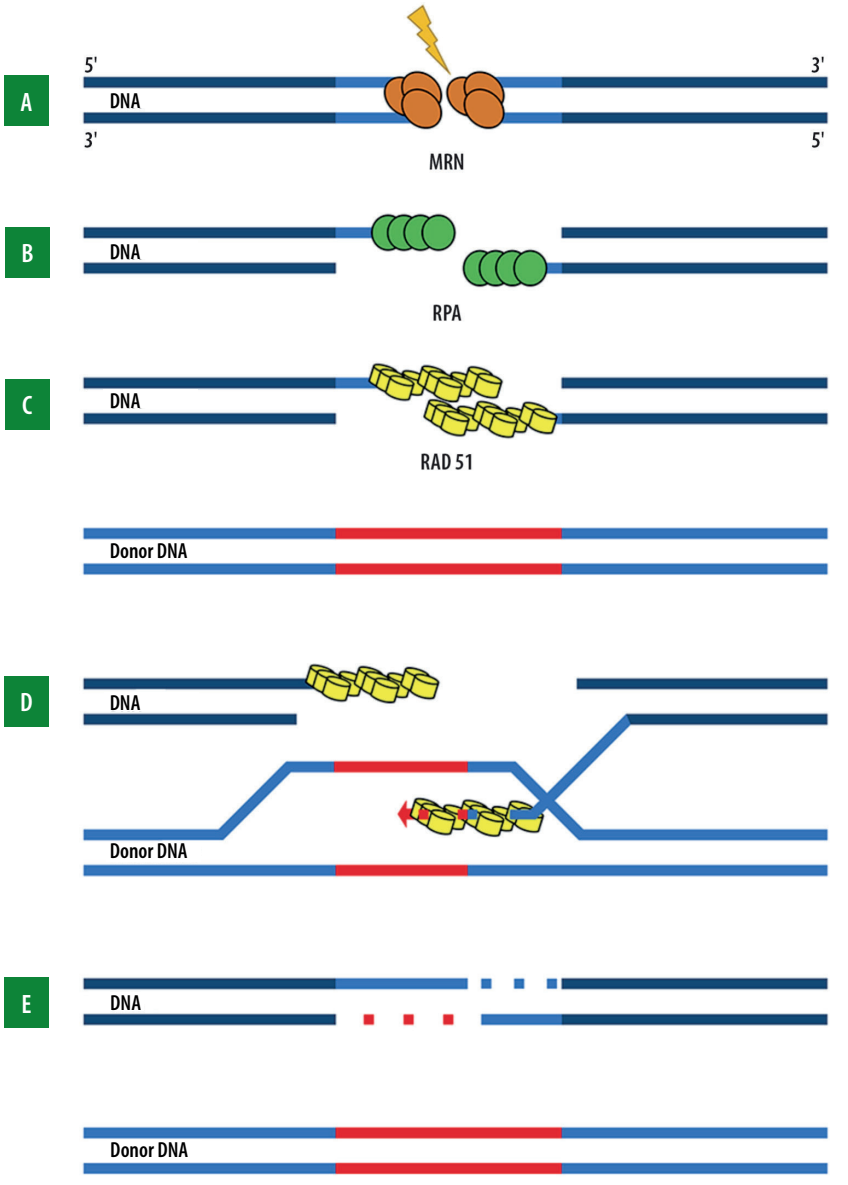

Figure 1. Repair mechanisms via HR by SDSA. A) HDR repair begins with a heterotrimeric complex (MRN) attaching to the damaged double-stranded DNA. B) Replication proteins (RPA) bind to the DNA after its resection and minimize the formation of secondary structures to facilitate the coupling of the RAD51 recombinase. C) RAD51 forms the presynaptic filament in the ssDNA, which, in the presence of a donor template, searches for homology. D) The filaments identify the homologous sequence, forming the D-loop, and DNA synthesis begins from the 3' end of the template strand. E) DNA polymerization occurs in the 5'-3' direction, and the 3' end of the template strand acts as an initiator (primer) of this process; after the repair, the ends of the DNA are joined, and the strand is repaired without loss of genetic information.

Source: adapted from Renkawitz et al. (2014).

Practical applications and examples of GT

GT has been recently employed in genome editing and it has the ability to promote highly precise and specific changes in the target genome – improving this technique may revolutionize the field of plant breeding. Understanding the repair mechanism via SDSA is key to further develop technologies that aim to increase the efficiency of GT in eukaryotes. In this topic, we will describe examples of studies that demonstrate the application of GT in species of agronomic interest, such as rice (Butt et al., 2017; Wang et al., 2017; Li et al., 2019), maize (Shi et al., 2017), and tomatoes (Vu et al., 2019).

Precise genome editing with CRISPR by HR can be used to achieve several objectives, such as promoting deletions, insertions, and exchange of small sequences or even whole genes (Gil-Humanes et al., 2017; Shi et al., 2017; Wang et al., 2017; Au et al., 2019; Huang et al., 2019; Li et al., 2019; Vu et al., 2020; Weisheit et al., 2020). Aiming to validate the strategy for inserting coding sequences (CDS), Wang et al. (2017) introduced the *green fluorescent protein* (*GFP*) gene in rice cells. For this, the CRISPR/Cas9 genome editing system was associated with the strategy of delivering the donor DNA in a geminivirus-based vector generated from the wheat dwarf virus (WDV). Two loci were selected as insertion targets, *actin 1* (*ACT1*) and *glutathione S-transferase* (*GST*). Thus, the *GFP* CDS was inserted into the target genes forming the proteins ACT1-GFP and GST-GFP. The transgenic plants, called WDV2-ACT1-SG1 and WDV2-GST-SG2, incorporated the expression cassette with 19.4% and 7.7% of efficiency, respectively.

Also carrying out gene editing via CRISPR by HR on rice, Butt et al. (2017) and Li et al. (2019) used RNA as donor templates. In both works, rice plants tolerant to acetolactate synthase (ALS)-inhibiting herbicides were successfully generated. In the first study, the guide RNA (gRNA) and the donor RNA sequences were added together. In the second study, the authors tested two strategies, in one they added ribozymes flanking the repair template and in the other, they explored the exonuclease activity of Cas12a. Both works were able to successfully use RNA as a template strand for GT.

The CRISPR genome editing strategy via HR was also used to generate drought-tolerant maize genotypes both by inserting and replacing the promoter region of the *auxin-regulated gene involved in organ size 8* (*ARGOS8*) gene since its constitutive expression increases grain yield in maize under water deficit (Shi et al., 2015). The authors used the CRISPR/Cas tool to generate new variants of the *ARGOS8* promoter, increasing the transcriptional activity of the gene (Shi et al., 2017). Two strategies were used, in the first, the constitutive *GOS2* promoter of maize was inserted in

the 5'-UTR of the *ARGOS8* gene with 3% efficiency, and, in the second, the *GOS2* promoter was used to entirely replace the native promoter of *ARGOS8*, with 1% efficiency. The *ARGOS8* events had greater grain yield under drought. These results have already been accomplished with transgenic plants overexpressing *ARGOS8* (Shi et al., 2015). But Shi et al. (2017) also showed how the genome-editing tool via CRISPR can be used to create genetic variability to develop drought-tolerant cultivars.

