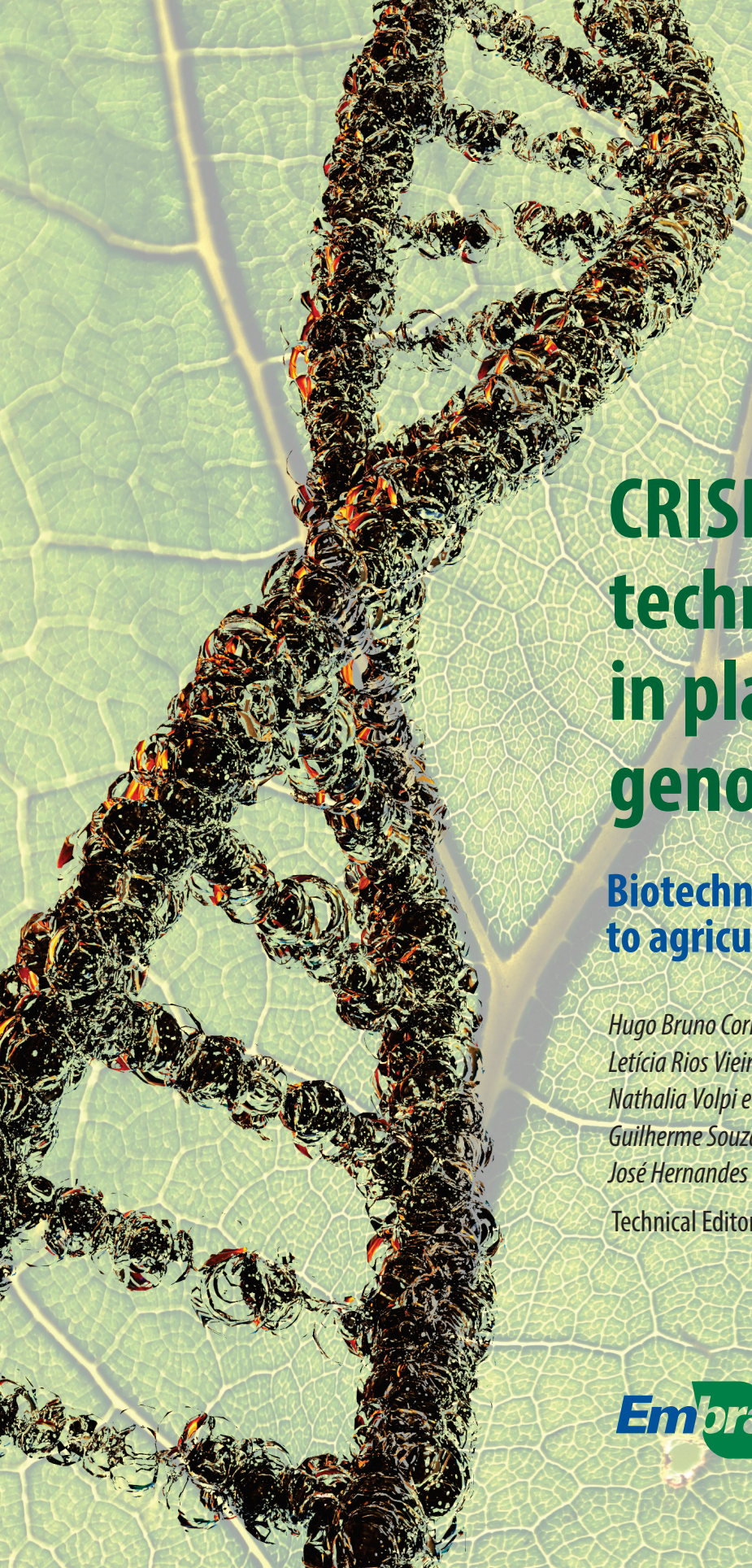

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Brazilian Agricultural Research Corporation
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Embrapa Agroenergy
Parque Estação Biológica (PqEB), s/n
Ed. Embrapa Agroenergy
70770-901, Brasília, DF, Brazil.
Phone: +55 (61) 3448-1581
Fax: +55 (61) 3448-1589
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www.embrapa.br/fale-conosco/sac/

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Rejane Maria de Oliveira (CRB-1/2913)

Authors

Adilson Kenji Kobayashi
Biologist, Ph.D. in Phytopathology, researcher at Embrapa Agroenergy, Brasília, DF, Brazil

Alexandre Lima Nepomuceno
Agronomist, Ph.D. in Molecular Biology and Plant Physiology, researcher at Embrapa Soybean, Londrina, PR, Brazil

Bruno de Oliveira Garcia
Bioprocess and Biotechnology Engineer, master's degree in Plant Biotechnology, Ph.D. student at Federal University of Lavras, Lavras, MG, Brazil

Bruno Paes de Melo
Biochemist, Ph.D. in Applied Biochemistry, Postdoc at Embrapa Genetic Resources & Biotechnology, Brasília, DF, Brazil

Carolina Vianna Morgante
Biologist, Ph.D. in Agronomy, researcher at Embrapa Semi-Arid Region, Petrolina, PE, Brazil

Clidia Eduarda Moreira Pinto
Biologist, Ph.D. in Molecular Biology, Postdoc at Embrapa Genetic Resources & Biotechnology, Brasília, DF, Brazil

Edivaldo Domingues Velini
Agronomist, Ph.D. in Agronomy, professor at São Paulo State University, Botucatu, SP, Brazil

Eduardo Romano de Campos Pinto
Biologist, Ph.D. in Molecular Biology, researcher at Embrapa Genetic Resources & Biotechnology, Brasília, DF, Brazil

Elton Gargioni Grisoste Barbosa
Biologist, master's degree in Genetics and Molecular Biology, Ph.D. student at Cambridge University, Cambridge, United Kingdom

Fabrício Barbosa Monteiro Arraes
Biologist, Ph.D. in Cellular and Molecular Biology, postdoc at Embrapa Genetic Resources & Biotechnology, Brasília, DF, Brazil

Fernanda Justen
Biologist, master's degree in Chemical and Biological Technologies, visiting specialist at Embrapa Agroenergy, Brasília, DF, Brazil

Fernanda Rausch Fernandes
Agronomist, Ph.D. in Agronomy, researcher at Embrapa Agricultural Informatics, and at the Genomics for Climate Change Research Center, Campinas, SP, Brazil

Galdino Andrade
Biologist, Ph.D. in Microbial Ecology, professor at State University of Londrina, Londrina, PR, Brazil

Guilherme Souza Prado
Biotechnologist, Ph.D. in Genomic Sciences and Biotechnology, visiting specialist at Embrapa Rice & Beans, Goiânia, GO, Brazil

Hugo Bruno Correa Molinari
Agronomist, Ph.D. in Agronomy, researcher at Embrapa Agroenergy, Brasília, DF, Brazil

Isabel Rodrigues Gerhardt
Agronomist, Ph.D. in Molecular Biology, researcher at Embrapa Agricultural Informatics, and at the Genomics for Climate Change Research Center, Campinas, SP, Brazil

Jéssika Angelotti-Mendonça
Agronomist, Ph.D. in Science (Phytotechnics), visiting specialist at Embrapa Soybean, Londrina, PR, Brazil

José Hernandes Lopes Filho
Biologist, Ph.D. in Science (Botany), visiting specialist at Embrapa Agricultural Informatics, and at the Genomics for Climate Change Research Center, Campinas, SP, Brazil

Josenilda Carlos dos Santos
Biologist, Ph.D. in Agricultural Microbiology, Postdoc at Embrapa Maize & Sorghum, Sete Lagoas, MG, Brazil

Josias Correa de Faria
Agronomist, Ph.D. in Phytopathology, researcher at Embrapa Rice & Beans, Goiânia, GO, Brazil

Juliana Erika de Carvalho Teixeira Yassitepe
Agronomist, Ph.D. in Agronomy, researcher at Embrapa Agricultural Informatics, and at the Genomics for Climate Change Research Center, Campinas, SP, Brazil

Larissa Girotto
Biologist, Ph.D. in Genetics e Molecular Biology, visiting specialist at Embrapa Soybean, Londrina, PR, Brazil

Letícia Rios Vieira
Biologist, Ph.D. in Plant Biotechnology, consultant at Embrapa Agroenergy, Brasília, DF, Brazil

Liliane Marcia Mertz-Henning
Agronomist, Ph.D. in Seed Science and Technology, researcher at Embrapa Soybean, Londrina, PR, Brazil

Maria Fatima Grossi-de-Sa
Biologist, Ph.D. in Molecular Biology, researcher at Embrapa Genetic Resources & Biotechnology, Brasília, DF, Brazil

Maria Lucia Zaidan Dagli
Veterinarian, Ph.D. in Experimental and Comparative Pathology, professor at São Paulo University, São Paulo, SP, Brazil

Maria Sueli Soares Felipe
Chemist, Ph.D. in Biochemistry, professor at Catholic University of Brasília and retired professor emeritus at the University of Brasília, Brasília, DF, Brazil

Mayla Daiane Correa Molinari
Agronomist, Ph.D. in Genetics and Molecular Biology, postdoc at Embrapa Soybean, Londrina, PR, Brazil

Natália Chagas Freitas
Biotechnologist, Ph.D. in Plant Biotechnology, visiting specialist at Embrapa Agroenergy, Brasília, DF, Brazil

Nathalia Volpi e Silva
Biologist, Ph.D. in Genetics and Molecular Biology, visiting specialist da Embrapa Soybean, Londrina, PR, Brazil

Patricia Machado Bueno Fernandes
Biologist, Ph.D. in Biochemistry, professor at Federal University of Espirito Santo, Vitória, ES, Brazil

Renata Fuganti-Pagliarini
Biologist, Ph.D. in Genetics and Crop Breeding, Cell Biology Platform Lead at Global Institute for Food Security (GIFS), Saskatoon, Saskatchewan, Canada

Ricardo Augusto Dante
Biologist, Ph.D. in Plant Sciences, researcher at Embrapa Agricultural Informatics, and at the Genomics for Climate Change Research Center, Campinas, SP, Brazil

Rosana Pereira Vianello
Biologist, Ph.D. in Molecular Biology, researcher at Embrapa Rice & Beans, Goiânia, GO, Brazil

Thaísa Tessutti Pinheiro
Biologist, Ph.D. in Sciences, visiting specialist at Embrapa Rice & Beans, Goiânia, GO, Brazil

Vívian de Jesus Miranda
Biologist, Ph.D. in Genomics Sciences and Biotechnology, visiting specialist at Embrapa Agroenergy, Brasília, DF, Brazil

Viviane Cristina Heinzen da Silva
Biologist, Ph.D. in Sciences (Functional and Molecular Biology - Biochemistry), postdoc at University of Campinas, and collaborator at the Genomics for Climate Change Research Center, Campinas, SP, Brazil

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involved in the
elaboration of this book.

Foreword

A few years ago, the possibility of promoting fast, precise and economical punctual alterations in sequences of the genomes of plants and animals of agricultural interest, in order to make them more suitable to a certain environment or condition, seemed something distant. Scientists were working on genetic transformation procedures that, in general, were too expensive, mainly due to the need of deregulating the elite events, and, moreover, were often too slow to respond to important changes in the socioeconomic scenario of agriculture activity. The immediate consequence of this was that only a few companies in the world were able to operate such a model efficiently.

This scenario began to change just over 8 years ago, when researchers Emmanuelle Charpentier, director of the Max Planck Institute for Infection Biology, and Jennifer A. Doudna, a biochemist at the University of California, published in the journal Science the seminal paper entitled "A Programmable Dual-RNA – Guided DNA Endonuclease in Adaptive Bacterial Immunity", in which they described how short and repetitive palindromic sequences, regularly grouped and interspersed, provided bacteria and archaea with adaptive immunity against viruses and plasmids, and showed that such bacteria/archaea use CRISPR RNAs to guide the cleavage of invading nucleic acids. Thereafter the field of Genetic Engineering entered a revolutionary new phase, in which it is possible to use CRISPR/Cas based systems with programmable RNAs, allowing scientists in almost any molecular biology laboratory to change or edit specific sequences of the eukaryotic cell genome. Thus, with these "molecular scissors", it is possible, for example, to "cut" a specific part of the DNA, inducing the cell to produce or not produce certain proteins. For this discovery Charpentier and Doudna were awarded the Nobel Prize in Chemistry in 2020.

Naturally, the technique is now widely used in agricultural research to develop crops with new attributes, such as resistance to pests and diseases, tolerance to drought, and other abiotic stresses. Embrapa has today in its project portfolio a large project, called CRISPRevolution, which consists in the company's most organized effort to use genome editing through CRISPR technology as a biotechnological solution to improve nutritional industrial quality and tolerance to water deficit in species of agronomic interest. Embrapa Agroenergy, which coordinates the project, has joined efforts to carry out this project, with Embrapa Maize & Sorghum, Embrapa Soybean, Embrapa Rice & Beans, and Embrapa Agricultural Informatics.

The idea of organizing and publishing this book came up in an informal conversation among the group of project leaders. For them, in addition to promoting

cutting-edge science and developing innovative products and processes based on the new technique, it was also necessary to act as a strategy for disseminating scientific knowledge. Well, this book is the result of this vision and brings a vast material that, without any doubt, will become a reference for professors, researchers, technicians and students who dedicate themselves to the study of biotechnology. We hope, finally, that this material can inspire many scientists to continue advancing in the field of biotechnology, work that in our view will continue to underpin modern agriculture for years to come.

We hope you enjoy this text. Good reading to all.

Alexandre Alonso Alves

Head of Embrapa Agroenergy

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Introduction to genome editing in plants

José Hernandes Lopes Filho
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Ricardo Augusto Dante
Isabel Rodrigues Gerhardt
Juliana Erika de Carvalho Teixeira Yassitepe
Fernanda Rausch Fernandes

Present and future challenges of modern agriculture

The global challenges faced by modern agriculture include increased food demand due to population growth, changes in eating habits, and climate changes. One of its biggest challenges is the sustainable increase in production with application of good agricultural practices, as well as the development of new crop varieties with both higher nutritional content and tolerance to biotic and abiotic stresses (DaMatta et al., 2010; Lobell; Gourdji, 2012; McCouch et al., 2013; Eisenstein, 2013; FAO, 2019). Also, another concerning topic is the major deforestation caused by the continuous increase of arable areas (Campbell et al., 2008).

Historically, the development of cultivars with desirable traits, such as higher productivity, resistance to pests, or greater nutritional value, has been mainly based on methods of selecting favorable alleles that naturally occur or that arise by non-specific mutagenesis techniques. Despite their enormous contribution, these methods have limitations, such as the phenotypic selection without knowledge of the molecular and physiological bases involved in the processes (Purugganan; Fuller, 2009).

With the advent of molecular biology, modern agriculture has benefited from several techniques that help plant genetic improvement, with emphasis on transgenics, marker-assisted selection, and genome selection. However, although genetically modified (GM) foods have an important role in the current agriculture scenario, they are strongly criticized by the public, and usually associated with the idea of "unnatural" since they contain genetic material from different organisms (Schmidt et al., 2020). Also, there are still many technical limitations for the development of commercial products, like being limited to the manipulation of characteristics controlled by few genes or the impracticality of choosing the genomic position where the exogenous DNA is integrated (Que et al., 2010).

Methods of gene introgression require several genetic crosses, extensive screening and large-sized populations, which are laborious, costly and time-

consuming (Mazur; Tingey, 1995; Jacobsen; Schouten, 2007; Harrison; Larson, 2014). An example is given in figure 1: An elite commercial germplasm has important agronomic traits, but is susceptible to a certain pest. To acquire resistance, such a cultivar is crossed with a donor variety, which is resistant to the pest but has other undesirable characteristics. The F1 (hybrid) generation is resistant to the pest thanks to the dominant allele from the donor parent but has intermediate traits. Several steps of backcrossing and selection are necessary to recover the characteristics of the elite variety while maintaining the resistance. Even after multiple backcrossing cycles, it is often impossible to recover 100% of the elite variety genomic sequences adjacent to the locus of interest, which are highly affected by the linkage drag effect (Figure 1) (Brown, 2002; Lin et al., 2014).

These methods are usually restricted to related species/cultivars since the crossing between distant species is not viable, or when it occurs, tends to produce infertile descendants (Moyle; Nakazato, 2008). Also, the extensive linkage drag associated with genome segmentation limits breeding programs, usually requiring many attempts to break the linkage of the target gene (Brown, 2002; Lin et al., 2014). The rate of recombination between a given marker close to the locus of interest and that locus itself is often low, being directly related to the distance between them (Li et al., 2015).

It is therefore urgent that new strategies and technologies are used to reduce the time and costs of conventional breeding. Such technologies could stack desirable traits by precise genetic modification into the elite material, avoiding the traditional random and uncontrolled mutagenesis of other strategies. In this context, genome editing in plants has emerged as an innovative tool with potential success for increasing food productivity, quality, and safety.

Gene editing tools for precision crop breeding

Efficient genome editing technologies represent a powerful tool for agriculture, allowing highly specific (non-random) manipulation of plant genomes in their natural chromosomal context (Chen et al., 2019). Among the leading gene editing techniques that have been used in recent years are those based on ZFNs (Zinc Finger Nucleases), TALENs (Transcription Activator-Like Effector Nucleases), and CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR associated protein).