The generation of gene variability for stress tolerance via genome editing was also achieved by Vu et al. (2020) in tomato plants. The authors demonstrated the applicability of CRISPR genome editing via HR using a bean yellow dwarf virus (BeYDV)-based replicon, to obtain tomato plants tolerant to osmotic stress. For this, *the high-affinity potassium (K+) transporter 1;2* (*HKT1;2*), which plays an important role in maintaining K^+ uptake under salt stress has been chosen as a target. Thus, a mutation (N217D) was induced in the tomato *HKT1;2* gene, using a selection marker-free construction. The edited plants inherited the mutation stably and were salt-tolerant in the presence of 100 mM NaCl during germination. Despite the low mutation efficiency obtained (0.66%), this strategy proved to be promising when using a selection marker free-system in genome editing in tomatoes (Vu et al., 2020).

The examples mentioned above represent some of the many applications of GT in plants. In the next topics, we will discuss the advantages and limitations of the technique and the main strategies that have been used to optimize them.

Factors that affect HR: what to take into account before starting?

Genome-editing via HDR has some limitations that should be considered. The greatest hindrance is the low efficiency of HDR, mainly owing to two factors: the competition with NHEJ and the availability of the donor template. Thankfully, some strategies can be applied to increase the success of GT. For example, the nuclease and the promoter used in the process may influence the efficiency of the technique. Moreover, an appropriate design of the gRNA and efficient strategy to deliver the donor template are other aspects that deserve special attention (Ran et al., 2013; Yang et al., 2013; Baltes et al., 2014; Wolter; Puchta, 2019; Vu et al., 2020).

Types of nucleases and recommendations for use in edition by HR

Two nucleases are commonly used for genome editing via CRISPR, Cas9, and Cas12a (previously called Cpf1). Cas9 has been chosen in most studies since it was the first RNA-guided enzyme to be described as a tool in CRISPR/Cas systems (Ran et al., 2013). This nuclease has already been used to generate plants edited by GT in several species such as *Arabidopsis thaliana* (Hahn et al., 2018), tomato (Dahan-Meir et al., 2018; Čermák et al., 2015), rice (Butt et al., 2017), and maize (Shi et al., 2017). However, considering HR editing, the nuclease Cas12a presents some advantages over Cas9. First of all, Cas12a has a staggered cut while Cas9 cuts the DNA abruptly (Huang; Puchta 2019; Zetsche et al., 2015; Swarts; Jinek, 2018). That is a very advantageous characteristic, taking into account that the formation of single-stranded DNA with cohesive ends favors the repair via HR over NHEJ. For HDR to occur, one of the 3' ends of the damaged DNA strand must invade the donor template (Zetsche et al., 2015). For this, the 3' end of the single strand must be long enough to anneal with the homologous sequence. After annealing, the single-stranded end is used to initiate DNA polymerization and, consequently, double-strand repair (Puchta, 1998; Huang; Puchta, 2019).

Another factor that makes Cas12a more suitable to GT, compared with Cas9, is the distal cleavage site. Cas12a cuts far from the PAM region, about 23 nucleotides distant on the non-target strand and 18 nucleotides away on the target strand, leaving the PAM and seed sequence unaffected by mutagenesis (Zetsche et al., 2015). Thereby, PAM is maintained until repair occurs using the donor template (Huang; Puchta, 2019). On the other hand, Cas9 creates a DSB of only 3-4 nucleotides upstream of the PAM, which can lead to indels (insertions and/or deletions) in the seed region, making the target site unrecognizable after editing by NHEJ. Considering that NHEJ is more likely to occur than HR, it would prevent further editing at this point by HDR. (Swarts; Jinek, 2018).

The third advantage of using Cas12a lies in its small gRNA. The mature gRNA from Cas12a has about 43 nucleotides. The 19 nucleotides at the 5' end compose the highly conserved directed repeated segment and the remaining ~ 24 make up the spacer-derived segment, the variable sequence that drives the nuclease cut. Additionally, Cas12a does not require transactivating crRNA (tracrRNA) and gRNA processing, which are essential for the activity of Cas9 (Zetsche et al., 2015; Swarts; Jinek, 2018).

Despite all the advantages of using Cas12a for editing via HDR, this enzyme has thermal limitations, being more sensitive to temperature changes than Cas9 (Schindele; Puchta, 2019). Among the 16 families of Cas12a, the three most

used for genome editing are those derived from *Francisella novicida* (FnCas12a), *Lachnospiraceae bacterium* ND2006 (Lb/Cas12a), and *Acidaminococcus* sp. BV3L6 (AsCas12a), whose sensitivity to temperature changes is variable; among them, AsCas12a is the most sensitive (Moreno-Mateos et al., 2017; Malzahn et al., 2019; Schindele; Puchta, 2019). For instance, Moreno-Mateos et al. (2017) observed that the activity of AsCas12a in zebrafish (*Danio rerio*) at 28°C was null and increased proportionally until the optimum temperature of 37°C. For this reason, this high temperature-dependent activity has limited the use of AsCas12a in plant editing since plants are usually cultivated under 20°C - 25°C (Malzahn et al., 2019).

Nevertheless, the FnCas12a and LbCas12a nucleases are less sensitive to temperature variations and have been widely used in plant genome editing, especially LbCas12a due to its greater editing efficiency (Endo et al., 2016; Malzahn et al., 2019; Schindele; Puchta, 2019). Malzahn et al. (2019) tested AsCas12a, FnCas12a, and LbCas12a under several temperatures in different plant species. In rice, all nucleases tested showed higher activity with increases in temperature until optimal function at 28°C. However, temperatures above 32°C reduced their activity. For this reason, Cas12a is not usually used for Arabidopsis transformation since this species is cultivated under ~22°C (Malzahn et al., 2019). These observations point out why the Cas9 nuclease is frequently used for GT in Arabidopsis (Malzahn et al., 2019).

Although the optimal temperature for Cas12a activity is a limitation for some species, in other cases, moderate heat stress may increase the efficiency of homologous recombination. Bokyo et al. (2005) demonstrated that subjecting plants to suboptimal temperature for a short period induces intrachromosomal recombination (Boyko et al., 2005). Aiming to increase GT efficiency in tomato, Vu et al. (2020) tested the effect of temperature on HDR in tomatoes using LbCas12a. The authors applied moderate heat stress of 31°C for 10 days and were able to increase the efficiency of HR without hindering plant regeneration.

How to enhance the frequency of HDR

Gene editing by HDR has two main conditioning factors that must be considered: 1) DSB occurs only during the S/G2 phases of the cell cycle; 2) the availability of the donor template during HDR (Puchta et al., 1996; Baltes et al., 2014; Vu et al., 2020). These points should be considered in order to delineate strategies to improve the efficiency of genome editing via HR.

To benefit the HDR route, some strategies blocked the activity of key enzymes for the NHEJ repair mechanism. In *A. thaliana,* mutants with silenced *ku70* and *lig4* – proteins involved in NHEJ repair – showed an HDR efficiency 10-fold higher (Qi et al., 2013). Similarly, in rice, *lig4* CRISPR-mutants showed an increase in the efficiency of HR repair compared with wild-type plants (Endo et al., 2016). These studies show that manipulating enzymes involved in the repair pathway may be an alternative to increase GT efficiency. The silencing of *FAS1* and *FAS2* genes, responsible for chromatin folding, has also been employed and led to an HR rate of up to 40 folds in somatic cells of *A. thaliana* mutants (Endo et al., 2006).

However, it is worth noting that not all alternatives to increase genome editing efficiency involve complex transformation steps, some of them have already been described. Therefore, one of the great limitations to apply GT is related to the efficiency of the donor template delivery to be used during the lesion repair. Currently, some alternatives can be used to increase the number of molecules available at the time of HR, enhancing editing efficiency.

Template strand delivery strategies

Even though the most common donor template used for genome editing by CRISPR via HR is dsDNA, different types of templates can be used, such as ssDNA, dsDNA (Baltes et al., 2014; Shi et al., 2017), and even RNA (Butt et al., 2017; Li et al., 2019). In the case of ssDNA-type donor template, single-stranded oligos are recommended for editions smaller than 50 bp (Ran et al., 2013). ssDNA has not been widely applied for genome editing of plants, being frequently applied in animal cells (Bai et al., 2020). On the other hand, the use of dsDNA was successfully carried out to edit plant genomes, mainly in the form of a plasmid (Shi et al., 2017; Hahn et al., 2018; Hayut et al., 2017; Schiml et al., 2014; Svitashev et al., 2015).

The components of the editing system can be delivered to the plant, either via Agrobacterium or particle bombardment, as a transformation vector to be integrated into the genome, or by the ribonucleoprotein particle technique (RNP; see Chapter 2). The dsDNA template can be delivered as a plasmid and it may or may not be in the same plasmid with the nuclease and gRNA (Shi et al., 2017; Hahn et al., 2018). Using the particle bombardment, Shi et al. (2017) successfully changed the promoter of the ARGOS8 gene in maize, and Hahn et al. (2018) reestablished the formation of trichomes in a glabrous Arabidopsis mutant by repairing the defective *glabrous1* gene.

Another strategy using dsDNA, proposed by Baltes et al. (2014), has gained prominence for genome editing by HR repair. The authors developed a vector based on geminivirus replicon (GVR) to deliver the donor template. The GVR-based vectors produce a high copy number of replicons through rolling-circle replication, significantly increasing the amount of donor template available during DNA repair, favoring the HDR pathway. This strategy is based on the viral infection mechanism of a member of the *Geminiviridae* family. This virus consists of an ssDNA, with a genome of approximately 2.8 Kb containing ORFs (Open Reading Frames) encoding proteins that control the virus replication, movement, and coating (Zaidi; Mansoor, 2017).

In order to use the geminivirus replicon as a biotechnology system, the sequences encoding the viral coat protein (CP) and the movement proteins (MP) are removed, allowing the insertion of the sequence of interest into the GVR-based vector. Once inside the host cells, the geminivirus replicon (ssDNA) is converted to double-strand (dsDNA) by intracellular polymerases, and this double-stranded genome commands the transcription and translation of proteins responsible for the formation of new GVRs. Two replication proteins (Rep and RepA), encoded by the same genomic sequence, are required to generate GVR. The Rep/RepA proteins identify two repeated regions of the viral dsDNA, called LIR (Large Intergenic Region), which flank the sequences that compose the replicon. Also, the formation of the circular viral replicon requires the presence of the SIR (Short Intergenic Region) sequence, located between the LIR (Baltes et al., 2014). After the formation of the circular replicon, the Rep/RepA proteins amplify the replicon copies. Reports show that the level of expression of exogenous proteins in plants can be increased by up to 80 folds using a GVR-based vector compared with the use of conventional plasmids (Zhang; Mason, 2006; Baltes et al., 2014). The mechanism of action of GVR is outlined in Figure 2.

In addition to providing a high number of copies of donor DNA, the use of GVR-based vector in genome editing has other advantages (Baltes et al., 2014; Zaidi; Mansoor, 2017):

1) Geminiviruses are capable of infecting a broad variety of plant species, making them potential vectors for editing a wide range of plants.
2) They only need Rep/RepA proteins to initiate replication within host cells, and this replication can be driven by their native geminivirus promoter or an engineered promoter of interest.

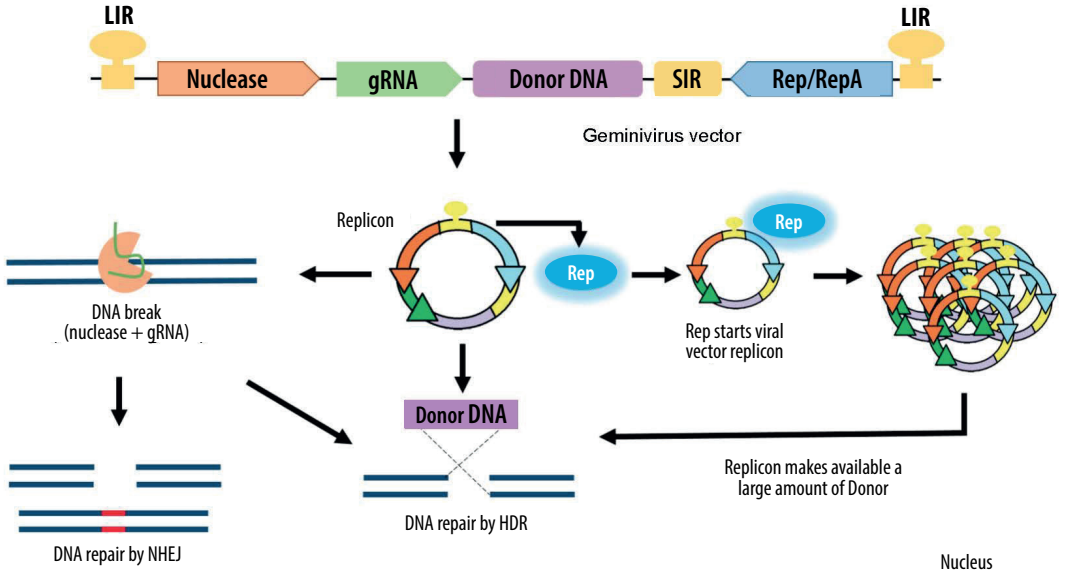

Figure 2. Mechanism of action of the geminivirus vector for genome editing in plants via CRISPR. Once in the cell nucleus, and after the translation of the Rep/RepA proteins, the entire sequence between the two LIRs will give rise to the geminivirus replicon through the circularization of DNA. The circular replicon is then amplified by the Rep/RepA proteins, making available a large number of copies of its components, in this case, nuclease, gRNA, and donor DNA. Upon DBS induction by the nuclease + gRNA complex, DNA may be repaired via NHEJ or HDR, using the donor DNA as a template. LIR - Large Intergenic Region; SIR - Short Intergenic Region; Rep/RepA - replication proteins.