One of the greatest advantages of using genome editing techniques for crop breeding is that it allows the simultaneous improvement of multiple traits

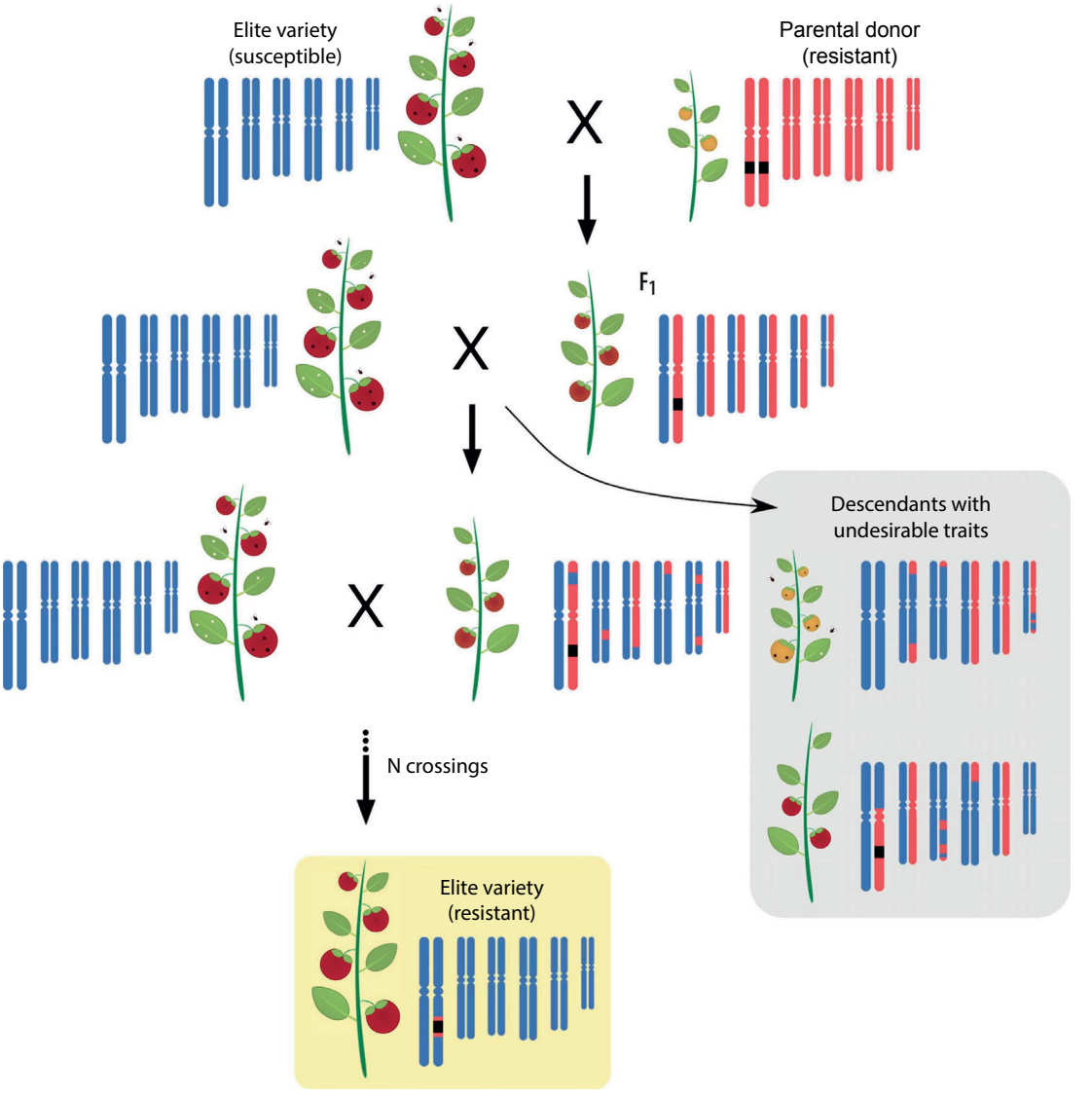

Figure 1. Representation of the introgression process in which an elite variety susceptible to a pest is crossed with a resistant donor. Blue and red chromosomes represent the genome of the elite and donor cultivars, respectively. The black region indicates the locus that confers resistance to the pest.

directly in elite lines. This possibility speeds up the development of commercial products, which is generally impractical when using conventional breeding (Gao et al., 2020b). Such strategies, usually referred to as multiplex editing, involve

the concomitant modification of several loci, which is especially important for the improvement of characteristics controlled by QTLs (Quantitative Trait Loci) (Rodríguez-Leal et al., 2017).

There are also alternative strategies, such as the *in vivo* Desired-Target Mutator (DTM) (Li et al., 2017), based on the crossing of transgenic lines (containing the gene editing machinery) with elite genotypes, so that the editing can occur directly into the elite recipient cultivar. These strategies minimize the linkage drag effect and accelerate the recovery of the elite genotype by requiring both a smaller number of backcrossings and a less intensive use of molecular markers.

In addition, approaches based on ribonucleoproteins allow genome editing without chromosomal insertion of exogenous DNA, thus potentially avoiding regulatory aspects (Figure 2) (Jansing et al., 2019). In this regard, different classifications have been adopted worldwide based on the type and extent of genomic modifications performed. Several countries have already chosen not to treat as Genetically Modified Organisms (GMOs) those obtained through genome editing (depending on the approach used). In Brazil, genome editing falls into the "New Breeding Technologies" category. These techniques were defined by the Normative Resolution No. 16, of January 15th, 2018 of the Brazilian National Technical Commission on Biosafety (CTNBio) and are characterized by the absence of recombinant DNA in their final products. Therewith, genome editing allows small and medium-sized companies, as well as research institutes, to overcome regulatory obstacles presently applicable to GMOs (Schmidt et al., 2020). The current regulatory scenario of organisms with an edited genome is discussed in Chapter 5 of this book.

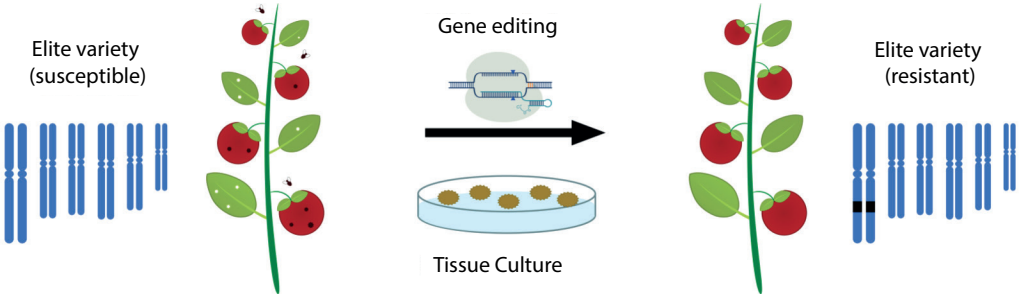

Figure 2. Improving resistance to a specific pest by genome editing without integrating exogenous DNA into the genome of an elite variety.

Genome editing methodologies

Methods used to induce specific modifications in the DNA of living organisms are often referred to as genome editing, gene editing, or genome engineering (Baltes et al., 2017). Genome editing (GE) techniques are based on the action of site-directed nucleases capable of cleaving the target DNA molecule, with subsequent activation of endogenous DNA repair mechanisms, which can be directed by homologous recombination (HR) or by non-homologous end joining (NHEJ) (Satheesh et al., 2019). This only became possible thanks to the development of recombinant DNA technology, which began in 1972 when Paul Berg's laboratory published the creation of the first recombinant DNA molecule (Jackson et al., 1972). Since then, molecular biology and genetic engineering techniques have evolved, allowing scientists to develop several genome editing methodologies. These include the construction of vector systems, methods of delivering genetic material into cells, and the application of engineered proteins (Jansing et al., 2019; Anzalone et al., 2020). Among the first generation of genome editing tools in plants are the ZFNs and TALENs.

ZFNs (*Zinc Finger Nucleases*)

ZFNs are engineered chimeric proteins composed of a nonspecific FokI cleavage domain, which promotes the DNA double-strand break, and a repeat of 3-5 Cys2-His2 zinc fingers, with DNA binding properties (Shah et al., 2018). Each zinc finger interacts with 3 adjacent nucleotides, forming a dimer. This dimer identifies a target sequence of 18 to 24 base pairs (bp) in the genome. Thus, the zinc fingers can be modified to recognize different regions of interest in the DNA molecule. The double-strand break can then be repaired via NHEJ or HR, which can result in gene editing through insertions or deletions (Satheesh et al., 2019). ZFNs were the first enzymes used in plant GE, having *Arabidopsis thaliana* as a model. Since then, several studies have been conducted applying this technique in other plant species (Davies et al., 2017). In maize, ZFN was used edit the *ipk1* gene, which led to herbicide-tolerant plants (bialaphos and quizalofope) (Shukla et al., 2009). This technique was also used to generate tobacco plants resistant to imidazolinone and sulfonylurea (Townsend et al., 2009). Another study showed the efficiency of ZFN associated with the HR mechanism in tobacco when used to replace a 7 kb genomic sequence with a 4 kb cassette encoding multiple markers (Schneider et al., 2016). In soybeans, the functional role of genes encoding a family of DICER-LIKE1 proteins, involved in the maturation pathway of small RNAs, has been confirmed through mutations generated by ZFN (Curtin et al., 2016).

TALENs (*Transcription Activator-Like Effector Nucleases*)

Transcriptional activator effectors are proteins synthesized by *Xanthomonas* phytopathogenic bacteria (Gaj et al., 2013). These proteins are composed of DNA-binding domains, which are formed by 13 to 30 amino acid repeats. Each array contains about 34 identical amino acids, except for variable residues repeats (VRR) at positions 12 and 13, which are responsible for the binding specificity to the target nucleotide sequence (Satheesh et al., 2019). Each VRR recognizes a single base pair and allows modifications to the specific DNA target (Shah et al., 2018). These repeat domains are artificially fused to the FokI nuclease, as with ZFNs, adding the cleavage function to transcriptional activator effectors.

TALENs have been used for gene editing in various crops to improve specific traits. For instance, TALEN was used to induce mutations in a highly conserved region of the sugarcane caffeic acid-o-methyl transferase (Sedeek et al., 2019). The mutant lines showed a reduction in lignin content, proving the efficiency of the editing technique for complex genomes such as sugarcane (Jung; Altpeter, 2016). In rice, TALEN was used to cause mutations in the *OsSWEET* and *OsBAHD2* genes, generating plants resistant to rust and with fragrance improvement, respectively (Li et al., 2012; Shan et al., 2015). Two genes, *FucT* and *XylT*, were knocked out in tobacco using TALEN to improve the plant's ability to produce glycoproteins (Li et al., 2016).

Limitations of classic techniques and advantages of the CRISPR/Cas System

Although the application of ZFNs for genome editing in diverse plant species has been successful, the technology has some limitations. For example, it is necessary to design a new zinc finger arrangement for each selected target (Chen et al., 2019). Also, the number of possible targets is limited and there is a risk of overlap between the catalytic and the DNA binding domains, which can affect specificity. TALEN, although more accurate than ZFNs, has its main limitation in the requirement for a large number of VRRs to act on a selected target (Satheesh et al., 2019). Thus, both ZFNs and TALENs need to be redesigned for each specific target, which is a great challenge since they are complex proteins. Also building chimeric proteins like these is costly and time-consuming. More recently, the CRISPR system emerged, which does not depend on the modification of proteins, but rather on the simple inclusion of RNA molecules that confer target specificity (Jinek et al., 2012).

Briefly, genome editing by the CRISPR system is based on two basic components: a nuclease (an enzyme capable of breaking the DNA) and guide RNA molecules (sgRNAs), which direct the nuclease activity to specific sites of the DNA (Anzalone et al., 2020). Thus, by simply exchanging the sgRNA molecules, it is possible to edit

different genomic regions. Furthermore, editing multiple loci does not necessarily depend on the use of a large number of sgRNAs. For example, several genes can be edited simultaneously by using sgRNAs that target a conserved sequence of a multigenic family. This approach facilitates the engineering of metabolic pathways rich in redundant enzymes. This same strategy can be used to edit several copies of the same gene, which is especially interesting for plants with polyploid genomes.

Origin of the CRISPR/Cas technology

The CRISPR system is a sophisticated mechanism of adaptive immunity against viruses and plasmids present in prokaryotes (bacteria and archaea) (Figure 3). The system allows microorganisms to cleave nucleic acids from invaders, disrupting their reproductive cycle (Wiedenheft et al., 2012; Koonin; Makarova, 2013). The acronym CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) refers to the genetic arrangement of repetitive regions containing small, non-coding RNA genes, which confer specificity to bacterial defense: the CRISPR RNA (crRNA) and the crRNA transactivator (tracrRNA), absent in some types of the CRISPR system. The CRISPR-associated proteins (Cas) are nucleases able to cleave and destroy invading DNA (Wiedenheft et al., 2012; Marraffini, 2015).

During the immunization process in prokaryotes, small fragments of exogenous DNA (from the invader) are integrated into the CRISPR locus of the host chromosome. This integration occurs as new spacers between repeats (Amitai; Sorek, 2016), promoting the adaptation of the host against new infection by the same invader (Barrangou et al., 2007). If a subsequent invasion by the same virus or plasmid occurs, the transcription of the CRISPR locus (now containing new spacers) and its processing to form mature crRNAs (sometimes dependent on tracrRNA) results in the recognition of the invader: the 5' region of the crRNA containing the spacer pairs with the exogenous DNA sequence in the spacer-precursor region (protospacer), which is then cleaved by the Cas nuclease (Figure 3) (Marraffini; Sontheimer, 2008; Hale et al., 2009; Garneau et al., 2010). The specificity and degradation of the invading element in most CRISPR/Cas systems are also determined by a small 2-5 bp sequence, located close to the target sequence (protospacer) in the invading DNA, known as the PAM motif (Protospacer Adjacent Motif) (Mojica et al., 2009; Anders et al., 2014; Jiang; Doudna, 2017).

Years of research have been carried out between the discovery of the CRISPR system to its adaptation as a genome editing tool in living organisms. In 1987, when analyzing the DNA sequence of *iap* gene from *Escherichia coli* k12, Ishino et al. (1987) noted the presence of an unusual region at the 3' end of the gene. This

region consisted of repeat sequences and interwoven spacer sequences (Ishino et al., 1987). A few years later, these same regions were found in the *Haloferax mediterraneii archea* genome (Mojica et al., 1993). In 2000, these genetic elements were identified in other 20 different microorganisms, including *Mycobacterium tuberculosis*, *Clostridium difficile* and *Yersinia pestis*. Finally, they were characterized and named as "short regularly spaced repeats" (SRSRs) (Mojica et al., 2000). Shortly thereafter, the name of this element was changed to CRISPR, but its biological function remained unknown (Jansen et al., 2002).

The discovery of four genes of the *Cas* family, located adjacent to the CRISPR locus, was crucial to unraveling its biological function. These genes encode proteins with characteristic nuclease and helicase motifs, indicating that they could be involved in DNA metabolism or gene expression, having a putative functional association with the CRISPR locus (Jansen et al., 2002). Thus, several hypotheses emerged for the

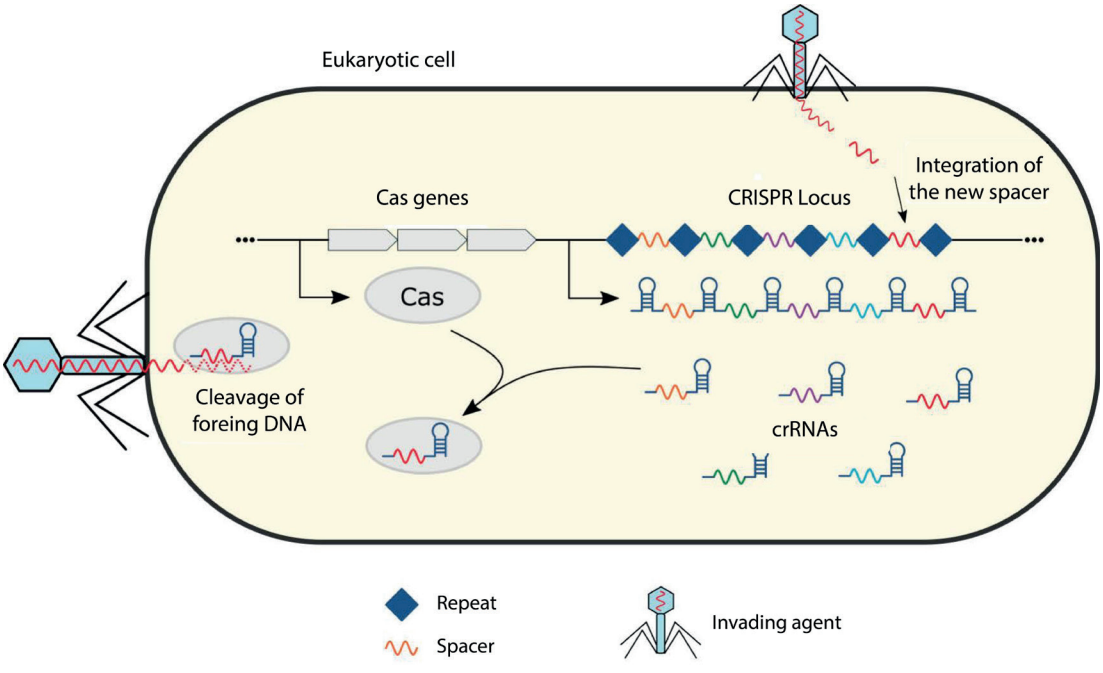

Figure 3. Schematic representation of the CRISPR/Cas immunity system in prokaryotes. A prokaryotic cell, when invaded by a virus or plasmid, can integrate part of the invading genome as a new spacer (in red) in its CRISPR locus. In a recurring infection, crRNAs derived from the CRISPR locus associate with Cas proteins, which start to recognize and cleave DNA molecules from the invader.

Source: adapted from Doudna Lab (2020).

functional role of CRISPR: it could participate in gene regulation, in the partitioning of replicons, DNA repair, among others.

In the following years, with the aid of bioinformatics tools, the first evidence that the CRISPR locus was involved with the prokaryotic immune system appeared. Searching for DNA sequences similar to the *E. coli* CRISPR locus, researchers found that it matched the P1 phage sequence, which infected many *E. coli* strains. From that information, 4,500 CRISPR spacers were identified in 67 bacterial strains, many of which were similar to known viruses or conjugative plasmids sequences (Mojica et al., 2005). At the same time, it was found that 61 strains of *Y. pestis* had identical CRISPR loci, except for their spacers, and that many of these spacers corresponded to phage sequences. The authors then suggested that the CRISPR locus would function as a defense mechanism and that it could represent the memory of previous infections (Pourcel et al., 2005).