Source: adapted from Zaidi and Mansoor (2017).

3) The viral replicon multiplies within the cell via HR-dependent replication, inducing the cells to pass from the resting state to the S and G2 phases of the cell cycle.

4) The number of copies of the viral replicon is hugely increased, allowing a large amount of donor DNA to be available at the time of HDR, which may increase the efficiency of HR editing.

Several works have been carried out using GVR-based vectors in diverse species. In dicots, it is common to use vectors based on the bean yellow dwarf virus (BeYDV) (Čermák et al., 2015; Butler et al., 2016; Dahan-Meir et al., 2018; Vu et al., 2020). In monocots, vectors based on the sequence of the wheat dwarf virus (WDV) are broadly used (Wang et al., 2017; Vu et al., 2019). Čermák et al. (2015) engineered a vector derived from the BeYDV replicon to obtain tomato plants capable of accumulating a large amount of anthocyanin pigment, obtaining HR efficiencies 10 folds greater than those obtained using conventional T-DNA to deliver the

components necessary for editing. Similarly, the use of targets that allow visual selection of edited plants has been applied, eliminating the need for selective agents in the plant regeneration process. For example, Dahan-Meir et al. (2018) sought to replace the *carotenoid isomerase* (CRTISO) gene, making the fruits of the edited plants orange. Whereas Vu et al., (2020) generated tomato plants with a purple color through editing aimed at the accumulation of anthocyanins.

To optimize the GVR-based systems in genome editing via CRISPR, strategies to develop smaller replicons have also been adopted. The smaller the size of the replicon, the greater the efficiency of the Rep/RepA proteins in amplifying them (Vu et al., 2020). Some approaches use the viral replicon to produce all the components necessary for editing – nuclease, sgRNA, and donor template – thus avoiding insertion into the genome (Čermák et al., 2015). Although it represents an efficient strategy, it generates a large replicon. Thus, to further increase the availability of donor template in the intracellular environment, it is possible to use GVR to carry only the donor (Dahan-Meir et al., 2018), or even to use multiple replicons to deliver all components necessary for editing without loss of efficiency due to the size of the formed GVR (Vu et al., 2020).

Although the delivery of donor DNA via GVR has shown encouraging results in several species, the success of this technique depends on the susceptibility of the target species to geminivirus. In *A. thaliana*, for example, Hahn et al. (2018) compared the efficiency of HR when the donor DNA is delivered via GVR-based plasmids or via binary plasmids. The results clearly showed that the use of viral replicons does not promote HR editing in *A. thaliana*. In this case, it is advisable to use other methods of donor template delivery, such as conventional plasmids used in plant transformation (Hahn et al., 2018).

Step by step – A GT strategy pipeline

Editing the genome of a plant using the CRISPR tool by HR involves a series of steps, from the selection of the genomic target to the selection of edited plants (Figure 3). The first step is to define the target species, as well as to outline the type of edition, and the genomic target. Once defined, you can proceed to the design of the gRNA and the donor template. To verify the efficiency of the designed gRNAs, it is possible to perform an optional *in vitro* validation step to select the best gRNAs. In parallel, the vector is synthesized in the case of plasmid-based strategies, or the gRNAs and Cas in the case of transgene-free systems (for more details, see Chapter 2). After acquiring these components, it is time to deliver the CRISPR/Cas system by the desired transformation method. Finally, after obtaining the transformed

plants, the last step is to select the plants that contain an edition of interest, which is commonly screened by sequencing.

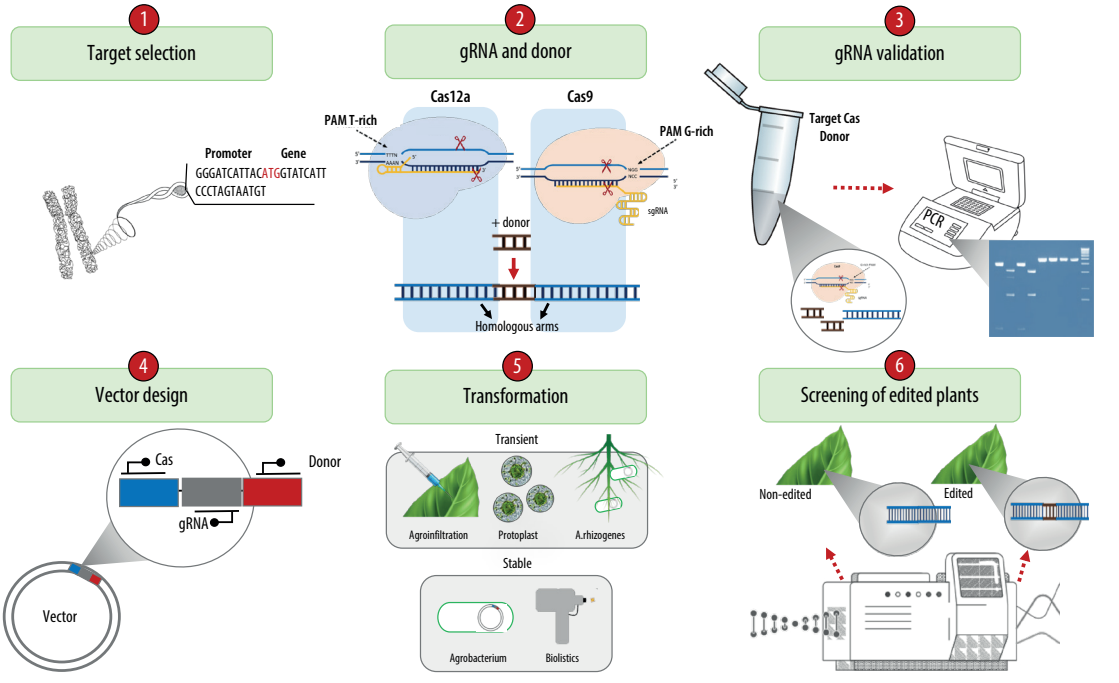

Figure 3. Pipeline of a gene-targeting strategy by CRISPR.

Source: adapted from Shan et al. (2020).

1° Step – Defining the target for editing: gene and species selection

The first step to carry out a genome editing project with the CRISPR tool via HDR involves defining the species to be studied. Characteristics such as ploidy and life cycle duration (annual or perennial) could affect the efficiency of gene editing in plants and, therefore, should be considered at this stage (Shan et al., 2020). Once the target species has been defined, its susceptibility to genetic transformation should be taken into account to decide whether a strategy based on transgene-free (such as RNP) or on stable transformation via plasmid will be used.

Bearing in mind the edition type you want to obtain (insertion, deletion, or replacement) and the target, which could be a promoter region or a CDS, attention

should be given to each case specifically. When designing a strategy for editing the promoter region via HDR, the first checkpoint is to verify if the target region overlaps with other adjacent regions (Kumar, 2009). The promoter sequence of one gene may overlap with the CDS of an adjacent gene or with an alternative transcript. The region considered for edition should be very well characterized and unique in order to only alter the target sequence and not changing others in the process.

In Figure 4, we show an example of how to observe these characteristics. In the example, it is possible to visualize the promoter region of a soybean gene. Representing three alternative transcripts each with a different promoter region. Light blue represents the region that should be considered for editing, in case the aim is to edit the region without overlap. In addition, attention should be paid to preserved motifs in promoter regions since they are crucial for gene regulation and should not be altered or disrupted. For this, software such as PLACE (Higo et al., 1999) and PlantCARE (Lescot et al., 2002) can be used to identify these regulatory motifs to avoid them as targets.

CRISPR editing via HDR may present different aims, such as inserting nucleotide sequences and even entire genes (Wang et al., 2017), replacing sequences, or altering specific nucleotides. This last approach was performed to obtain rice plants resistant to ALS-inhibiting herbicides (Butt et al., 2017). In all cases, the degree of chromatin folding in the chosen region should be verified, since the editing of genes located in heterochromatin is hampered since the nuclease has less access to create the DSB (Dillon; Festenstein, 2002; Noman et al., 2016).

2° Step – gRNA and donor template design

The gRNA and donor template can be designed using specific software or even manually. Most of the parameters used to design a gRNA for gene editing via NHEJ apply to HR (see Chapter 2). However, beyond these default parameters, in the case of GT, the distance between the gRNA-induced DSB and the target location must also be considered. Due to the limitations of performing GT in plants, gRNA and donor template design are based on parameters established in other eukaryotic species, such as mammals.

Studies show that even though DSBs between 30 and 100 nucleotides away from the target point allow HR to occur, DSBs 10 to 30 nucleotides from the target optimizes editing efficiency (Yang et al., 2013). Therefore, the greater the distance between the DSB and the target region, the lower the GT efficiency (Ran et al., 2013; Yang et al., 2013; Singh et al., 2015). In HDR via SDSA, the damaged DNA undergoes

>Clyma. 15G061400 A

GTATACATTTTCTAATCTTTATTTTCTATTTAATCCATGATTAAACCTTCCATAGTAATTAAACGACTCTAAATATAGGATAAATTTCCTTAATTTAGACTTGGAATGTGTCATTTTTTAATTATTACTTTGAAATT
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>Glyma.15G061400.3 D

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Figure 4. The promoter region of a soybean gene and three alternative transCcripts. (A) 1,000 bp sequence of the promoter region obtained by the RSATplant software. 1,000 bp sequence of the promoter region of the primary (B), secondary (C), and tertiary (D) transcript, retrieved from the Phytozome database.

Caption: Dark blue: promoter region without overlaps in adjacent genes. Light blue: promoter region without overlapping with alternative transcripts of the same gene. Black: Promoter region overlapping with 5′ UTR of alternative transcripts. Green: 5′ UTR sequence.
Source: adapted from Contreras-Moreira et al. (2016).

5' end resection, forming a 3' single strand that pairs with the donor template, acting as a primer to initiate DNA polymerization, as previously mentioned. Thereby, only one of the template strands will be able to induce the edition as planned, allowing the polymerization of the DNA containing the mutated PAM and the desired edition sequence (Huang; Puchta, 2019).

DNA polymerization only occurs in the 5'– 3' direction, for this reason, extra care should be taken when designing the ssDNA or RNA template to ensure that the new sequence will be incorporated correctly during the repair. When the donor template is similar to the antisense strand, the target must be positioned upstream of the DSB, only then, after the invasion by the 3' end of the DNA, the edition will be incorporated (Figure 5C) (Huang; Puchta, 2019). Whereas when the donor is a dsDNA, this is not an issue, as both strands can be used as a template during the repair (Figures 5A and 5B) (Huang; Puchta, 2019).

Another point to consider is that gRNA efficiencies to guide the nuclease to create DSB vary depending on the target region and the nuclease used, directly affecting GT efficiency. This could be mitigated by previously testing several gRNAs for the same target using transient transformation, proceeding only with the best-validated guide(s) to generate plants (Zhang et al., 2019). Table 1 describes some software available to design gRNAs and donor templates and shows the genomes available in the software database to check possible off-targets.

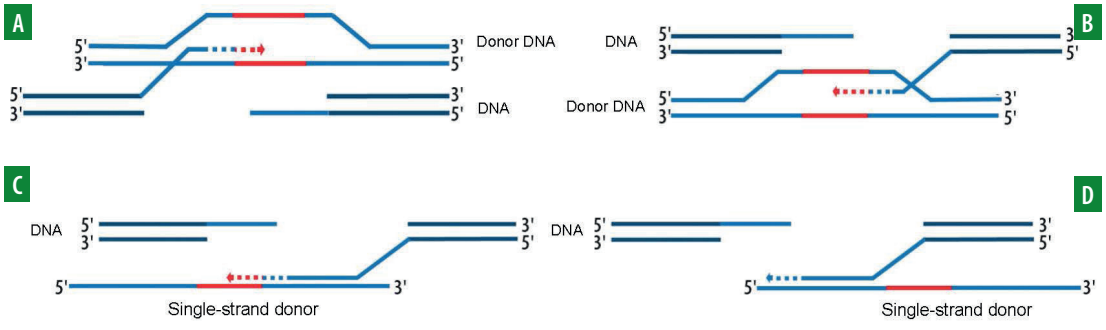

Figure 5. Difference between the use of double-stranded and single-stranded donors regarding the positioning of the edited sequence incorporated by the SDSA repair mechanism. A-B) Using a double-stranded donor, regardless of the location, the mutation will be incorporated. C) Incorporation of the edition using a donor template homologous to the DNA antisense strand placed upstream of the cut site. D) No edition is incorporated, only repair via HDR occurs, because the desired mutation is located downstream of the DSB.

Source: adapted from Huang and Puchta (2019).

Table 1. Web-based software for gRNA and donor strand design, highlighting for which prominent species genomic databases are available for reference, as well as the possibility of designing guides for Cas9 and/or Cas12a.