The hypothesis that CRISPR would be involved in the immune response of prokaryotes gained support after Rodolphe Barrangou and collaborators published their findings in 2007. They analyzed the variation within the CRISPR locus sequence of several strains of *Streptococcus thermophilus* and observed that after facing bacteria and phages, new spacers corresponding to the gene sequences of the phages were integrated into the bacterium's genome. The bacteria that integrated the DNA sequence of the phage became resistant to it, demonstrating that CRISPR performs functions related to the bacterial immune system (Barrangou et al., 2007). In the following years, based on bioinformatics, genetics, and molecular biology, the function of each component and the CRISPR/Cas mechanism was elucidated. In 2012, the effectiveness of the CRISPR/Cas system *in vitro* was proven, opening the door for eukaryotic genome editing based on endonucleases guided by programmable RNAs (Gasiunas et al., 2012).

Thus, the CRISPR system represented a major breakthrough in genome editing technology, especially because it does not depend on the long and costly process of protein modification to confer target specificity. Since the reprogramming of the system for editing different targets depends, in general, only on the exchange of molecules of guide RNAs, the technology was quickly spread among laboratories around the world. Since then, CRISPR/Cas genome editing technology has become increasingly efficient and applied to a wide range of organisms (Chen et al., 2019; Anzalone et al., 2020; Li et al., 2020c).

Overview of mechanisms and enzymes

There are different CRISPR/Cas mechanisms in prokaryotes, which can be divided into two classes, each subdivided into three types based on the different Cas genes and the nature of the effector complex. Class 1 (types I, III and, IV) employs multiple Cas proteins in the effector complex, while Class 2 (types II, V and, VI) has only a single effector protein. The CRISPR/Cas9 System of *Streptococcus pyogenes* (*SpCas9*) belongs to Class 2, type II, and was the first system adapted for gene editing in eukaryotic genomes. Unlike types I and III, in type II the crRNA and tracrRNA molecules hybridize to form unique structures, which guides the Cas9 to the target sequence. Thus, it cleaves any DNA molecule containing a complementary target sequence adjacent to a PAM (Gasiunas et al., 2012; Jinek et al., 2012). The eukaryotic genome editing platform is simplified by the synthesis of a single chimeric molecule containing both the crRNA and tracrRNA, referred to as sgRNA (single guide RNA) or gRNA (guide RNA) (Jinek et al., 2012; Koonin et al., 2017). Thus, the sgRNA molecule contains the crRNA sequence, with its spacer complementary to the target DNA sequence (protospacer), fused to the tracrRNA, which contains a secondary structure in the form of three hairpins necessary for the recognition of the Cas enzyme, in addition to a hairpin structure to stop its transcription (Figure 4) (Jinek et al., 2012). This simplified two-component system can be programmed to recognize virtually any specific sequence of interest in the genome, as long as it is adjacent to a PAM site.

Initially, the Cas9 enzyme recognizes the sgRNA through its recognition lobe (Rec). Once formed, the Cas9-sgRNA complex scans the DNA molecule for a PAM

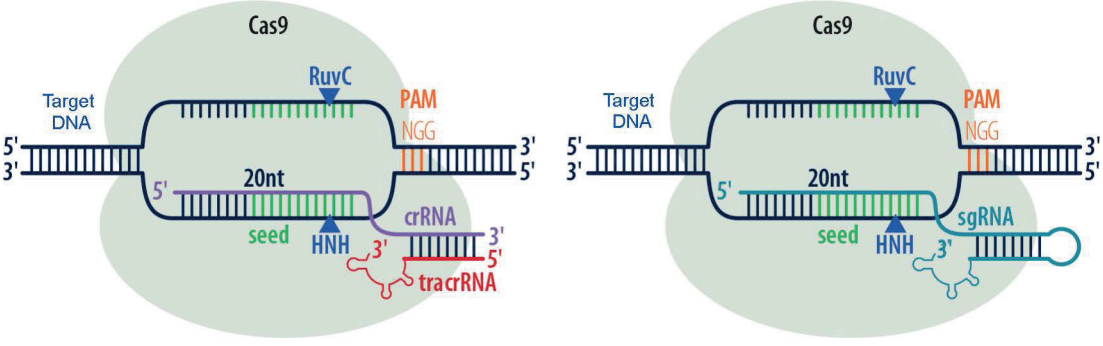

Figure 4. Comparison between the native CRISPR/Cas9 complex of *Streptococcus pyogenes* (left), containing crRNA and tracrRNA, and the optimized complex used for genome editing in eukaryotes (right) with a single guide molecule (sgRNA). Blue triangles indicate the cleavage positions in the target molecule, performed by the active sites RuvC and HNH.

Source: adapted from Doudna and Charpentier (2014).

site, which is also recognized by the Cas9 Rec lobe. Then, Cas9 opens the double-stranded DNA molecule immediately upstream of the PAM, allowing the pairing of 20-24 nucleotides between the complementary target DNA and the sgRNA. In most cases, the Cas9-sgRNA complex is unable to recognize DNA sites with more than three non-complementary nucleotides and is also incapable to recognize and cleave the target DNA that contains any non-complementary nucleotides in the 10-12 nucleotides near the PAM site (region also known as seed). Only after complete pairing, HNH and RuvC Cas9 nuclease activity domains cleave the complementary and non-complementary strands of DNA, specifically in the third nucleotide upstream of the PAM site (Figure 4) (Cong et al., 2013; Hsu et al., 2013; Jiang et al., 2015). Thus, the Cas9-binding specificity to the target DNA is determined by the conserved PAM sequence in the target DNA and by the pairing of its protospacer region with the spacer region (mainly the seed region) of the sgRNA. Finally, the double-strand cleavage leads to the recruitment of DNA repair mechanisms by the organism being edited. In general, the repair system can follow two different routes: (1) non-homologous ends joining (NHEJ), or (2) homologous recombination (HR). While the repair by NHEJ tends to produce small insertions and/or deletions (indels) around the cleavage site, repair by HR is directed by homology, allowing the

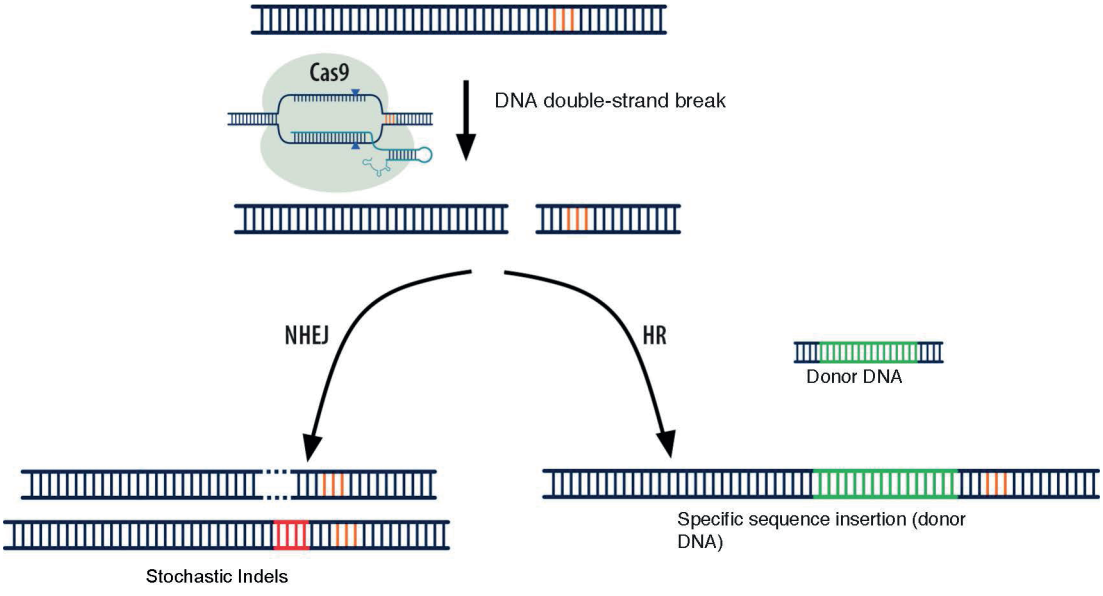

Figure 5. Schematic representation of the DNA repair mechanisms by NHEJ or HR. Dashed and red regions indicate deletions and insertions, respectively. The green region represents a sequence of interest to be integrated into the target DNA.

insertion of sequences of interest in the edited region with high fidelity (Figure 5) (San Filippo et al., 2008; Chen et al., 2019; Anzalone et al., 2020).

Modification and alternatives to SpCas9

Despite CRISPR/Cas9 being the most used system for genome editing (Doudna; Charpentier, 2014; Sander; Joung, 2014), the sequences it can recognize is restricted by the need for an adjacent NGG PAM motif (Mojica et al., 2009; Jinek et al., 2012; Shah et al., 2013; Sternberg et al., 2014). It has been reported, however, that the SpCas9-sgRNA complex is also able to recognize alternative PAM sequences (NAG and NGA), potentially increasing the probability of off-target mutagenesis (Zhang et al., 2014; Kleinstiver et al., 2015). In addition to the requirement for specific PAM sites and the probability of off-target cuts, other characteristics of Cas9 such as its size and mode of action may limit its use in editing eukaryotic genomes. Such restrictions led to the search for alternative Cas proteins, as well as to the development of diverse modifications of Cas9, aiming to improve its accuracy, efficiency, and versatility of applications.

SpCas9 orthologues such as *Staphylococcus aureus* (SaCas9), *Streptococcus thermophilus* (StCas9) and *Neisseria meningitides* (NmCas9) (Gasiunas et al., 2020), recognize PAM sites with different sizes and sequences and have also been successfully used as a tool in genome editing (Ran et al., 2015; Cebrian-Serrano; Davies, 2017). The SpCas9 has also been modified to recognize different PAM sites, such as the VQR-Cas9 (NGA), EQR-Cas9 (NGAG), VRERCas9, (NGCG), SaKKH-Cas9 (NNNRRT) (Kleinstiver et al., 2015), xCas9 (NG, GAA and GTA) (Hu et al., 2018) and SpCas9-NG (NG) (Nishimasu et al., 2018). Other modifications such as the fusion of Cas9 to the DNA-binding domains of other proteins have also resulted in the cleavage of sequences with alternative PAM motifs (Bolukbasi et al., 2015).

The SpCas9-sgRNA system can also tolerate some pairing errors between sgRNA and the target DNA, which can lead to undesired off-target mutations (Cradick et al., 2013; Fu et al., 2013; Hsu et al., 2013). The relevance of off-target mutations in genome editing is still questionable (Iyer et al., 2015; Zhang et al., 2018b) and, although limiting the applicability of CRISPR/Cas9 for human therapies (Li et al., 2020b), it is not a hindrance for using the technology in plants (Young et al., 2019; Gao et al., 2020a; Graham et al., 2020; Herbert et al., 2020; Zhang et al., 2020).

Great advances have been achieved in increasing the Cas9 specificity. An example with a wide application is the use of Cas variants known as nickases (nCas9), which had one of their nuclease domains inactivated by point mutations: D10A mutation at the HNH site, or H840A mutation at the RuvC site (Cong et al., 2013; Mali et al.,

2013; Ran et al., 2013; Cebrian-Serrano; Davies, 2017). Nickases continue to recognize the target DNA but cleave only a single DNA strand. This single-strand cutting approach increases repair fidelity and the probability of obtaining the desired mutation (Dianov; Hübscher, 2013; Cebrian-Serrano; Davies, 2017). Also, nickases are highly specific in human cells, reducing off-targets without compromising editing efficiency (Cho et al., 2014). The inactivation of both nuclease domains led to the development of deactivated or "dead" Cas9 (dCas9), capable of recognizing specific target sequences and usually fused proteins with other catalytic activities (Brezgin et al., 2019). Another approach to achieve greater specificity without losing efficiency is the mutation in residues involved in the energy reduction of the SpCas9-sgRNA-DNA complex (Kleinstiver et al., 2016; Slaymaker et al., 2016). These modified proteins became known as high-fidelity and enhanced CRISPR-Cas9 nucleases (SpCas9-HF1 and eSpCas9, respectively), which are continuously optimized in their "plus" versions (Kulcsár et al., 2020).

Besides the Cas9 protein, the sgRNA is also subject to improvements aiming at increased specificity. Modifications in its 5' region, with the addition of a small target region or the addition of two extra guanine nucleotides, led to a reduction in off-target mutations (Cho et al., 2014; Fu et al., 2014; Kim et al., 2014; Kim et al., 2015). However, such modifications also decrease mutagenesis efficiency for some targets (Cho et al., 2014).

Finally, the delivery of CRISPR/Cas9 components into cells must also be efficient, and the large size of the Cas9 protein (160 kDa) is a limiting factor (Mout et al., 2017). The diverse delivery mechanisms, their advantages and disadvantages will be covered throughout this book. In general, the size of the system components can influence the success of genome editing in plants, both in *Agrobacterium*-mediated transformation for stable or transient expression and in biolistics delivery of ribonucleoprotein complexes (Murovec et al., 2017). Thus, the development of SpCas9 mutants with deletions in redundant regions as well as the discovery of smaller orthologues, or even of alternative CRISPR systems which do not require tracrRNA, for example, have been reported (Cebrian- Serrano; Davies, 2017; Murovec et al., 2017). The discovery of other Class 2 effector enzymes opened up new application possibilities. The enzyme originally described as Cpf1 (now known as Cas12a), as well as its orthologues and modified variants (Shmakov et al., 2017; Chen et al., 2019), are of special interest due to some key differences when compared to Cas9. Cas12a does not require tracrRNA, its gRNA being almost half the size of that needed for Cas9 (~ 43 versus ~ 80 nucleotides, respectively) (Zetsche et al., 2015). Also, the Cas12a-crRNA complex promotes staggered cuts in a distal position to the T-rich PAM sequence (TTTN), which can facilitate the reduction of off-targets in GC-rich genomes (Zetsche et al., 2015; Fonfara et al., 2016; Chen et al., 2019). Since

the cleavage promoted by Cas12a results in cohesive ends in the double-stranded DNA, these can increase the efficiency of HR strategies (Figure 6) (Zaidi et al., 2017).

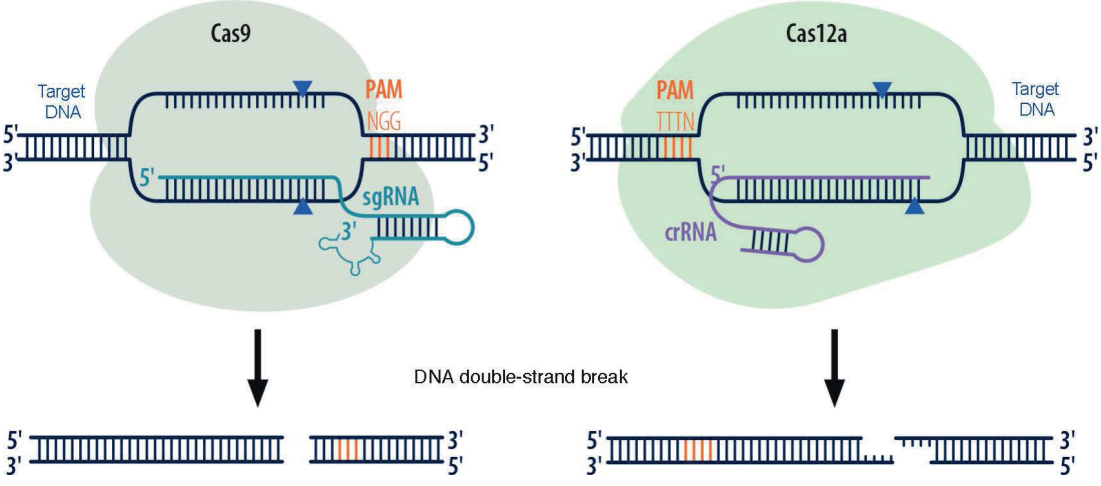

Figure 6. Representation of the main differences between the Cas9 (left) and Cas12a (right) nucleases. Blue triangles represent the cut position in the target molecule.

Source: adapted from Doudna and Charpentier (2014) and Zaidi et al. (2017).

Other Class 2 enzymes, such as Cms1 (*Microgenomates* and *Smithella*) and AaCas12b (*Alicyclobacillus acidiphilus*), also have interesting characteristics, such as smaller size than Cas9 and Cas12a, AT-rich PAM site (Begemann et al., 2017), or optimal activity at high temperatures (Teng et al., 2018). Finally, new effector proteins such as C2c2 (known as Cas13) and their variants have been modified to recognize and edit RNA targets (Abudayyeh et al., 2017) with the emerging potential for application in viral RNA interference in plants (Mahas et al., 2019).

CRISPR derived applications

The continuous development of the CRISPR technology, either by the improvement of engineered nucleases, the optimization of the sgRNA scaffold molecule, or yet by new delivery mechanisms into eukaryotic cells, has been successful in enhancing its efficiency and specificity. In addition to these improvements, new combinations of CRISPR with other biotechnological tools are bringing surprising advances to genome editing systems (Anzalone et al., 2020).

For example, new technologies are based on the ability of nickases and dCas9 to recognize specific genomic sites (guided by gRNAs) without causing double-strand breaks on the DNA molecule. The fusion of dCas9 to transcription factors can lead to the transcriptional activation and repression of target genes without promoting changes in the DNA itself (Bikard et al., 2013; Perez-Pinera et al., 2013). There are currently two major promising techniques based on nickases and dCas9: base editing and the emerging prime editing.

Base editing allows the direct and irreversible conversion of one nucleotide to another in a programmable way. The technique was first developed in 2016 and relies on a fusion between a defective Cas9 (dCas9/nCas9), a cytosine or adenosine deaminase, and an uracil glycosylase inhibitor (UGI). The modified Cas9 acts together with the sgRNA to bind to the DNA molecule at the target site. Then the (cytosine or adenosine) deaminase converts a single nucleotide at the desired target site (Anzalone et al., 2020; Mishra et al., 2020), while the UGI subverts the cell uracil excision repair pathway (Molla; Yang, 2019), assuring the conclusion of the correct edit. The technique allows the four types of transition mutations (C→T, G→A, A→G, and T→C) and has been used in rice, maize, wheat, potato, tomato, watermelon, and cotton (Mishra et al., 2020; Qin et al., 2020). Although it has been successfully applied in plants, the technique also has its limitations, including the unfeasibility of the eight types of transversions (C→A, C→G, G→C, G→T, A→C, A→T, T→A, and T→ G), which may restrict the targets it can mutate (Anzalone et al., 2020). In addition to DNA conversions, a variant of the technique also allows RNA mutations, converting adenine (A) to inosine (I). This technique is known as RBE (RNA base editor) (Cox et al., 2017).

On the other hand, the prime editing technique was recently developed aiming to make genome editing safer for human therapies (Anzalone et al., 2019). The technique, however, has already been successfully employed in the editing of wheat and rice genomes (Li et al., 2020a; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020). Its main advantages are the reduction of off-target mutations, and the flexibility to promote a greater variety of edits, rendering the technique more precise and versatile than other CRISPR alternatives. In summary, the technique uses a nCas9 fused to a modified reverse transcriptase (RT) enzyme. In this case, the guide RNA molecule also acts as a template for precise editing at the target location and is called pegRNA (prime editing guide RNA). The pioneering work demonstrated that the system is capable of recognizing and modifying the human genome through insertions, deletions, and precise single base editing (including the 12 possible base conversions), constituting a possible advantage over the single base editing method that uses cytosine or adenosine deaminase (Anzalone et al., 2019). However, because the template RNA molecule is more susceptible to damage from cellular

enzymes, the technique is not able to generate large DNA insertions or deletions such as those achieved by homologous recombination in which a donor DNA is added to the conventional CRISPR/Cas9 system. Therefore, prime editing is more of a complementary technique, like the other variations discussed previously, in which different types of editing require different genome editing tools.

Despite all efforts to achieve greater precision, efficiency and versatility in genome editing, one of the bottlenecks for its success in plants lies in the efficiency to deliver the system into cells. This factor is especially important when considering the diverse crop varieties that are little or inefficiently transformable, in addition to the long and laborious process of tissue culture. Therefore, the development of methodologies that associate transformation with the transient expression of morphogenic regulators such as *Wuschel* (WUS), *Baby Boom* (BBM), and *Shoot Meristemless* (STM) represents a breakthrough in reducing tissue culture time and increasing the efficiency in obtaining regenerant plants (Lowe et al., 2016; Zhang et al., 2019; Maher et al., 2020). This technique has already been successfully applied in maize, sorghum, sugar cane, rice, tobacco, tomato, grape, and potato (Steinwand; Ronald, 2020). Using CRISPR/Cas systems associated with the expression of these regulatory genes is one of the great promises to expand the use of genome editing tools in different crop varieties.

Application of CRISPR in agriculture

In February 2019, the first product derived from a crop with an edited genome began to be commercialized in the United States. High oleic acid soybean oil, is extracted from a variety developed through the TALEN system by Calyxt Inc. and entered the North American market without the regulation applicable to GMOs (Kim; Kim, 2019).

Over the past few years, site-directed mutation techniques such as ZFN and TALEN have been used to achieve desired traits in various crops, (e.g., rice, maize and soybean) (Jansing et al., 2019). Now, given the ease and versatility of genome editing via CRISPR/Cas, other techniques are becoming less and less used, whereas CRISPR-based studies are rapidly increasing.

Proof of concepts and technique improvements account for most of the current efforts on plant genome editing, being few the reports on field trials. Nevertheless, many of these studies have market-oriented applications, both in major crops and in less widespread plant species (Metje-Sprink et al., 2020). We will discuss next

some applications of CRISPR into the improvement of diverse agronomic traits, such as productivity, quality, and increased resistance to biotic and abiotic factors.

Productivity increase

Productivity is a complex trait, involving several factors that are often specific to each crop. The number and size of fruits and/or grains, plant architecture, and biomass are all examples of attributes influencing productivity (Chen et al., 2019). As many of these factors are quantitative and controlled by QTLs, genome editing via CRISPR/Cas presents itself as a powerful tool for productivity improvement. This is especially true since multiplex strategies can be easily used to edit different QTLs simultaneously (Rodríguez-Leal et al., 2017; Sedeek et al., 2019).

For example, the simultaneous knockout of three genes (*GW2*, *GW5,* and *TGW6*) that negatively regulate grains weight in rice led to an increase of approximately 30% of this trait. The potential of the tool is evident in the study: although individual mutants for each of these genes were already known, they were present in different genetic backgrounds. Moreover, mutant strains were obtained for the three genes still at the T_0 (primary transformant) generation, making it possible to segregate the T-DNA used for expression of the CRISPR system as early as T_1, thus resulting in non-transgenic mutant lines for the three genes (Xu et al., 2016). Similarly, grain length and weight were improved in two wheat (*Triticum aestivum*) genetic backgrounds via the knockout of three *TaGASR7* homologues. Using only one sgRNA targeting a conserved region, the authors were able to simultaneously knockout all six alleles of these genes in T_0 (Zhang et al., 2016).

In addition to CRISPR-mediated knockout, other strategies have already been used to improve productivity, such as promoter editing. In tomato (*Solanum lycopersicum*), the editing of cis-regulatory elements in genes from the *CLAVATA-WUSCHEL* circuit, responsible for controlling the size of meristems, led to different effects on fruit size (Rodríguez-Leal et al., 2017). This work demonstrates that modulating the expression of individual genes through promoter variants could enhance quantitative traits.

Quality improvement

Crop quality also involves a wide range of factors such as color, aroma, nutritional content, and shelf life. Nutritional content is especially important since vegetables

can be both directly used as food and as source of raw material for processed products. Thus, there are efforts to increase this trait through genome editing. For instance, the genome of *Camelina sativa* and *Brassica napus* were edited, resulting in plants that produce seeds with high oleic acid content (Jiang et al., 2017; Morineau et al., 2017; Okuzaki et al., 2018). In another example, the pathways for converting lycopene into α-carotene and β-carotene were inhibited in tomato through a multiplex approach, resulting in lycopene enriched fruits (Li et al., 2018a).

Other approaches seek to modulate metabolic pathways, increasing or decreasing the biosynthesis of certain molecules. For example, starch is the main reserve carbohydrate in plants and is widely used in industry. It is composed of two polysaccharides: amylose and amylopectin. In maize grains, starch is usually 75% amylopectin and 25% amylose (Ricroch, 2019). However, differences in this proportion are found in rice and maize varieties and reflect directly on the properties of the grains. Thus, varieties of rice with high amylose content develop firmer and well-separated grains after cooking, while varieties with a low amylose content result in softer and glutinous grains (Zhang et al., 2018a).

Two independent groups knocked out the *OsWaxy* gene via CRISPR in three rice varieties (T65, XS134, and 9522) to reduce their amylose content. Both groups achieved similar results: a reduction in amylose content from approximately 20%-15% to 2.5% (Ma et al., 2015; Zhang et al., 2018a). The rapid development of these lines is especially interesting as the XS134 and 9522 elite varieties did not show changes in other traits of agronomic interest (plant height, number of grains per panicle, number of panicles per plant, size and grain weight) after *OsWaxy* gene edition (Zhang et al., 2018a). Also by knocking out the *Wx1* gene via CRISPR, DuPont Pioneer produced a variety of maize with starch composed only by amylopectin (Waltz, 2016; Ricroch, 2019). The reverse effect was also achieved in rice: by knocking out the *SBEIIb* gene via CRISPR, plants with high amylose content were developed. These plants are a potential source of resistant starch, important for reducing risks of non-infectious diet-related chronic diseases (Sun et al., 2017).

Elimination of undesirable traits

Plant quality can often be improved by eliminating undesirable characteristics. Gluten proteins, for example, are known to trigger celiac disease in 0.7% to 2% of the world population (Rewers, 2005). In wheat, the largest gene family that encodes gluten proteins (α-gliadin) has almost 100 genes and pseudogenes (Ozuna et al., 2015), hindering the use of conventional mutagenesis and selection methods to generate wheat varieties with low immunogenic gluten activity. Although a large number of target genes may suggest a multiplex approach, one group was able

to simultaneously knockout most of the conserved α-gliadin domains using only two sgRNAs. As a result, wheat non-transgenic lines with low gluten content were created, presenting a reduction of up to 85% in their immunoreactivity (Sánchez-León et al., 2018).

Genome editing by CRISPR was also carried out in potato (*Solanum tuberosum*) aiming the complete elimination of steroidal glycoalkaloids, substances that have a bitter taste and can be toxic if ingested in large quantities (Nakayasu et al., 2018). In this case, the study still represents an initial step in the development of a variety with commercial potential. Given the low editing efficiency resulting from the potato tetraploid genome, the work was developed in a system known as hairy roots. However, it was an important step in identifying a gene that, when mutated, interrupts the glycoalkaloid synthesis pathway.

Another trait eliminated by genome editing is the browning caused by polyphenol oxidase enzymes (PPO). Although PPO silencing via RNAi had already been performed on apples by Okanagan Specialty Fruits (Waltz, 2015), there are now PPO knockout initiatives based on targeted mutagenesis. This is the case of Calyxt, which using TALENs, developed a non-browning potato variety (Ricroch, 2019). The CRISPR/Cas9 system was used to knockout a PPO in champignons (*Agaricus bisporus*), improving the product's visual appearance and shelf life (Waltz, 2016; Ricroch, 2019).

Resistance to biotic factors

Diseases and pests are among the greatest threats to modern agriculture. Thus, improving tolerance to biotic stresses is an important demand for the application of genome editing (Ricroch et al., 2016). Given their significance, the molecular and genetic basis of numerous plant diseases (as well as their resistance pathways) are already known, which facilitates the use of CRISPR to improve resistance to bacteria, fungi, and viruses.

A common approach to promote plant immunization is the knockout of the host plant factors used by pathogens to establish its infection and replication (Sedeek et al., 2019). For example, the *CsLOB1* gene confers susceptibility to the citrus bacterial canker, caused by *Xanthomonas citri* (Hu et al., 2014). By editing both promoter and coding regions of this gene, citrus canker resistant orange (*Citrus sinensis*) and grapefruit (*Citrus x paradisi*) lines were obtained (Jia et al., 2017; Peng et al., 2017). In rice, bacterial blight is caused by *Xanthomonas oryzae*, which uses the effector PthXo2 to induce the expression of the *OsSWEET13* gene in the host plant. This gene, related to sucrose transport, seems to be paramount for the *Xanthomonas*

infectious process. Accordingly, mutations promoted by CRISPR/Cas9 in the coding region of *OsSWEET13* resulted in plants resistant to the infection (Zhou et al., 2015).

Similar strategies have been used to promote resistance to fungal diseases. Powdery mildew (*Blumeria graminis*) resistant wheat was obtained after editing (by CRISPR/Cas9 and TALEN) three homologues of the *MILDEW-RESISTANCE LOCUS O* gene (*TaMLO-A1*, *TaMLO-B1,* and *TaMLO-D1*) (Wang et al., 2014). Similarly, mutation of their tomato orthologue (*SlMlo1*) conferred resistance to *Oidium neolycopersici*. As the work points out, although spontaneous *slmlo1* mutants are available, the introgression of such allele into elite cultivars would be a long and laborious process whereas mutation of *SlMlo1* via CRISPR led to the development of non-transgenic plants of the elite "Moneymaker" variety in just 10 months (Nekrasov et al., 2017). In another example, resistance to rice blast disease (*Magnaporthe oryzae*) was achieved after editing the ethylene-responsive transcription factor *OsERF922* (Wang et al., 2016). Again, non-transgenic resistant plants were obtained without any changes in other agronomic traits.

Finally, CRISPR/Cas can also perform its original role: conferring resistance to viruses. Several studies demonstrate the potential of CRISPR/Cas9 against different DNA viruses, such as the Tomato Yellow Leaf Curl Virus (TYLCV), Beet Curly Top Virus (BCTV), Merremia Mosaic Virus (MeMV), Bean Yellow Dwarf Virus (BeYDV), and Beet Severe Curly Top Virus (BSCTV) (Ali et al., 2015; Baltes et al., 2015; Ji et al., 2015; Ali et al., 2016). Interestingly, interference with different geminiviruses was achieved targeting a conserved region with a single sgRNA (Ali et al., 2015). However, it is worth mentioning that these are mostly proof-of-concept studies, based on the *Nicotiana benthamiana* and *Arabidopsis thaliana* models. Also, the interference mechanism depends on the presence of both Cas9 and sgRNAs in plant tissues, so that application of the system requires the endogenous expression of such components, thus implying in a GMO. An alternative to transgenics would be the direct delivery of RNPs (ribonucleoproteins), a methodology that will be addressed in Chapter 2. There is also evidence that the RNA-cleaving CRISPR/Cas13 system (Class 2, type VI) has the potential to be used against RNA viruses (Aman et al., 2018; Mahas et al., 2019).

Resistance to abiotic factors

Though abiotic stresses were always significant threats to the global food production, the current climate change scenario is greatly concerning given that drought and extreme temperatures have been consistently rising (Tong; Ebi, 2019; Sippel et al., 2020). Although there is a strong demand for improved abiotic stress tolerance, such initiatives are hampered by the complexity of metabolic pathways

involved in stress response in plants. Nonetheless, there are some successful efforts in crop improvement of abiotic stress resistance.

For instance, researchers at DuPont Pioneer were able to improve drought tolerance in maize by editing the *ARGOS8* gene, an inhibitor of the ethylene response that is usually expressed in low levels. Through an HR approach, the promoter of another gene, *GOS2*, was inserted in the 5'UTR region of *ARGOS8*, thus increasing its expression. As a result, the lines developed survive longer and have improved productivity under drought conditions (Shi et al., 2017).

In rice, CRISPR/Cas9 was used to knockout genes from the *OsPYL* family, which is involved in abscisic acid (ABA) response. The triple *pyl1/4/6* mutant has higher grain yield and improved tolerance to high temperatures. However, the same triple mutant appears to be more sensitive to drought (Miao et al., 2018). This work demonstrates how the response pathways to different stresses can be related, representing a challenge for plant breeding even with the aid of genome editing tools.

Accelerated and *de novo* domestication of wild species

So far, we have covered the application of genome editing to improve specific traits. However, CRISPR has also been applied in a different and increasingly interesting approach: the *de novo* domestication of wild species.

One of the negative effects of classical breeding is the loss of genetic variability. This genetic erosion occurs as a consequence of prioritizing traits of agronomic interest (such as grain and biomass yield), at the expense of quality characteristics (e.g., tolerance to pests and diseases) (Doebley et al., 2006). Thus, over time the process creates cultivars that are more susceptible to pathogens and/or abiotic stresses. To overcome this problem, elite lines are often crossed with their wild parental species, leading to the introgression of genes that may confer the desired resistance or trait (Mazur; Tingey, 1995; Jacobsen; Schouten, 2007; Harrison; Larson, 2014). This process, however, requires long periods to carry out backcrosses that aim to segregate unwanted loci.

The *de novo* domestication applies the inverse rational: key genes are edited in wild species to make them more interesting for large-scale cultivation. Therefore, whereas desirable traits are introduced in such species, their genetic variability is preserved (Zsögön et al., 2017).

The idea to use genome editing for rapid *de novo* domestication of wild species dates from the mid-2010s. An example is a well-structured proposal for the domestication of pennycress (*Thlaspi arvense,* Brassicaceae) as an oilseed crop. The extensive knowledge about the model plant *Arabidopsis thaliana* (also a Brassicaceae) was extrapolated to select genes which could be edited in *T. arvense* in a fast and efficient manner to obtain the desired agronomic traits (Sedbrook et al., 2014).

The first publication describing the *de novo* domestication of a wild species by CRISPR/Cas occurred in 2018. A wild tomato species (*Solanum pimpinellifollium*) had six loci of its genome simultaneously targeted. Such loci were chosen based on their recognized importance for yield in the commercial tomato species (*S. lycopersicum*). Accordingly, after few generations a *S. pimpinellifollium* line with improved characteristics was obtained. Among the enhanced traits, fruit size and yield were increased three and ten-fold, respectively. Also, this line presents advantages over the current tomato crop, such as fruits with 500% higher lycopene content, in addition to the parental stress tolerance retention (Zsögön et al., 2018).

In a similar effort, four *S. pimpinellifollium* accessions were edited by CRISPR/Cas9 through a multiplex approach, resulting in plants with domesticated phenotypes (compact morphology, increased number of flowers and fruits, as well as increased ascorbic acid content). Remarkably, such plants retained the parental tolerance to pathogens (Li et al., 2018b).

Finally, genome editing has the potential to improve orphan crops, which are species of regional importance but not commercialized internationally on a large scale, thus receiving little or no attention from researchers when compared to the most widespread crops (Varshney et al., 2012). This is the case of *Physalis pruinosa*, another Solanaceae species, which was also edited via CRISPR/Cas9 for improvement of plant architecture, increased flower production, and fruit size (Lemmon et al., 2018).

Perspectives and conclusions

The development of genome editing techniques, such as TALENs, ZFNs, and CRISPR/Cas, brought extraordinary contributions to modern agriculture over the past three decades. The CRISPR/Cas system stands out for being an unprecedented technology in terms of simplicity, specificity, versatility, and low cost. It has been successfully applied to quickly generate plants with enhanced productivity and quality, in addition to increased resistance to biotic and abiotic factors, bringing benefits to producers, consumers, and the environment.

Constant improvements and adaptations of the CRISPR/Cas systems have expanded the technology applications, such as the mutagenesis of an increasing number of specific loci, transcriptional regulation, epigenome editing, single base editing, multiplex mutations, replacement of target sequences, among others.

Unlike the GMO approach, introducing a desired trait via gene editing does not require the integration of exogenous DNA into the genome of interest. Consequently, this technology is also leading to changes in food and agricultural products regulation, which can facilitate the applied research at public universities or small companies.