Software	*A. thaliana*	Soybean	Maize	Sorghum	Sugarcane	Wheat	Rice	Common bean	Cas9	Cas12a	*Donor template*	Link	Reference
ATUM	•	x	x	x	x	x	x	x	•	x	x	https://www.atum.bio/eCommerce/cas9/input	ATUM, CA
Benchling	•	•	•	•	x	x	x	x	•	•	•	https://www.benchling.com	Benchling, CA
Breaking-Cas	•	•	•	•	•	•	•	•	•	•	x	https://bioinfogp.cnb.csic.es/tools/breakingcas	Oliveros et al. (2016)
CC-TOP	•	•	x	x	•	x	•	x	•	•	x	https://crispr.cos.uni-heidelberg.de	Stemmer et al. (2015)
CHOPCHOP	•	•	•	•	•	•	•	•	•	•	x	https://chopchop.cbu.uib.no	Labun et al. (2019)
CRISPOR	•	•	•	•	•	•	•	•	•	•	x	http://crispor.tefor.net	Concordet e Haeussler (2018)
CRISPRdirect	•	•	•	•	x	•	•	x	•	•	x	https://crispr.dbcls.jp	Naito et al. (2015)
Crispr-GE	•	•	•	•	x	x	•	x	•	•	x	http://skl.scau.edu.cn	Xie et al. (2017)
CRISPR-P	•	•	•	•	x	•	•	x	•	•	x	http://crispr.hzau.edu.cn/CRISPR2	Liu et al. (2017)
E-CRISP	•	x	•	x	x	•	•	x	•	•	x	http://www.e-crisp.org/E-CRISP	Heigwer et al. (2014)
GT-Scan	•	•	•	x	x	•	•	x	•	•	x	https://gt-scan.csiro.au	O'Brien e Bailey (2014)
Horizon Discovery	x	•	•	x	x	x	•	x	•	•	•	https://horizondiscovery.com/en/products/tools	Horizon Discovery, UK
RGEN	•	•	•	•	•	x	•	•	•	•	x	http://www.rgenome.net	Bae et al. (2014)
Synthego	•	x	•	x	x	•	x	x	•	x	x	https://www.synthego.com/products/bioinformatics/crispr-design-tool	Synthego, CA

Besides a well-designed gRNA, the design of the donor template is also essential for HR to occur efficiently. The donor consists of a single or double-stranded segment of nucleotides, containing the desired edition flanked by regions homologous to the target region in the genome, known as homologous arms (Rozov et al., 2019). After successful editing, the continuous generation of DSBs in the target DNA should be avoided. For this, two strategies can be used, involving either the gRNA or the donor design. In the first, the gRNA is positioned in such a way that when the new sequence is incorporated the guide loses complementarity and can no longer pair with this region (Figure 6A). In the second, a silent mutation in the PAM region can be included in the donor template, making the DNA target region now unrecognizable by the nuclease (Figure 6B) (Huang; Puchta, 2019). These two strategies (Figure 6) can be applied using different nucleases, whether they generate abrupt or cohesive ends.

When designing the donor template, the size of the homologous arms should also be taken into account, and it may vary according to the size of the expected edition. Similar to the gRNA design, there is no specific information regarding plant cell editing, and the parameters used to design the donor are based on HR edits performed on other types of eukaryotic cells (Renaud et al., 2016; Rozov et al., 2019).

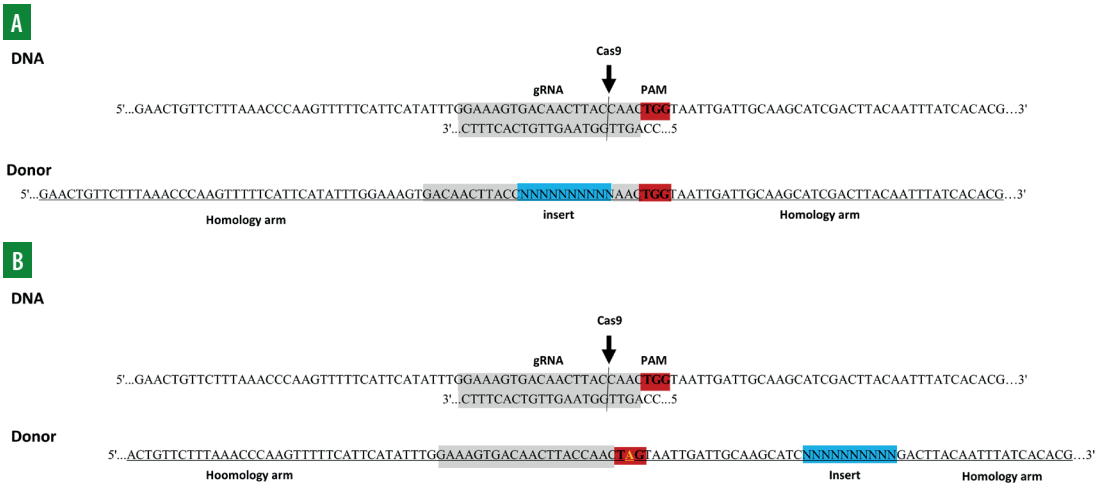

Figure 6. Donor template design strategies to avoid future DSBs after target editing by HR. A) Preventing the gRNA recognition site by editing. B) Silent mutation in the PAM region (cut by the Cas 9 nuclease). Legend: in gray, gRNA; in red, PAM; in yellow, silent mutation in PAM; in blue, edition (insertion).

Source: adapted from Horizon Discovery Ltd. (2020).

For changes smaller than 50 bp, it is recommended that the homologous arms have between 50 bp and 80 bp, and the total size of the donor template should not exceed 200 bp (Renaud et al., 2016). Whereas, for mutations greater than 100 bp, the homologous arms should have at least 500 bp (Ran et al., 2013).

3° Step – Delivery vector design

After rationally designing the gRNA and donor template, the next step is the correct design of the delivery vector. The main types of vectors used for GT are traditional binary vectors, used to obtain genetically modified organism (GMOs), and vectors based on geminivirus replicons, which are binary vectors adapted with the geminivirus replicon sequences (Schiml et al., 2014; Baltes et al., 2014; Svitashev et al., 2015; Čermák et al., 2015; Shi et al., 2017; Gil-Humanes et al., 2017; Dahan-Meir et al., 2018; Vu et al., 2020).

Most vectors used for GT have genetic elements resembling those used for editing by CRISPR/Cas via NHEJ. However, in the case of GT, in addition to the sequences that encode the nuclease, gRNA, and selective agents, it is necessary to include the donor template, either in the same vector (Figure 7A) or separately. The donor is generally used by the cell as dsDNA, and it does not need to be transcribed; thus, no promoter or terminator flank this region (Figure 7 A) (Schiml et al., 2014; Svitashev et al., 2015). Nevertheless, this strategy has the limitation of delivering a low amount of donor templates, hampering GT (Čermák et al., 2015). For this reason, the GVR-based donor delivery system has been gaining attention, as it produces a greater amount of templates, making it available during the repair via HR.