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CHAPTER 2

Guilherme Souza Prado
Thaísa Tessutti Pinheiro
Josias Correa de Faria
Rosana Pereira Vianello

Genome editing via non-homologous end-joining (NHEJ) and ribonucleoproteins (RNP)

Introduction

Genome editing is a tool that allows the manipulation of genetic material to induce mutations in regions of interest so that the organism presents a desirable phenotype, and, in some cases, may not be considered transgenic. Because of this, its application in plants of agronomic interest has aroused great interest, mainly for simplifying regulatory steps.

Four main classes of nucleases are used in new gene-editing technologies, namely: meganucleases, zinc-finger nucleases (ZFNs); transcription activator-like effector nucleases (TALENs); and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) -associated (Cas) (Gaj et al., 2016). Meganucleases are endonucleases that cleave DNA in specific regions, recognizing sequences larger than 12 bp (base pairs). The LAGLIDADG family of meganucleases comprises I-CreI and I-SceI, which are the first enzymes that were used for gene editing. As only a few amino acid residues make contact with nucleotides, these enzymes have been manipulated to cleave genes at specific locations (Paques; Duchateau, 2007). Also, ZFNs are artificial enzymes that were also one of the first to be used to induce targeted mutations in plants. These enzymes were generated from the fusion between Zinc-Finger domains and FokI restriction enzyme domains. Like the other nucleases used in gene editing, the ZFN inserts breaks in the double-strand (DSBs- double-strand break) at specific locations in the DNA that are going to be repaired and, through failures during the repair mechanism, mutations can arise (Carroll, 2011). The main problem with the use of this system is the high toxicity of this enzyme and also the fact that it generates many off-target effects (Cornu et al., 2008; Ramirez et al., 2008), which can impair functions of genes that should not have its functioning changed (Zhang et al., 2015). With the consolidation of gene editing as a biotechnological tool and the search for more viable alternatives, the TALEN technique was developed from the fusion of TAL effectors (transcription

activator-like), proteins derived from pathogens that infect plants and can bind to DNA, to the FokI endonuclease domain (Moscow; Bogdanove, 2009). TALENs can be generated from a plasmid library that allows the assembly of the protein of interest in a combinatorial way. Although this technique has advantages over ZFN, because it uses proteins that are less toxic and easier to manipulate (Mussolino et al., 2014), it is still considered more laborious when compared to CRISPR (Nemudryi et al., 2014).

Among the techniques that have made genome editing more accessible and revolutionary, the CRISPR/Cas system stands out. This is due to the simplicity, low cost, and high efficiency of the technique when compared to other editing tools, such as ZFNs and TALENs. Due to this, the CRISPR/Cas system has become widely used in molecular biology laboratories on a routine basis in recent years and is currently the main genome editing tool used. The CRISPR system is based on the induction of mutations in DNA through cuts in the double-strand, in an extremely specific and targeted manner. These cuts are promoted by endonucleases from the Cas family and directed by the so-called guide RNAs (gRNA). They will be repaired by one of the two main repair pathways that the cell presents when the double-strand is damaged: homologous recombination (HR) or non-homologous end-joining (NHEJ) (Cardona; Morales, 2014). More details can be found in Chapter 1.

We will focus here on the use of the CRISPR/Cas system to induce mutations via NHEJ. The NHEJ mechanism acts in most of the cell cycle and is not based on homology to repair DNA, causing mutations such as insertions or deletions (indels) or even substitutions. In this way, the expression of the genes in the place where the repair occurred by NHEJ can, in general, be interrupted, and the encoded proteins may not be functional (Cui et al., 2019). To clarify the process of genome editing by CRISPR, this chapter aims to address the details of the NHEJ mechanism, as well as the use of ribonucleoprotein systems (RNPs) to promote editing, in addition to providing a practical procedure for the design of experiments.

Mechanisms of genetic repair

The CRISPR system acts promoting specific cuts in the double-stranded DNA in genomic regions in a targeted manner using a guide RNA (gRNA) which is then repaired, may leading to the modulation of genes or promoter regions. Thus, clarifying the main repair systems used by the cell to reconstruct the double-strand becomes essential for understanding the technique. The importance of the genetic information being transmitted through the generations in a precise way is evident, both by the efficient replication system and by the complexity and investment of

the cell in a repair system specialized in the correction of the diverse types of DNA disorders.

With this objective, to minimize the modifications in DNA, the cells have a mechanism of response to damage. One of them is the DDR (DNA Damage Response) system, which is efficient in DNA repair and acts by several pathways and enzymes, depending on the type of injury that has occurred. In this mechanism, when cells suffer damage, the replication process is suspended until the repair is carried out (Silva; Ideker, 2019). For this, a signaling cascade occurs generating changes in the chromatin, leaving the damaged end accessible to DNA repair proteins (Riches et al., 2008). With the damaged DNA end exposed, the Mre11-Rad50-NBS1 (MRN) complex binds to the DSB, recruiting proteins members of the MAT (Mutated Ataxia Telangiectasia) family, which regulate the response to the damage through phosphorylation (Lavin; Kozlov, 2007). Thus, cell cycle checkpoints and chromatin remodeling are activated; everything so that the DNA is repaired before the cell cycle continues (Silva; Ideker, 2019). However, in multicellular animals, if the damage to the genetic material is irremediable, apoptosis is activated so that the lesion is not perpetuated (Norbury; Zhivotovsky, 2004).

For the cell to repair the lesion in the genetic material, five main repair pathways are activated at different moments in the cell cycle. When the damage to the genetic material occurs in only one of the DNA strands (SSB, single-strand break), the repair mechanisms are of the mismatch repair (MMR) type, base excision repair (BER), and nucleotide excision repair (NER). However, when DNA is damaged in both strands (DSB), repair pathways can occur by homologous recombination (HR) or by non-homologous end-joining (NHEJ) (Cardona; Morales, 2014), which are the molecular basis of CRISPR technology.

Non-homologous end-joining (NHEJ)

The NHEJ pathway does not require template DNA and is responsible for 75% of repairs to the genetic material of animal cells (Mao et al., 2008). This repair path takes about 30 minutes to complete, while HR is completed within 7 hours or more (Mao et al., 2008). In the NHEJ pathway, when the DSB is repaired, indels are generally formed which, by modifying the original DNA sequence, can cause gene inactivation (Her; Bunting, 2018). NHEJ repair employs, for this, a series of essential factors that work to detect the DSBs, as well as align and repair the ends so that they can be connected again (Her; Bunting, 2018).

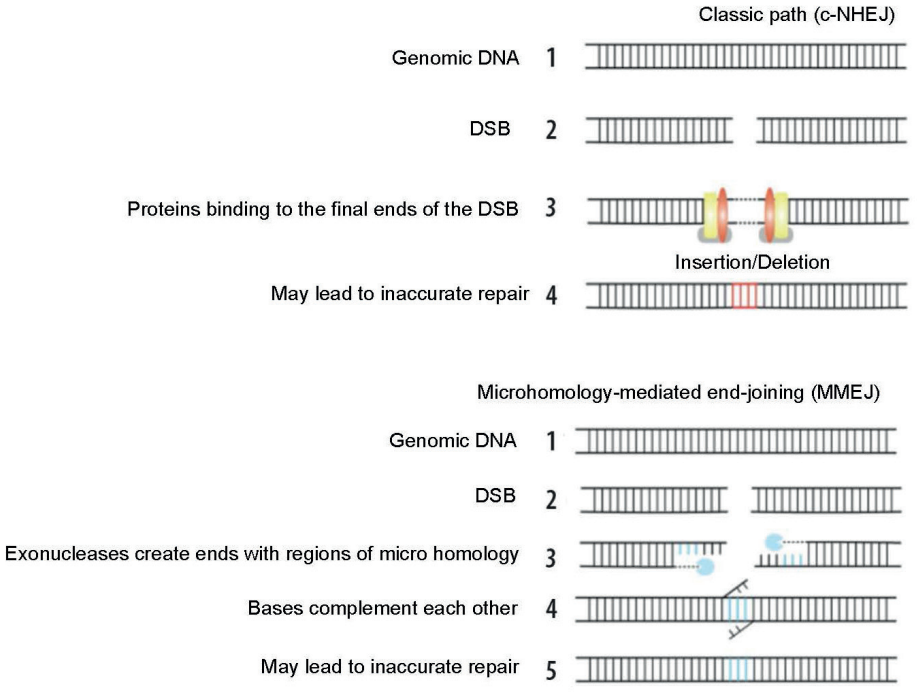


Figure 1. Repair pathways by non-homologous end-joining (NHEJ). (A) Classic non-homologous end-joining (c-NHEJ), which can lead to insertions or deletions (indels). (B) Final union mediated by microhomology (MMEJ), which always results in deletions.

Source: adapted from Deriano and Roth (2013).

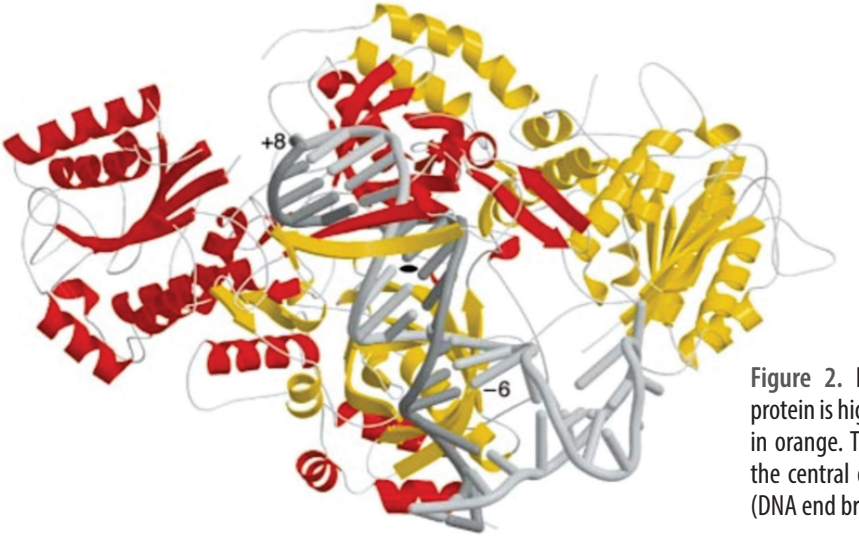


Figure 2. Ku complex: DNA. Ku70 protein is highlighted in red, and Ku80 in orange. The terminal base pairs of the central duplex are numbered +8 (DNA end broken) and -6.

Source: adapted from Walker et al. (2001).

Biochemical mechanism

In the presence of the DSB, the DDR damage response mechanism is activated. Thereby, a cascade of phosphorylation is activated, generating changes in chromatin, which leave the damaged end accessible to DNA repair proteins (Riches et al., 2008). In plants (Steinert et al., 2016) and mammals (Hartlerode; Scully, 2009), both HR and NHEJ are triggered.

NHEJ, in turn, can be subdivided into two routes: classic (c-NHEJ), which is the main chromosomal repair and rearrangement route (Lieber, 2010), and backup (b-NHEJ), also called alternative (a-NHEJ) or microhomology-mediated end-joining (MMEJ) (Deriano; Roth, 2013). This second pathway is activated if functional problems occur with c-NHEJ. However, it does not have such a precise repair mechanism, which generally leads to chromosomal translocations and excessive deletions (Deriano; Roth, 2013) (Figure 1).

The c-NHEJ pathway can be divided into three main stages: DSB recognition, DNA processing, and ligation (Yang et al., 2016). DSB recognition depends on Ku70-Ku80 (Ku) proteins, a heterodimer with two subunits of 70 kDa and 80 kDa, which intertwine in a ring that surrounds the end of the DSB, forming the Ku:DNA complex (Figure 2). This complex keeps the ends of the DSB relatively close so that there are no additional translocations or mergers (Downs; Jackson, 2004).

As Ku:DNA complex is formed at each end of the DSB, protein factors are recruited, such as the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), complementary transversal X-ray repair protein 4 (XRCC4), factor type XLF, PAXX (paralogous to XRCC4 and XLF) and DNA-Ligase IV (Lig4), which keep the ends of the DNA close, forming a bridge between them for processing and ligation to occur (Davis et al., 2014).

If the DSB generates simple ends, they can be directly connected after the DSB is recognized. However, in most DSBs there are incompatible ends due to chemical modifications or incompatibility of the ends of the DNA strands, which end up preventing the strands from being connected directly. Thus, the DNA must be processed, and, for that, exonucleases degrade the 5'or 3' ends, generating regions of microhomology. This process, known as a recession, allows the two ends to become compatible.

During the recession of the final ends of the DSB, the DNA-PKcs act in a complex with the endonuclease Artemis which, after being phosphorylated by the DNA-PKcs, cleaves bonds from the single-strand and between the double-strands. Other factors involved in this process are polynucleotide kinase phosphatase (PNKP),

aprataxin (APTX), APTX-polynucleotide kinase-phosphatase-like factor 1 (APLF or PALF), werner (WRN), DNA polymerases, and deoxynucleotidyl terminal transferase (TdT) (Lieber, 2010).

After DSB processing, the ends are ready to be connected. All DNA processing and binding are coordinated by DNA-PKcs, which phosphorylate several proteins (Jiang et al., 2015). These proteins can also be autophosphorylated or trans-phosphorylated by other factors, such as ATM and ataxia related to telangiectasia (ART) (Davis et al., 2014). For double-strand binding to occur, specific ligases and the XRCC4-XLF-Lig4 complex are recruited, which is the main factor involved. For the formation of this complex, XRCC4 is anchored to the Ku:DNA-PKcs complex and, together with the XLF factor, forms a bridge with Lig4, promoting the connection of the DSB (Andres et al., 2012).

Genome editing via NHEJ – practical application

The application of the CRISPR/Cas system for genome editing in plants has been used successfully to obtain genetically improved cultivars. The characteristics manipulated with this tool range from nutritional improvement, efficient use of nutrients (Liang et al., 2014), disease resistance (Wang et al., 2014, Peng et al., 2017), tolerance to abiotic stresses (Mushtaq et al., 2018) until the increase in productivity (Ma et al., 2016). Thus, one of the aspects of improvement using CRISPR is the knockout, which eliminates gene function through indels derived from NHEJ repair.

Aiming at the resistance of wheat plants (*Triticum aestivum*) to the powdery mildew fungus (*Blumeria graminis* f. Sp. *Tritici*), gene editing was carried out via TALEN to introduce targeted mutations in three homologous genes encoding the Molo-Resistance Locus (MLO) proteins (*TaMLO-A1*, *TaMLO-B1,* and *TaMLO-D1*), and also editing via CRISPR to knockout the *TaMLO-A1* gene. MLO proteins act as negative regulators, repressing the plant's defense pathways against powdery mildew in uninfected tissues (Piffanelli et al., 2002). Thus, when the homologous MLO genes were edited via TALEN and CRISPR, there was an increase in the resistance of wheat plants to powdery mildew when compared to unedited plants. For the CRISPR construct, an expression vector containing the Cas9 CDS, the Ubiquitin 1 corn promoter, and the sgRNA was used. The vector was introduced into the wheat genome via biolistics, resulting in a mutation efficiency in regenerated plants equal to 5.6% (Wang et al., 2014).

In another study, researchers worked with maize (*Zea mays*), which has a high concentration of phytic acid, an antinutrient that is not digested by monogastric

animals and impairs the absorption of nutrients. With the use of CRISPR, a knockout was performed on the gene *Zea mays* Inositol Phosphate Kinase (*ZmIPK*), generating edited plants with a low concentration of phytic acid. In this study, the genetic transformation via protoplast transfection using two guides constructed to direct Cas9 to the *ZmIPK* gene showed a mutation efficiency of 16.4% and 19.1% for each guide used (Liang et al., 2014).

For soybean (*Glycine max* (L.) Merrill), the first successful work conducted as a proof of concept using the CRISPR system to perform directed mutagenesis in roots (hairy roots) demonstrated the effectiveness of the technique by turning off endogenous genes and the *bar* gene (Cai et al., 2015). In this study, seven vectors were constructed, one with gRNAs for the transgene *bar* and other six with gRNAs targeting two endogenous soybean genes (*GmFEI2* and *GmSHR*), with three guides for each gene (*GmFEI2-SP1*, *GmFEI2-SP2,* and *GmFEI2- SP3* for the *GmFEI2* gene and *GmSHR-SP1*, *GmSHR-SP2* and *GmSHRSP3* for *GmSHR*). Cas9 expression was driven by the maize Ubiquitin promoter, the gRNAs were driven by the *Arabidopsis* U6 promoter, and the GFP gene expression, for rapid visualization of transgenic hairy roots, was driven by the CAMV 35S promoter. The genetic transformation method used was based on *Agrobacterium rhizogenes* and, as plant material, transgenic homozygous plants were used for the *bar* gene (for editing the *bar* gene) and wild plants, not genetically transformed, for the editing of the *GmFEI2* and *GmSHR* genes. For the transgene *bar*, 11 mutations were identified in the 30 independent transgenic hairy roots analyzed, with a frequency of 1.3% to 21.0%. As for endogenous genes, in the *GmFEI2-SP1* guide, mutations were identified in 18 of the 30 independent transgenic hairy roots, with an indel frequency between 0.6% and 18.8%. Mutations at the site of the *GmFEI2-SP2* guide were identified in 28 of the 30 hairy roots, with the frequency of indels ranging from 1.0% to 95.0%. For the *GmFEI2-SP3* guide, 3 mutations were identified in the 30 independent transgenic hairy roots analyzed, with indels frequency between 5.4% and 27.0%. As for the *GmSHR-SP2* gene, 10 out of 22 hairy roots were identified, with an indel frequency of 8.7% to 30.0%. For *GmSHR-SP1*, mutations were found in 15 of the 30 roots analyzed, with the frequency of indels ranging from 2.3% to 21.3%. At the *GmSHR-SP2* site, mutations were found in 10 of the 22 roots analyzed, with the frequency of indels ranging from 8.7% to 30.0% (Cai et al., 2015). In *GmSHR-SP3* mutations were identified in 10 of the 28, with frequency ranging from 2.8% to 28.7%.

Promoter modulation and optimization

The use of NHEJ to modulate gene expression can also be applied to regulatory elements, such as promoter regions. This strategy makes the transcriptional activity, based on the modulation of transcription factors access to the promoter, be altered and, as a consequence, there is a change in the expression of genes (Cong et al., 2013). When promoter sequences are modified using genome editing techniques, it is called in vivo promoter engineering (IPE) (Pandiarajan; Grover, 2018). Some studies have conducted this approach aiming at generating variants of promoters with altered transcriptional activity (Li et al., 2020).

To obtain rice plants more adapted to soils with high salinity content, CRISPR technology was used to generate indels in regions of functional promoter units, the cis-regulatory elements (CREs) of the *OsRAV2* gene. This system was established to confirm that deletions in CREs of the promoter sequence of the *OsRAV2* gene (in GT-1 elements) would be related to resistance to saline stress. The *RAV* (Related to ABI3/VP1) genes encode proteins involved in the response to abiotic stress, with *OsRAV2* being specific in the response to environments with excess salinity in rice. In this way, a vector containing Cas9 and a gRNA directed to the GT-1 elements of the promoter region of the *OsRAV2* gene was constructed, and the genetic transformation was carried out via *Agrobacterium tumefaciens*. In this study, 12 plants were edited, and all showed the desired targeted mutations, which promoted the resistance of the plants to soils with high salt concentration (Duan et al., 2016).

In a study involving citrus canker, caused by *Xanthomonas citri*, a pathogen responsible for significant losses in the cultivation of *Citrus* spp., CRISPR was used in *Citrus sinensis* Osbeck, aiming at the modulation of the Lateral Organ Boundaries 1 (*CsLOB1*) gene promoter, associated with susceptibility to the pathogen. The gene had its induction in response to the infection stopped, and this approach generated plants with greater resistance to citrus canker compared to the wild type. To obtain the plants, a vector was coded for Cas9, in addition to the AtU6-1 promoter directing the expression of the gRNA directed to the promoter region of the *CsLOB*1 gene. The genetic transformation was carried out by *A. tumefaciens*, and the mutation efficiency was between 32.5% and 90.7% (Peng et al., 2017).

Ribonucleoprotein (RNPs) nuclease-sgRNA

RNPs: DNA-free system

Genome editing or engineering is, in most cases, conducted using plasmid vector systems carrying genes that, when integrated into the host's genome, encode the expression of the products necessary for the editing mechanism, being a nuclease and an RNA-guide (gRNA), in addition to a transformant selection marker gene, which confers resistance to an antibiotic or herbicide, when the target organism is a plant, and, eventually, reporter genes (Xie; Yang, 2013).

With this, new characteristics are incorporated into the host both due to the mutagenesis triggered in the host through the editing and due to the exogenous genes, that are introduced, which configures the event obtained as a transgenic GMO. In this case, concerns and problems related to regulation in biosafety and bioethics can become obstacles for the developed biotechnological product to become a market reality. To circumvent and/or avoid barriers that delay the arrival of the edited organism to the market, researchers usually appeal to methods that employ the elimination of inserted sequences utilizing hybridization and Mendelian segregation techniques, so that the edited organism is characterized only as GM, but not as transgenic, since the exogenous genetic material is lost (Cyranoski, 2015).

However, these techniques may become unfeasible for obtaining non-transgenic edited plants that have asexual reproduction processes, such as grapes (*Vitis* spp.), bananas (*Musa* spp.), and potatoes (*Solanum tuberosum*), due to the absence of recombination that would promote the elimination of transgenes in the offspring. They may also be little viable or little advantageous in the case of perennial plants, such as passion fruit (*Passiflora* spp.), eucalyptus (*Eucalyptus* spp.), and cherry (*Prunus* spp.), due to the impracticality of the transgenic elimination process, which becomes too long. Thus, plants with a short life cycle are more likely to be obtained through this method due to their greater practicality (Woo et al., 2015).

Given this scenario, the list of target plants becomes narrow, existing the need to develop or find alternatives to circumvent this limitation. Therefore, one of the strategies is the use of DNA-free gene-editing systems, from which ribonucleoproteins (RNPs) are the most well-known and its use in recent years has continued to expand to several species (Kanchiswamy, 2016).

In genome editing, RNPs are ribonucleoprotein complexes composed of a nuclease and one or more gRNAs. The gRNA is obtained by in vitro or chemical

synthesis, and it can also eventually be optimized so that its non-specificity is reduced, by truncating 17 nucleotide residues, unlike the 20 residues normally used for Cas9, for example. The gRNA is also stabilized when protected against degradation by RNases due to complexation with the nuclease (Kanchiswamy, 2016). The nuclease, in turn, can be obtained commercially from suppliers of biotechnology companies or expressed in recombinant form in a heterologous system, commonly bacterial systems based on *Escherichia coli*. After expression, the recombinant protein is purified, preferably by bioaffinity in a nickel chromatographic column, based on a 6-His-tag fused to one end of the nuclease.

According to reports in the scientific literature, RNPs are generally used to edit the genome of animal cells and, in comparison to plasmid vectors, they present very low cytotoxicity to the host. This is because cytotoxicity is often associated with the plasmid transfection process itself and with some reagents used to conduct the transfection.

Some advantages of RPNs compared to plasmids are highlighted below:

a) The plasmid mechanism is more complex, since it depends on the molecular machinery of transcription (for the nuclease and the gRNA) and translation (for the nuclease), since there is a need to integrate the genes of interest in the target genome, in the nucleus, so that the transcripts are produced and, in the case of genetic information for the nuclease, exported to the cytoplasm for gene translation, with the nuclease returning to the nucleus to perform the editing. With this, a continuous and complex transit is observed between different subcellular compartments, while in the use of RNPs the mechanism occurs directly since the preformed complex immediately goes to the nucleus and performs its function (Figure 3) (Amirkhanov; Stepanov, 2019; Wilbie et al., 2019).

b) The use of plasmids leads to gene integration, which culminates in the persistence of the expression of the editing agents, who remain functionally active for a long time, maintaining the editing activity, which increases the likelihood of off-target effects. On the other hand, RNPs do not depend on gene integration to carry out the editing, which occurs immediately after the delivery of the complex in the nucleus, with a peak of mutagenesis after about 24 hours, being rapidly degraded and therefore reducing the occurrence of off-target effects. (Amirkhanov; Stepanov, 2019).

c) In addition to the integration of the genes of interest, the plasmid backbone can also be integrated into the host genome and persist, even after the use of hybridization techniques, leaving molecular "footprints" (Mao et al., 2018),

which can become another problem in the regulation of the edited plant, as it characterizes a transgenic organism.

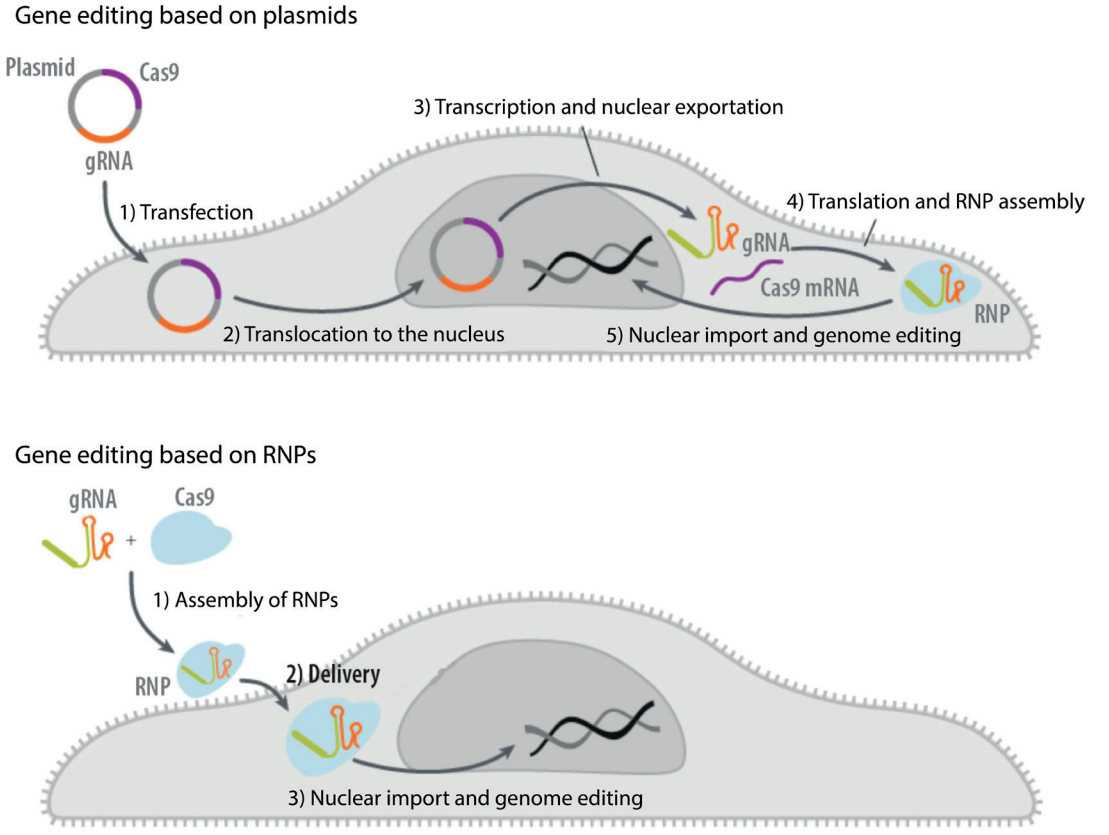


Figure 3. Plasmids versus RNPs in genome editing. (A) Plasmid vector carrying genes for the encoding of a Cas9 protein and a gRNA, showing stages of a more complex transit between different cellular compartments until the genome editing. (B) RNP is composed of a Cas9 complexed with a gRNA, showing a simple step of direct transit to the nucleus to carry out the editing of the genome.

Source: adapted from Spencer (2020).

RNPs are not the only DNA-free strategy for genome editing. Other systems have also been developed and their applications have been demonstrated not only for animal cells but also for plants of economic interest. Among them, IVTs (in vitro transcripts), and vectors based on viral replicons are alternatives that also dispense genomic integration, each of which has advantages and disadvantages

(Gil-Humanes et al., 2017; Liang et al., 2018). For the various advantages offered by vectors based on viral replicons, see Chapter 3.

Through the IVT system, mRNA molecules encoding the nuclease are delivered and, although IVT-derived mRNAs have the advantage of not integrating into the genome, mRNA molecules are more subject to degradation by RNases in the host's intracellular environment, resulting in less system stability. Also, long RNA molecules, such as those encoding the nuclease, are easily degraded during laboratory manipulation processes, making preparation difficult. Viral vectors, which are often used in replicon-based systems, require additional steps for cloning and genetic engineering that are unnecessary in the RNPs system (Gil-Humanes et al., 2017; Liang et al., 2018). While some viral vectors for animal cells lead to the integration of the viral genome into the host's DNA, in plants this does not occur, and the replicon remains active in the episomal form in the cytoplasm, dispensing the transgenic elimination steps of the edited plant.

As an example of reports of the application of RNPs in plants, Woo et al. (2015) established genome editing in plant systems via RNP for the first time, having as host organisms: *Arabidopsis thaliana*, rice (*Oryza sativa*), lettuce (*Lactuca sativa*) and tobacco-coyote (*Nicotiana attenuata*). The authors performed the transfection of protoplasts via PEG with pre-assembled RNPs, obtaining editing efficiency of up to 46% (8.4% to 44% for *A. thaliana*, rice, and tobacco, and 46% for lettuce). No off-target effects were detected and editing remained stable for the next generation. The mutations generated proved to be indistinguishable from the genetic variations that occurred naturally.

Svitashev et al. (2016) and Young et al. (2019), in turn, demonstrated the applicability of RNPs in maize (*Zea mays*), using particle bombardment for the transfection of the complex in immature embryos, aiming at the knockout of the *LIG1*, *Ms26,* and *Ms45* genes, located on different chromosomes. More specifically, Young et al. (2019) studied the off-target effects of this strategy compared to the use of plasmids, demonstrating the absence of these effects in the corn genome. Liang et al. (2017) compared RNPs with plasmid vectors in wheat (*Triticum aestivum*), verifying similarity of on-target activity, but five times less off-target effect when using RNPs, corroborating what was mentioned about the correlation between prolonged editing activity in the cell and the increase in off-target cases. Finally, Liang et al. (2018) compared RNPs with IVTs for *T. aestivum*, demonstrating that both were equivalent in terms of efficacy while eliminating genomic integration and giving rise to DNA-free (non-transgenic) edited plants. Other plant species have also been transfected with RNPs, such as grape (*Vitis vinifera*), apple (*Malus*

domestica) (Malnoy et al., 2016), petunia (*Petunia x hybrida*) (Subburaj et al., 2016), and potato (*S. tuberosum*) (Andersson et al., 2018)

In this case, the authors of these works used protoplasts as explants for transfection. Of these, only Andersson et al. (2018) regenerated tissues from the transfected protoplasts, obtaining sprouts derived from induced calli. For this work, the analysis of the editing efficiency was conducted using the leaf tissue itself as a source of genomic DNA sample, in which they concluded that 9% of the events obtained contained the expected mutation in at least one of the alleles of the target gene. On the other hand, both Malnoy et al. (2016) and Subburaj et al. (2016) analyzed genomic DNA samples obtained directly from protoplasts, without undergoing seedling regeneration. With that, Malnoy et al. (2016) reached an editing efficiency of 0.1% for grapes and 0.5% to 6.9% for apples, while Subburaj et al. (2016) obtained an efficiency of 2.4% to 21% in petunia.

As it is possible to observe, RNPs have wide-ranging applicability, with the potential to encompass other useful approaches for the development of edited and economically important plants that are DNA-free. Thus, like plasmids, IVTs, and vectors based on viral replicons, RNPs are suitable for generating knockout via indels from the NHEJ mechanism, as well as they can be adapted to knock-in from the HDR mechanism, which will be detailed in the next chapter. Besides, transcriptional regulation for editing and modulation of promoters via indels, aiming at overexpression or knockdown of genes, as well as for base editing (base editing) and the latest genome-accurate editing technology, prime editing, it is possible to be executed in plants using RNPs, which opens opportunities for research that demonstrate for the first time this viability and its success.

However, the limitation of the use of RNPs arises with approaches that require the persistence of the nuclease-sgRNA complex acting in the nucleus. This is because the nucleases of RNPs are degraded by intracellular proteinases, losing their biological activity as soon as their task of genome editing is completed. Therefore, studies involving modulation and epigenetic engineering, using activators and inhibitors via dCas9 in CRISPRa/CRISPRi system or studies aimed at imaging based on genomic marking with fluorescent probes, for example, become unfeasible through RNPs. Additionally, obtaining RNPs requires the in vitro production of sgRNA transcripts, which, once produced, have low durability due to their high sensitivity (Liang et al., 2018). Also, due to the factor mentioned concerning degradation, there is a short window available to RNPs to perform the editing, which usually leads to low mutagenesis efficiency by this mechanism. Finally, when working with RNPs, there is no use of selection marker genes that would assist in the discrimination of transformed explants, which makes RNPs a more laborious technique, as it

requires an additional step of molecular characterization to identify edited plants (Kanchiswamy, 2016).

In short, deepening and extending the use of RNPs in species already tested and in others not yet evaluated, respectively, is of great importance, within the technical limits of the approach, and can significantly impact world agriculture and, consequently, the quality of human life.

Delivery strategy and internalization of RNPs

The delivery of the molecular agents of genome editing is a fundamental step towards obtaining edited plants, and the parameters associated with it are directly linked to the success and practicality in the development of these events. Based on this, a wide variety of delivery methods for RNPs is already used or can be complemented with strategies not yet established for plants, although they are already widely applied to animal cells (Figure 4). These methods can be classified into:

1) **Simple transfection:** covers techniques for direct introduction of RNPs - although it is also used for plasmid systems and other mechanisms - in the host cell by physical or chemical methods, without the participation of complex and sophisticated reagents or systems to facilitate transfection. Examples:
   a) Electroporation: in the case of plants, it consists of the application of electric pulses in protoplasts, to promote the rapid opening of pores in the cell membrane, allowing the introduction of RNPs in the cell.
   b) PEG: polyethylene glycol is a polymer that promotes the internalization of RNPs and is often used for transfection of protoplasts as explants.
   c) Particle bombardment (biolistics): method mostly used for plants, consisting of the acceleration of microparticles composed, generally, of gold or tungsten, at speeds above 1.500 km/h, using the gene gun equipment. In this procedure, the RNP complex is prepared and precipitated on the microparticles, following well-established protocols. It is a technique classically used in the genetic transformation of plants. More information about this technique can be found in Chapter 5 of the book "Manual of genetic transformation of plants" (Vianna et al., 2015).
2) **Nanostructured systems**: includes polymers or other substances, produced in the form of particles on a nanometer scale, which act as nanocarriers for RNPs (Zuris et al., 2015). Li et al. (2015) reviewed potential non-viral vectors