Vectors based on GVRs were developed by Baltes et al. (2014) by removing genes related to viral infection and leaving only the sequence that encodes the replication initiator proteins (Rep/RepA) flanked by the DNA sequences of intergenic LSL regions (LIR-SIR-LIR), which are essential for the circularization and self-replication of the GVR. This structure was assembled in a T-DNA format to be delivered to plants via Agrobacterium-mediated transformation. The LIR sequence functions as a bidirectional promoter activated by the Rep/RepA proteins, strongly inducing vector replication. To ensure higher rates of circular replication, a strong promoter can be positioned upstream of the LIR sequence, considering the circularized vector. Generally, Rep/RepA is positioned downstream of the complementary sense sequence of the LIR promoter, as found naturally in geminiviruses (Baltes et al., 2014) (Figure 7 B). Using GVRs, it is possible to delineate more than one vector construction strategy. Vu et al. (2020), for example, delivered all parts of the vector via GVR, while Dahan-Meir et al. (2018) delivered only the donor template by this system.

Another possibility is to use RNA as donor templates. In this case, the structure of the vectors is similar to when the DNA template is delivered by traditional vectors; however, it contains some specificities that deserve to be detailed. Butt et al. (2017) engineered a vector that differs from the default (Figure 7A) only by the donor template location, fusing it to the gRNA (gRNA - pre-tRNA), being driven by the OsU3 promoter (Figure 7C). Whereas Li et al. (2019) used RNA as a donor repair template and their vector differs from the default only in the donor template structure, designing two delivery systems. In the first, two units called RCR (Ribozyme - crRNA - Ribozyme) and one unit called RDR (Ribozyme - Donor repair template - Ribozyme) were used in tandem under control of the OsUbi3 promoter and NOS terminator (Figure 7D). In the second strategy, two RCR units were used in tandem with a DTT unit (Target 1 - Donor repair template - Target 2), where the donor is flanked by two crRNA targets (Figure 7E). The second system exploited the ability of Cas12a to process pre-crRNA to produce RNA templates. Both strategies were designed to ensure that the RNA templates were kept in the nucleus and used as a template for GT. This work by Li et al. (2019) elegantly shows that RNA can be used efficiently as a donor repair template in HDR.

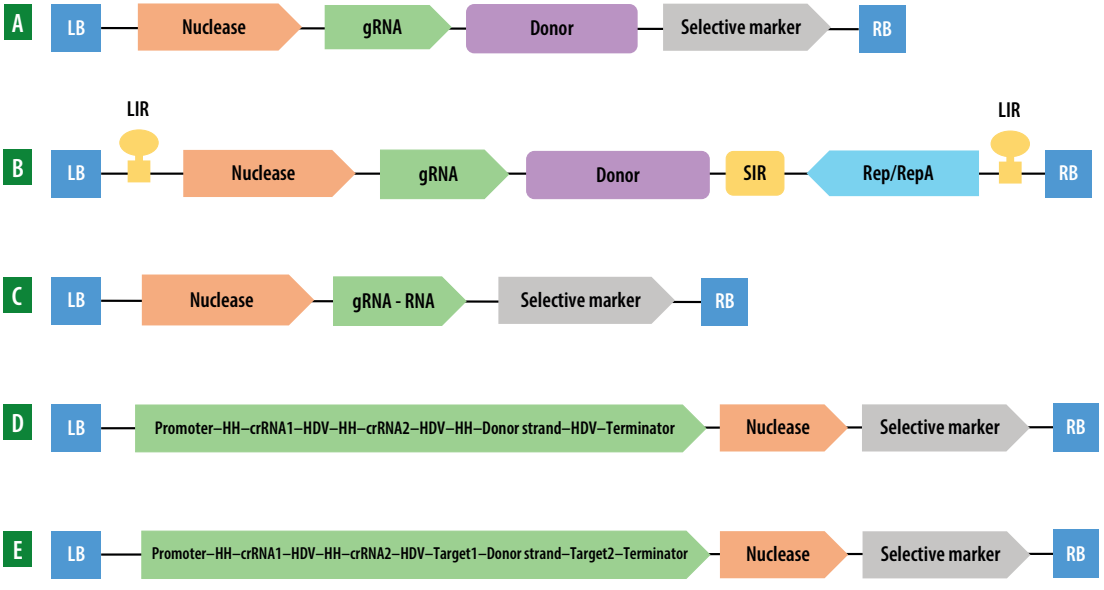

Figure 7. Examples of vector types used in plants. A) Vector with donor template being delivered as double-stranded DNA – a strategy used by Schiml et al. (2014). B) Vector based on Geminivirus viral replicon (GVR) – system proposed by Baltes et al. (2014). C) Binary Agrobacterium vector with RNA donor template fused to gRNA – design according to Butt et al. (2017). D) Binary agrobacterium vector with RNA donor template flanked by ribozyme –strategy traced by Li et al. (2019). E) Binary agrobacterium vector with flanked RNA donor template, crRNA targets –system developed by Li et al. (2019).

4° Step – Guide and donor template validation

After concluding the vector design, the next step is to verify the gRNA efficiency in guiding the nuclease to create the DSB. gRNAs may be validated using transient expression assay or using commercial kits, such as Guide-itTM sgRNA Screening (Takara Bio, 2018) (Figure 8).

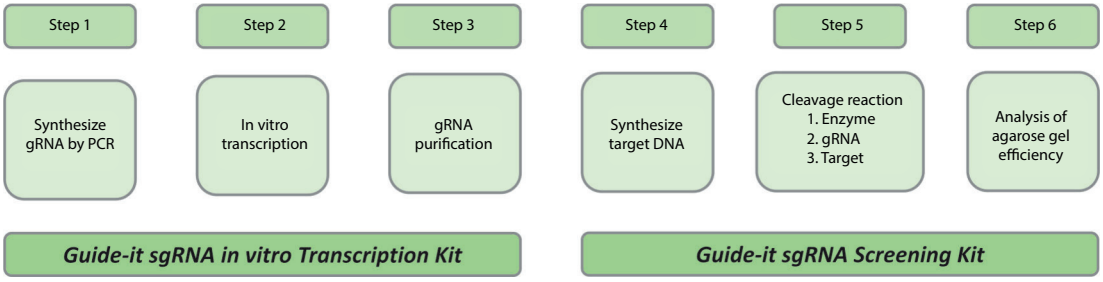

Figure 8. Scheme of steps for synthesis and validation of gRNA *in vitro* (Takara Bio, 2018).

Validating gRNA by transient expression is cheaper and presents great reproducibility to proceed to stable transformation, as it is conducted *in vivo*. Moreover, it can also be used to check the circularization of GVR-based vectors, which is crucial for self-replication (Baltes et al., 2014). Results can be obtained in a few days, ranging from 3 to 6 days, depending on the species (Shan et al., 2018). For plants, there are two main approaches to perform transient assays, agro-infiltration (Bortesi; Fischer, 2015) and protoplast transfection, which can also be used for plant regeneration in some species (Woo et al., 2015; Andersson et al., 2017; Collonnier et al., 2017; Osakabe et al., 2018). Furthermore, in legumes, the transient transformation of roots may also be mediated by *Agrobacterium rhizogenes* (Jacobs et al., 2015; Iaffaldano et al., 2016; Wang et al., 2016; Li et al., 2017; Bernard et al., 2019; Yuan et al., 2019).