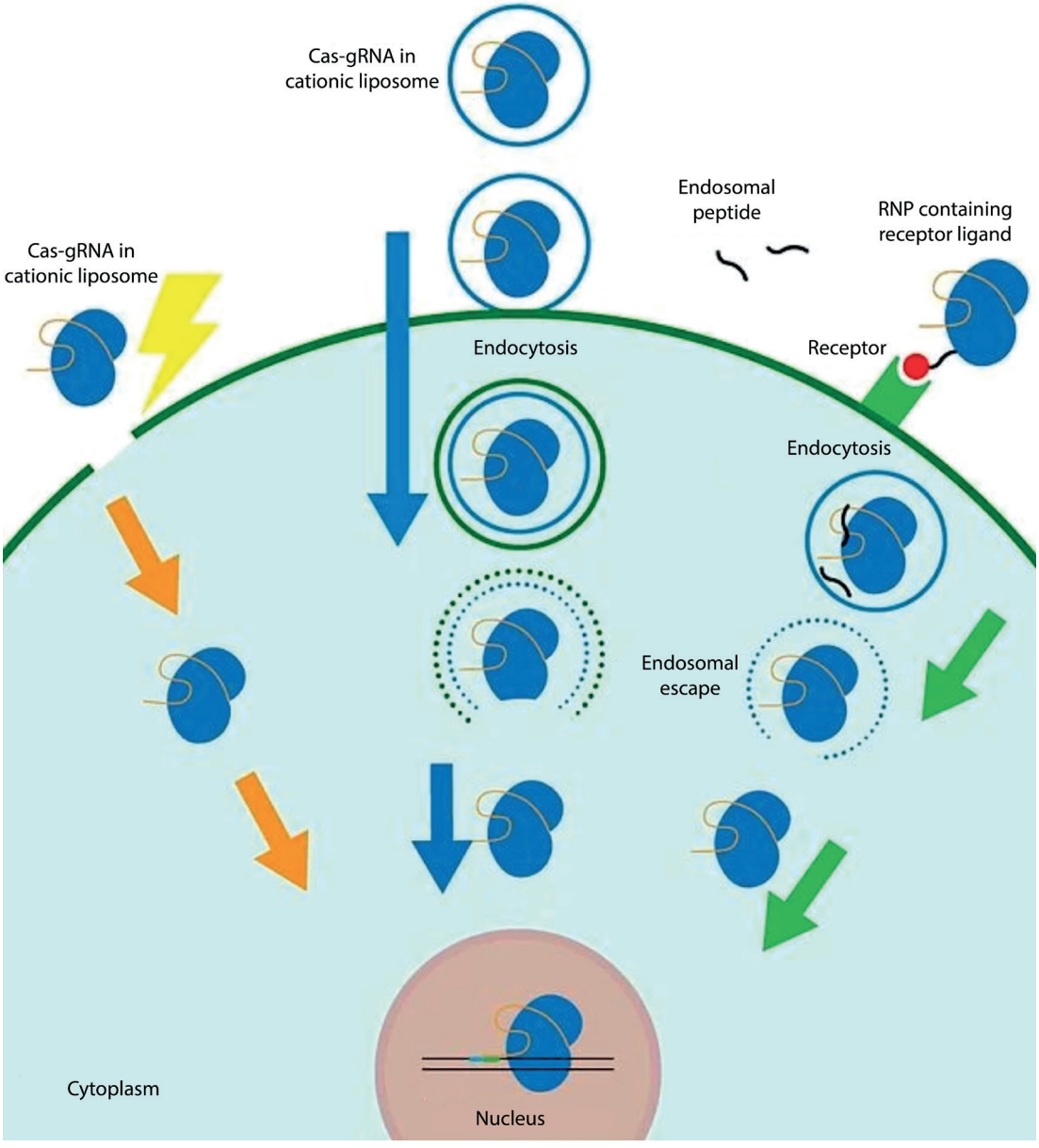


Figure 4. Delivery methods and internalization of RNPs. (A) Simple transfection - exemplified by electroporation. (B) Nanostructured systems - exemplified by the encapsulation of RNP in cationic liposomes. (C) Ligand-receptor mechanism - exemplified by RNP fused to a peptide and endosomal ligand. All methods culminate in editing the nuclear genome, with or without endocytosis as an intermediate step.

Source: adapted from Hampstead (2020).

in the form of nanostructured systems or nanomaterials for the delivery of CRISPR/Cas, which can perfectly be used shortly for editing in plants via RNPs, such as:

a) Cationic liposomes: spherical lipid bilayers with positive charges, enabling the binding to negatively charged nucleases, which, in turn, are complexed with sgRNA. They can be composed of DOTAP - N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate, DOTMA - N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride, DOSPA - 2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl -1-propanaminium trifluoroacetate and DMRIE - 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide.

b) Solid lipid nanoparticles (SLNs): nanoparticles consisting of lipids with a high melting point, especially fatty acids (e.g. stearic acid and palmitic acid), with a solid core and covered by surfactant molecules.

c) Polymeric nanoparticles: polymers in the form of nanometric particles, which may be composed of PEI (polyethyleneimine), PLL (poly-L-lysine), and PDMAEMA - poly [2- (dimethylamino) ethyl methacrylate, for example.

d) Chitosan nanoparticles: nanoparticles composed of a polysaccharide derived from chitin deacetylation and with cationic properties.

e) Dendrimers: highly positively charged polymers, functioning as "proton sponges", with a high affinity for negatively charged molecules. Poly(amidoamine) (PAMAM) is one of the main examples.

f) Nanoparticles of silica (Torney et al., 2007).

g) Magnetic nanoparticles (Zhao et al., 2017).

h) Carbon nanotubes (Demirer et al., 2019).

Although the use of RNPs for genome editing in plants is sporadic, their reports in the literature mostly involve the use of simple transfection. Only recently, Liu et al. (2020) conducted the transfection of tobacco protoplasts (*Nicotiana tabacum* cv. Bright Yellow-2) based on nanostructured systems for lipofection, using two lipid reagents - Lipofectamine 3000 and RNAiMAX- for the assembly of cationic liposomes, obtaining delivery efficiency of 66% and 48% for these reagents, respectively. For Lipofectamine 3000, the editing efficiency was 6%. Although lipofection is well established for genome editing in animal cells, only in this study a transition from nanomaterials to CRISPR/Cas was established in plant systems.

3) **Ligand-receptor mechanism**: cover the logic that the nuclease responsible for the editing process can be fused to peptide or protein ligands of specific cell receptors (Rouet et al., 2018). Although there are reports of the use of this approach for editing in animal cells, its applicability to CRISPR in plants has not yet been demonstrated, and neither do authors in the scientific literature have perspectives in this regard.

Excluding particle bombardment, for all methods and techniques used for delivery, it is important to emphasize the need to establish protocols that facilitate the use of these systems, mainly for in vitro regeneration of plants from protoplasts, since they are explants most used to conduct these procedures. However, knowing that some species are recalcitrant to regeneration from protoplasts, the development of such protocols would be unfeasible, and other strategies based on alternative types of explants should be chosen. Anyhow, for those species for which this procedure is possible, this benefit arises when working with RNPs.

Experimental procedure

Part 1: in silico

The first step to perform genome editing via CRISPR/Cas is to choose genomic targets of the desired species to be edited. This choice will serve as a basis to subsequently identify, within the respective genes, sites with sequences complementary to the gRNA used to guide the nuclease, since they meet the requirements of the respective PAM sequence required by the enzyme for cleavage. Also, it is essential to perform the prediction of potential off-target sites, to propose safer candidate gRNAs regarding genomic specificity. Finally, choosing a suitable delivery strategy, in particular, depending on the type of explant required for transfection, as well as an appropriate expression vector with appropriate configuration of its genetic elements, is the final and fundamental step - although they vary according to the objectives and the type of each study - a set of procedures that must be conducted in a complementary way to the *in-silico* analysis, as will be detailed below.

Identification and selection of genomic targets

Aiming at knockout in coding sequences (CDS) or transcriptional regulation of promoters (generating variants of promoters), both via NHEJ, the target gene must

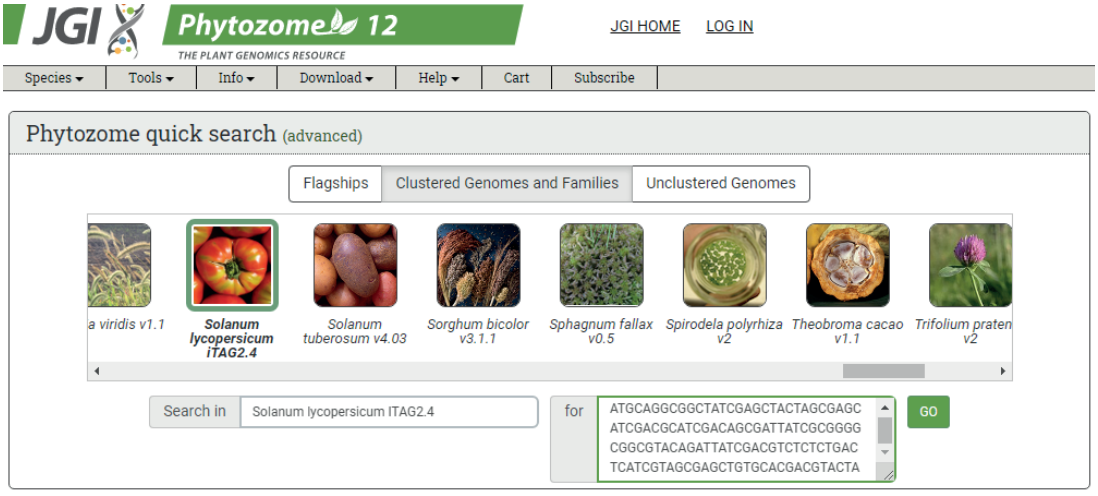


Figure 5. Phytozome v12.1. Search for sequences across the genome of different possible plant species. A search bar for inserting the nucleotide sequence of the selected species, on the right, is shown, enabling BLAST-mediated search, using the "GO" function.

Figure 6. Search for mature transcript sequences in Phytozome v12.1. Differentiation stage of the genetic elements that compose the nucleotide sequence of the gene after transcription, which will serve as a model for similarity in the analysis of candidate gRNAs. In green, the sequence related to the 5'-UTR region (useful for the selection of gRNAs for the transcriptional modulation of promoters). In blue, sequences related to neighboring exons (useful for the selection of gRNAs aiming at the knockout of the gene), separated by light and dark tones.

be characterized in terms of the nucleotide sequences that compose it, including its regulatory elements. In this case, it is possible to obtain the nucleotide sequences in the annotated database of the National Center for Biotechnology Information (NCBI)[1], using terms that identify the species and/or the sequence, as well as accession numbers, when possible. Another possibility is to obtain the complete gene sequence from smaller sequences (query), through similarity with sequences deposited (subject) in the database. For that, this information can be obtained using the different modalities of the Basic Local Alignment Search Tool (BLAST)[2] for different types of sequences.

However, the BLAST resource can be accessed indirectly and in a personalized way for many of the main plant species of economic importance, as, for example, in the Phytozome v12.1[3] database (Figure 5).

With the identification of the target sequence (Figure 6), the nucleotide composition can be detailed in the "Sequence" tab. The subsection "Transcript Sequence" provides the components of the mature transcript differentiated by colors between 5'-UTR, CDS, and 3'-UTR, which can be useful for the design of gRNAs for both approaches (CDS and regulatory elements). As in the NCBI, the sequence can be obtained in FASTA format and stored for gRNA analysis.

At the same time, it is recommended that in the case of CDS as a target, the sequence of the polypeptide chain should also be accessed and stored in any text editing tool, for another type of analysis, as will be shown below. In this case, in Phytozome it is possible to access the respective sequence in the subsection "Peptide Sequence", within the same tab "Sequence", while in NCBI the respective sequences are shown on the page of the characteristics table of each access, in the section "CDS".

gRNA design

Having the target site delimited in silico and containing its genetic elements, one should, depending on the strategy, design one or more gRNAs complementary to a regulatory element or the CDS, taking into account the target sequence at the genomic level. To promote the knockout of a gene, the functionality of the expression product must be eliminated by truncating the encoded polypeptide chain. Therefore, one must start from two fundamental premises:

[1] Available at: https://www.ncbi.nlm.nih.gov/nuccore

[2] Available at: https://phytozome.jgi.doe.gov/pz/portal.html

[3] Available at: https://phytozome.jgi.doe.gov/pz/portal.html

a) The knockout should occur based on mutagenesis in more distal 5' regions, that is: the more upstream the CDS cleavage occurs, the less likely that native and functional protein domains will remain expressed based on the remaining sequence in the frame.

b) The pairing of the gRNA with the genome must occur in regions restricted to specific and unique exons, that is: the PAM sequence and the cleavage site must be located in the same exon. Therefore, locating these sequences in introns or exon-intron junctions can make the candidate sequence irrelevant to effective genome editing with concrete results.

Considering these factors to start the second stage, a very practical, intuitive, and broad tool for the search and prediction of candidate gRNAs is CRISPOR[4] (Concordet; Haeussler, 2018), which will be detailed below, instructing the reader to select the best guides for the aforementioned purposes:

1) When accessing CRISPOR (Figure 7), the target sequence must be inserted in the nucleotide sequence bar in "Step 1". If you prefer, you can assign a name to the sequence in that same section.

   Note: the sequence to be searched can be genomic or cDNA. In both cases, one must pay attention to the second premise exposed above, discarding candidate gRNAs that match introns or that cover more than 1 exon, respectively.

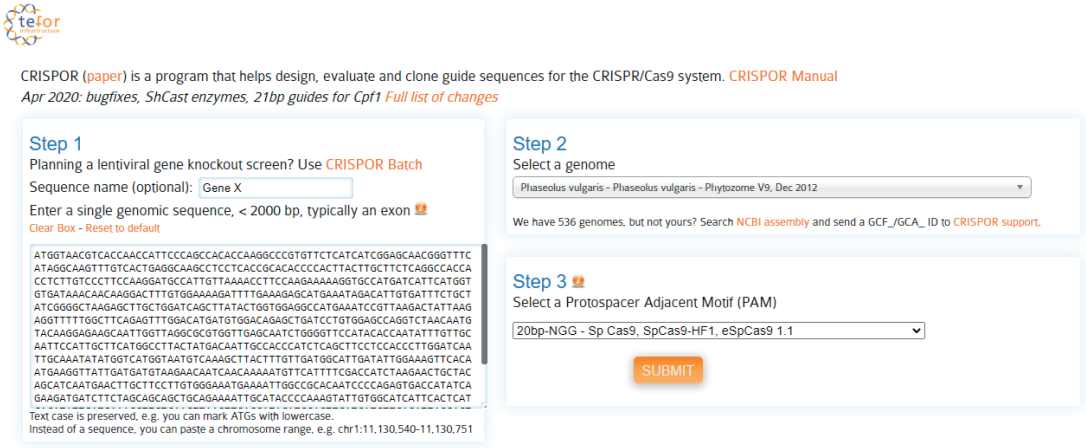


Figure 7. Search for candidate gRNAs at CRISPOR. Input window of the target sequence for the design of the candidate gRNAs, detailing the bars of insertion of the sequence (Step 1), selection of the target genomes options (Step 2), and selection of the PAM sequence (Step 3).

[4] Available at: http://crispor.tefor.net/

2) Then, in the "Step 2" section, from the 536 available genomes, select the target host genome to be edited. If the genome of the species is not available, it is possible to disable the selection of a specific genome by selecting the option "No genome". In this case, the output will have less data richness, without specific details.

3) Finally, in the "Step 3" section, select the PAM corresponding to the PAM sequence of the nuclease with which you will work on the bench and whose abbreviation is shown next to each generic sequence. The PAM selection already includes the standard length of the gRNA sequences to be returned as an output, which varies according to the nuclease used in the editing. After that, click on "Submit" to start the search.

4) Right after, new information will be shown on a new page (Figure 8). First, the target sequence will be made available in a gray box, with residues identified every 10 nucleotides. Under the residues, the initial gRNA sequences overlaid on the target will appear. Depending on the specificity of each gRNA, they will appear in three different colors: green (sequences with high specificity), yellow (medium specificity), or red (low specificity). It is strongly recommended that only green colored sequences be selected. The sequences can be aligned to the sequence Forward (starting with nucleotides and ending with dashes), or Reverse (starting with dashes and ending with nucleotides) to the target sequence of the input.

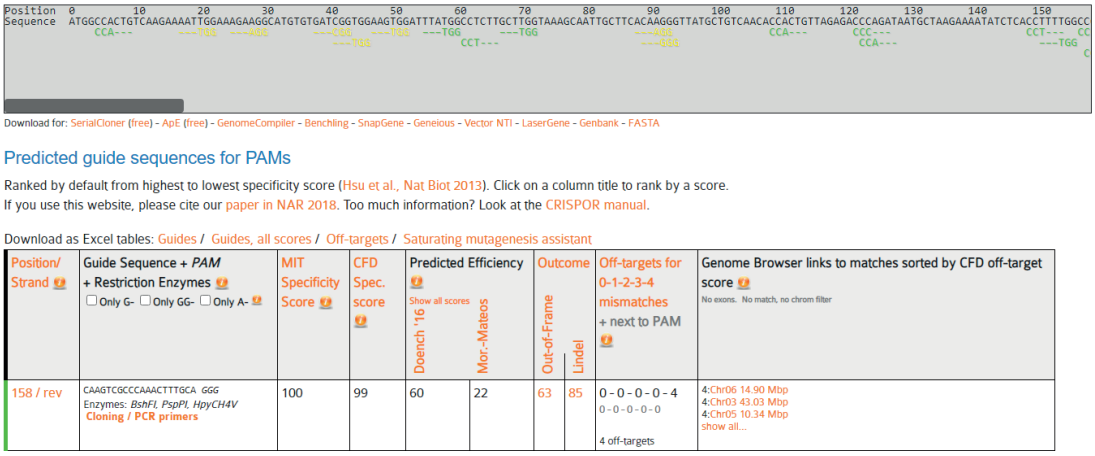


Figure 8. Result of the prediction of candidate gRNAs in CRISPOR. Output window of candidate gRNA sequences, showing the overlap of each in the target sequence provided, its nucleotide composition, as well as the location on the respective strand and on-target and off-target parameters, detailing each of the cases of potential offs -targets.

Note 1: when working with nucleases that cleave the target sequence in both strands, it is irrelevant to select Forward or Reverse candidates.

Note 2: it is possible to click on each sequence of the gray box to be directed to the respective information in the table that contains all the gRNAs.