5° Step – Stable transformation

A crucial and often limiting step in the GT process is tissue culture. Some plant species are recalcitrant to regeneration and/or transformation, and tissue culture represents a bottleneck for applying the CRISPR technology (Altpeter et al., 2005).

Plant regeneration methodologies by tissue culture generally occur via somatic organogenesis or somatic embryogenesis, and their applicability and efficiency vary according to the species (Kamle et al., 2011; Phillips; Garda, 2019). Although somatic organogenesis is widely used, somatic embryogenesis is especially advantageous for genome editing via CRISPR, as the chances of generating chimeric plants for the transgene are lower when compared with organogenesis. However, not all species have well-established and efficient protocols for this procedure (Duclercq et al., 2011; Kamle et al., 2011). In general, the transformation protocols used for GT are the ones normally employed for traditional GMO generation and genome editing via NHEJ, being mainly agrobacterium and biolistic-mediated strategies (Schmil et al., 2014; Svitashev et al., 2015; Shi et al., 2017).

One way to optimize GT efficiency via tissue culture is by inducing moderate stress to plants, such as short periods of heat stress to increase HDR occurrence in the cells (Boyko et al., 2005). Vu et al. (2020) demonstrated that the application of heat stress at 31°C for 10 days optimized the efficiency of HR in tomatoes. This approach is especially interesting when using Cas12a, which exhibits optimal activity at 31°C. Nevertheless, it is worth noting that the ideal temperature for applying moderate stress to plants may vary according to the species and it may not coincide with the optimal temperature for the nuclease.

Additionally, the photoperiod may also influence the nucleases activity; Cas12a, for instance, works better under both short – 8/16 h light/dark regime – and long – 16 h of light and 8 h of dark– photoperiods than in complete darkness (Vu et al., 2020); and the promoter CaMV 35S works better under long photoperiod (Boyko et al., 2005). On the other hand, these conditions may not be favorable to HR in Arabidopsis, considering that intrachromosomal recombination occurs more frequently in short photoperiods for this species (Boyko et al., 2005). However, these observations are likely to vary according to the type of study. Therefore, despite the limitations related to each species, tissue culture strategies can be tested for the species of interest and have the potential to help increase the efficiency of GT in plants.

6° Step – Screening and selection of edited plants

After plant regeneration by tissue culture, two steps must be taken to ensure the success of GT, identifying the transformed plants and then the edited ones. The fact that a plant is transformed does not necessarily mean this plant has been edited. Transformed plants are usually identified via Polymerase Chain Reaction (PCR) to identify the sequences present in the transformation cassette. To verify the edition, different strategies may be applied, and they are summarized below.

The best strategy to screen the CRISPR-mutants will vary depending on the size of the mutation. When the mutation consists of very small insertions or SNPs (Single Nucleotide Polymorphisms), an alternative is to use the PCR strategy based on loss of restriction enzyme sites – PCR-RE (Nekrasov et al., 2013). After the PCR-RE, the samples are sequenced to confirm the presence of the mutation and to characterize it, comparing with the wild sequence. Other options to identify point mutations such as SNPs are the High-Resolution Melt technique and sequencing (Sanger or next-generation sequencing; NGS) (Fauser et al., 2014; Ma et al., 2015; Svitashev et al., 2015; Zhang et al., 2019).

Larger insertions can be identified by conventional PCR and visualized on an agarose gel. After this step, sequencing of the amplified fragments is recommended to identify and confirm the expected mutation, reaching a nucleotide-level characterization and ensuring that it is not a false positive (Zhou et al., 2014; Zhang et al., 2019). Another strategy, recently described for identifying indels above two bp, is the use of 4-6% agarose gels (Bhattacharya; Van Meir, 2019). This approach proved to be a simple and inexpensive method; further details are described in Chapter 2. In all cases, the amplification primers, both for sequencing and PCR, need to be designed to complement sequences outside the homologous arms to avoid false positives derived from the donor template amplification (Ran et al., 2013).

Another important checkpoint is to select plants that are not chimeric for the mutation, which could result in plants with an unwanted phenotype. Similarly, the zygosity of the mutant (heterozygous or homozygous) must be characterized; even when the plants are genomic mutants, they can have undesired phenotypes due to heterozygosity. The most widely used method to characterize zygosity is sequencing, which, in addition to identifying mutations, enables the identification of different types of alleles (monoallelic or biallelic mutations). It may be performed using Sanger and NGS chromatograms assisted by software such as DSDecode (Liu et al., 2015), TIDE, Hi-TOM (Liu et al., 2019), MaGeCK-VISPR, MaGeCKFlute PinaPl-Py, CRISPRCloud2, CRISPRanalyzeR (Hanna; Doench, 2020), and scripts like Cris.py (Connelly; Pruett-Miller, 2019).

Conclusions and perspectives

The use of CRISPR/Cas technology via HDR for genome editing has been highly promising. However, its use is still largely limited to studies on model plants. This can be attributed to the low frequency of HDR in somatic cells, which happens mainly because it is not the predominant repair mechanism, and due to the difficulty to maintain the donor template in sufficient quantity to be used during the repair

instead of the sister chromatid. However, as the use of this technology advances, new strategies to optimize it emerge. For instance, the use of the Cas12a nuclease instead of Cas9 (Zetsche et al., 2015; Huang; Puchta, 2020), and the application of moderate heat stress, which may lead to cell cycle arrest at stages that favor HR (Boyko et al., 2005; Vu et al., 2020). The use of GVRs has also been shown to be a promising strategy, increasing the availability of the donor repair templates and inducing the cell to enter the S/G2 phases by the Rep/RepA proteins, thus favoring the HDR pathway (Baltes et al., 2014).

As this area is relatively new, GT still has enormous potential and is expected to advance hugely in the next few years, making this technique even more efficient. In this regard, several studies have been conducted in animal cells, yeasts, and plants (Gil-Humanes et al., 2017; Shi et al., 2017; Au et al., 2019; Liu et al., 2019; Yan et al., 2019; Jin et al., 2020; Vu et al., 2020). An application still little explored in plants is the deletion of whole genes. Although more challenging, it will certainly be enhanced since studies are being developed in the most diverse organisms (Au et al., 2019; Huang et al., 2019; Weisheit et al., 2020). Another crucial point is the development of efficient protocols for plant transformation and regeneration, which is still one of the bottlenecks for the GT technology to be applied routinely for many species. Thus, it is believed that, in the coming years, many advances will enable genome editing by CRISPR/Cas via HR to become a technique commonly used in plant breeding laboratories.

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