5) The information table, presented in the Predicted guide sequences for PAMs section, contains the main data on all candidate gRNAs found according to the established parameters. This table can be exported and saved as a file with .xls extension, by clicking on the different options just above it (Download as Excel tables). As for the table parameters, the listed gRNAs are arranged in decreasing order of specificity (on-target, MIT Specificity Score, which ranges from 0 to 100), which is inversely proportional to the probability of occurrence of off-target effects using the respective guide. In the first column of the table (Position/Strand), the position and the strand of the target sequence that contains complementarity to the gRNA are informed. In the second column (Guide sequence + PAM + restriction enzymes), the sequences of each guide, containing the PAM, are shown, as well as the restriction sites present and capable of cleaving each guide. This column also indicates, when appropriate, an observation that the guide may not be adequate because it contains termination signs in the form of sequential thymine residues (T_n). It is also possible to restrict guides initiated with specific nucleotide residues (this restriction is generally used when working with U6 and U3 promoters to direct the expression of sgRNA, although even when selecting such promoters to direct expression, these residues can be added artificially to the 5'end of any sgRNA to make them compatible with the respective promoter). The CFD and Specificity Score columns are related to the specificity and cleavage efficiency scores, but less useful for choosing gRNAs. The Outcome column represents parameters related to the result of the cleavage, such as out-of-frame and frameshifts, caused by deletions and indels, respectively. In this case, the values also vary from 0 to 100, with the number representing the theoretical percentage of events/clones having these editing results. The column Off-targets for 0-1-2-3-4 mismatches indicates the number of cases of non-target sequences in the host genome that contain 0 to 4 mismatches, in that order, serving as a crucial parameter to define the best guides that represent less risk of generating off-target effects. The smaller the number of cases, especially if there are few or no cases with the least number of mismatches, the more specific the guide will be. The selected guides should have no cases with less than 3 mismatches, especially in the seed region of the gRNA. The last column details the genomic position of each of the off-target cases, detailing the pairing when hovering the mouse over each case and marking the mismatches

between the residues, which makes it easier to verify if the occurrence of the mismatches is within or outside the seed.

In addition to CRISPOR, for some plant species - especially those of great economic importance, such as soy, rice, and maize, it is possible to design gRNAs with other computational tools, such as CRISPR-P v2.0[5] (Lei et al., 2014). In this case, just access the main page of the program and click on the Submit tab. In the new window (Figure 9), you must select the target species, the PAM sequence, the U6 or U3 promoter and the length of the desired gRNA sequence to be returned (it can vary from 15 to 22 residues according to the program default), also inserting the nucleotide sequence to be edited. The sgRNA scaffold sequence is already pre-established. After that, click on Submit to search for gRNAs.

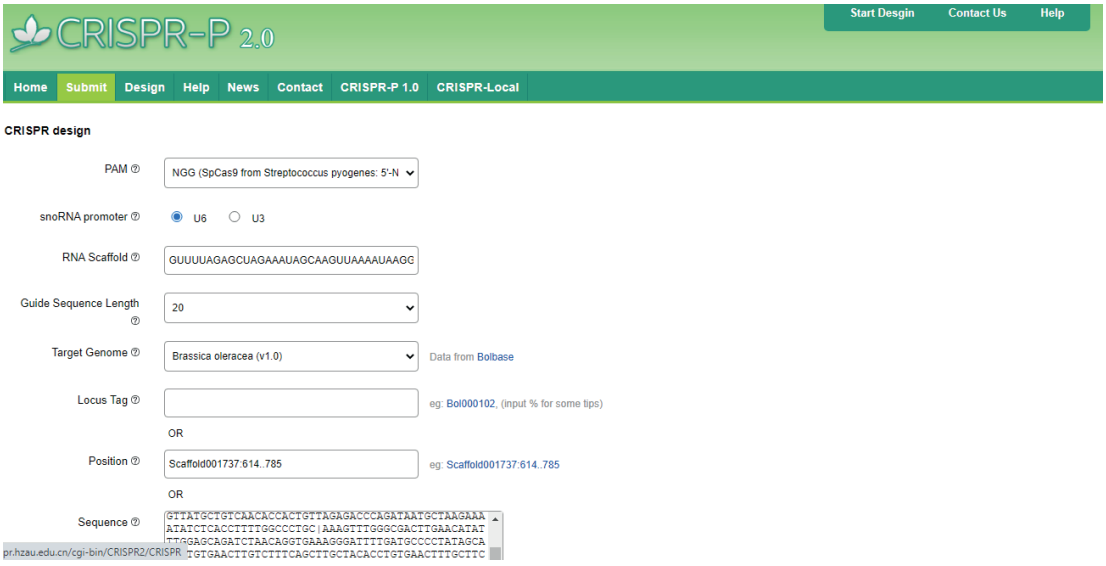


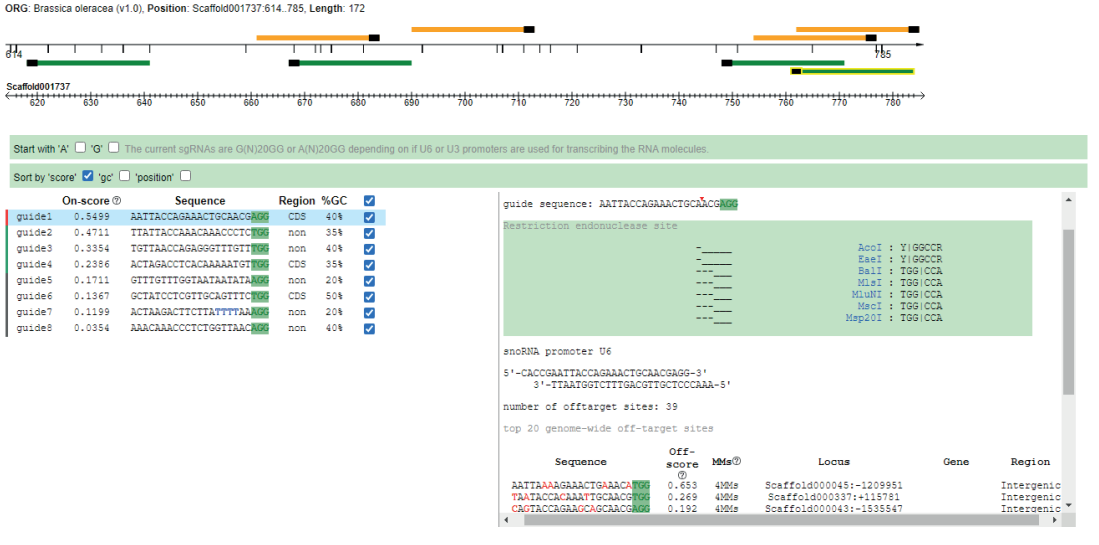
Figure 9. Search for candidate gRNAs in CRISPR-P v2.0. Input window of the target sequence for the design of candidate gRNAs, in the Submit tab of the page, detailing the selection bars of the PAM sequence, the length of the guide, the target genome (species), and the insertion of the nucleotide target sequence.

After submission, a new page (Figure 10) will open with details of the candidate guides for selection. On this page, the gRNAs will be represented graphically in line with the sequence in the upper panel, with the Forward and Reverse sequences in different colors. In the lower panel, the candidate sequences are listed, with several

[5] Available at: http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/SCORE

parameters informed on the left (% GC, on-target score, nucleotide composition, the presence or not in the coding region). On the right, the number of off-target cases is reported, with details of each of them regarding the nucleotide composition (with mismatches highlighted in red), the number of mismatches, chromosomal location, and the presence or not in a coding region (each case is classified as intergenic or CDS and, in the latter case, the corresponding gene ID is indicated in the column on the side). The restriction sites present in the sequence of each predicted gRNA are also informed, which can be useful in the stage of subcloning the guides in the expression vector, for example.

Additionally, it is possible to design more than one gRNA for the same target gene, which ensures a greater probability of occurrence of the knockout of that gene, in addition to being able to excise fragments of great length due to the production of at least two DSB sites. Going further, multiple gRNAs can be designed and used, usually cloned into the same expression vector, and not necessarily directed to the same gene, but to different target genes (Cong et al., 2013), in a strategy called "multiplex genome engineering". Multiplex engineering, therefore, allows modulating the expression of several genes associated with highly divergent or related characters converging to a common character.



	On-score	Sequence	Region	%GC
guide1	0.5499	AATTACCAGAAACTGCAACGAGG	CDS	40%
guide2	0.4711	TTATTACCAAACAAACCCTCTGG	non	35%
guide3	0.3354	TGTTAACCAGAGGGTTTGTTTGG	non	40%
guide4	0.2386	ACTAGACCTCACAAAAATGTTGG	CDS	35%
guide5	0.1711	GTTTGTTTGGTAATAATATAAGG	non	20%
guide6	0.1367	GCTATCCTCGTTGCAGTTTCTGG	CDS	50%
guide7	0.1199	ACTAAGACTTCTTATTTTAAAGG	non	20%
guide8	0.0354	AAACAAACCCTCTGGTTAACAGG	non	40%

Sequence	Off-score	MMs	Locus	Gene	Region
AATTAAAGAAACTGAAACATGG	0.653	4MMs	Scaffold000045:-1209951		Intergenic
TAATACCACAAATTGCAACGTGG	0.269	4MMs	Scaffold000337:+115781		Intergenic
CAGTACCAGAAGCAGCAACGAGG	0.192	4MMs	Scaffold000043:-1535547		Intergenic

Figure 10. Result of the prediction of candidate gRNAs in CRISPR-P v2.0. Output window of candidate gRNA sequences, showing the overlap of each one in the target sequence provided, its nucleotide composition, as well as present restriction sites and on-target and off-target parameters, detailing each of the cases of potential off-targets.

In order to conduct the multiplex approach, it is common for the designed gRNAs to be inserted into the expression vector so that they are arranged on polycistronic cassettes. For this purpose, in addition to the classic configuration of different gRNAs arranged in different expression cassettes (that is, in a monocistronic form), with expression directed by the respective promoters, the gRNA units can be interspersed by sequences of transfer RNA (tRNA), in a strategy based on the endogenous processing of tRNA molecules by RNases for excision of transcripts *in vivo* conditions, promoting the release of fused gRNAs and thus allowing them, once free in the nucleus, to be able to associate with the nuclease to guide them to different target sites in genomic DNA, also increasing the efficiency of genome editing (Xie et al., 2015; Wolabu et al., 2020). However, the tRNA-gRNA-based processing system is based on the use of promoters for RNA Polymerase III to direct the expression of these synthetic genes (He et al., 2017). The use of these promoters, however, like the U6 and U3 variants, has some limitations (Zhang et al., 2017): they are constitutively active and, therefore, lacking cellular or tissue specificity; they are poorly characterized for some organisms and are not suitable for use in in vitro transcription due to the lack of commercial versions of RNA Polymerase III.

To circumvent these obstacles, an alternative way to conduct multiplex engineering is to use promoters for RNA Polymerase II. These promoters, in principle, also have some limitations in their use for genome engineering (Zhang et al., 2017), such as the transcripts they direct in the expression undergo post-transcriptional processing (capping, splicing, and polyadenylation), modifications in the transcript that can functionally inactivate the gRNA; the mature RNA is transported to the cytosol, being confined in a distinct cell compartment from where it must act together with the Cas nuclease, which constitutes a physical barrier that blocks the editing activity. However, when these promoters are associated with a second processing strategy, they can become sufficient to promote editing. This strategy, called "RGR" (Ribozyme-gRNA-Ribozyme), has as a principle the use of gRNAs flanked by ribozymes at both 5′ and 3′ ends of the guides (Lee et al., 2016), being generally hammerhead ribozymes (HH) and Hepatitis D Virus (HDV).

Based on this, the principle of this mechanism is similar to the principle of the tRNA-gRNA strategy, although it is based on the autocatalytic activity of ribozymes from the moment they are transcribed, which also promotes the release of gRNAs in the cell nucleus, bypassing the processes that a mature RNA would receive, as mentioned above. Thus, it becomes possible to produce gRNAs from any promoters in any organism, making the choices of promoters to conduct genome editing virtually unlimited (He et al., 2017). Although this strategy can also be used for a single gRNA with a promoter-driven expression for RNA Polymerase II, using monocistronic cassettes similar to those mentioned on promoters for RNA

Polymerase III, it is quite convenient for the multiplex approach, as mentioned earlier, in a polycistronic configuration.

Identification of possible off-targets

Although both tools presented in the previous topic are capable of predicting potential off-target sites for each designed gRNA, there are specialized tools in this task and with greater accuracy. Among them, the Cas-OFFinder[6] tool (Bae et al., 2014) is quite complete, intuitive, with a simple and more widely used layout, and is therefore recommended by the authors for this procedure, which is detailed below:

1) Accessing Cas-OFFinder (Figure 11), the gRNA sequences selected in the previous step must be input simultaneously. In this window, the tool is subdivided into 3 sections: (a) PAM Type: first, the PAM sequence corresponding to the nuclease to be worked with must be selected; (b) Target Genome: here the group of organisms within which the species containing the target genome is located must be selected, being the groups: Vertebrates, Insects, Plants, Others and Non-reference Vertebrates, and then the species with its reference genome; (c) Query Sequences: in this section, the residue sequences of the gRNAs are inserted one below the other (in separate lines), without the PAM sequence, and a maximum limit of mismatches can be established, giving greater objectivity to the analysis. In the end, click Submit to start the search.

2) Then, new information will be shown on a new page (Figure 12). It presents two sections: (a) Summary: presents, for each sequence of gRNA, a line with the number of on-target cases and another line with the number of off-target cases, according to the number of mismatches; similar to what is shown in CRISPOR; (b) Details: for both on-target and off-target cases, the pairing between the gRNA and the genomic target is shown, as well as the indication on which chromosome the target is located, in addition to its starting position showing the nucleotide residue and sequence orientation on the strand (sense +, or anti-sense -). With this information, it is possible to return to Phytozome and locate the sequence of the off-target site, characterizing it in terms of expression. In this case, it is recommended to check first if the off-target sites are in intergenic or transcribed sequences. Being in transcribed sequences, it is important to determine the region of the sequence, evaluating its importance in the performance of the final product, so that, once the knockout is performed, the result does not generate harmful effects to the organism

[6] Available at: http://www.rgenome.net/cas-offinder/

or interfere with the analysis. For example cleavages in 5'-UTR regions and exons are often critical; therefore, a gRNA designed with a potential off-target in these cleavages should not be prioritized, unlike others located in intronic regions (except in consensus regions that are preponderant for splicing).

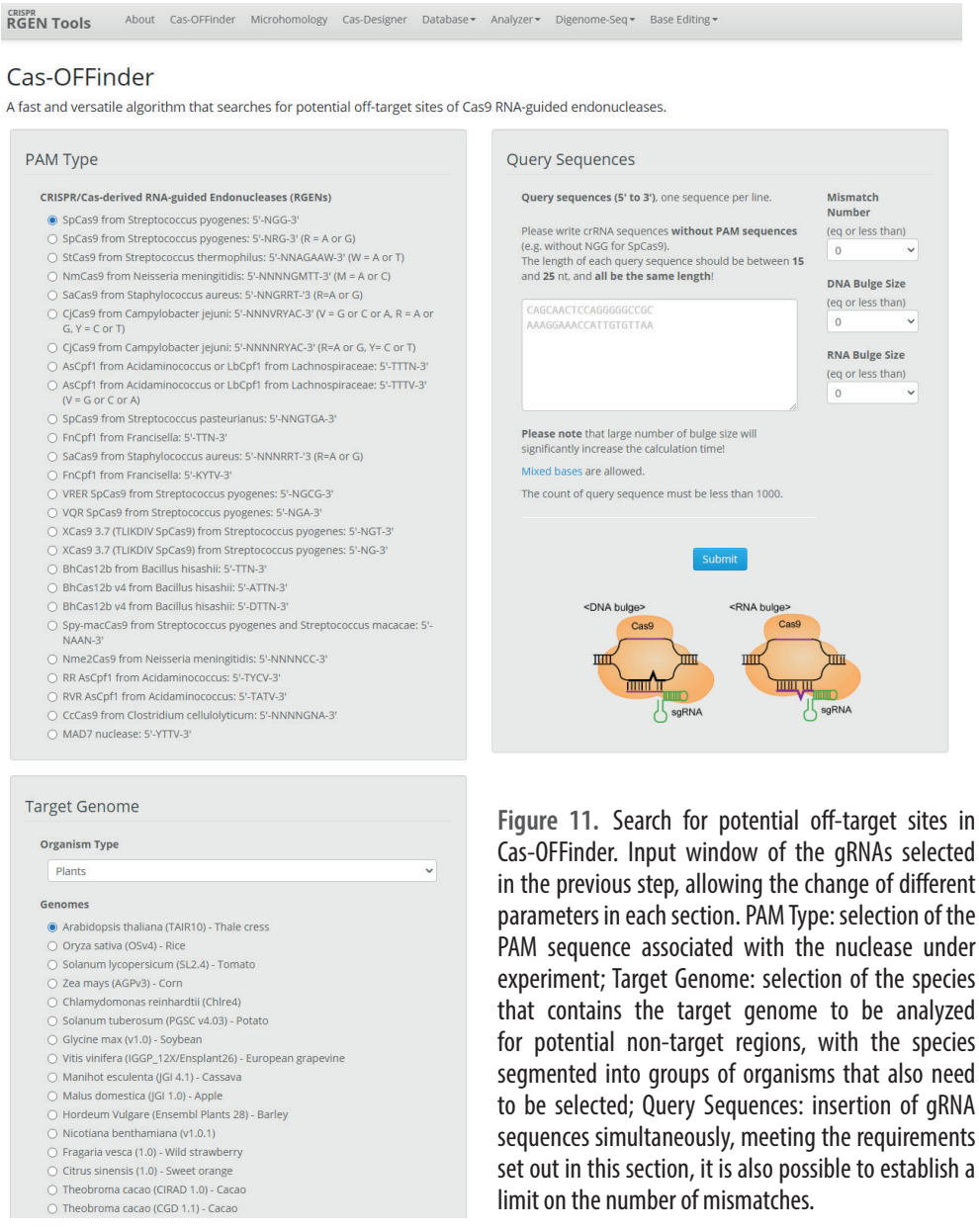


Figure 11. Search for potential off-target sites in Cas-OFFinder. Input window of the gRNAs selected in the previous step, allowing the change of different parameters in each section. PAM Type: selection of the PAM sequence associated with the nuclease under experiment; Target Genome: selection of the species that contains the target genome to be analyzed for potential non-target regions, with the species segmented into groups of organisms that also need to be selected; Query Sequences: insertion of gRNA sequences simultaneously, meeting the requirements set out in this section, it is also possible to establish a limit on the number of mismatches.

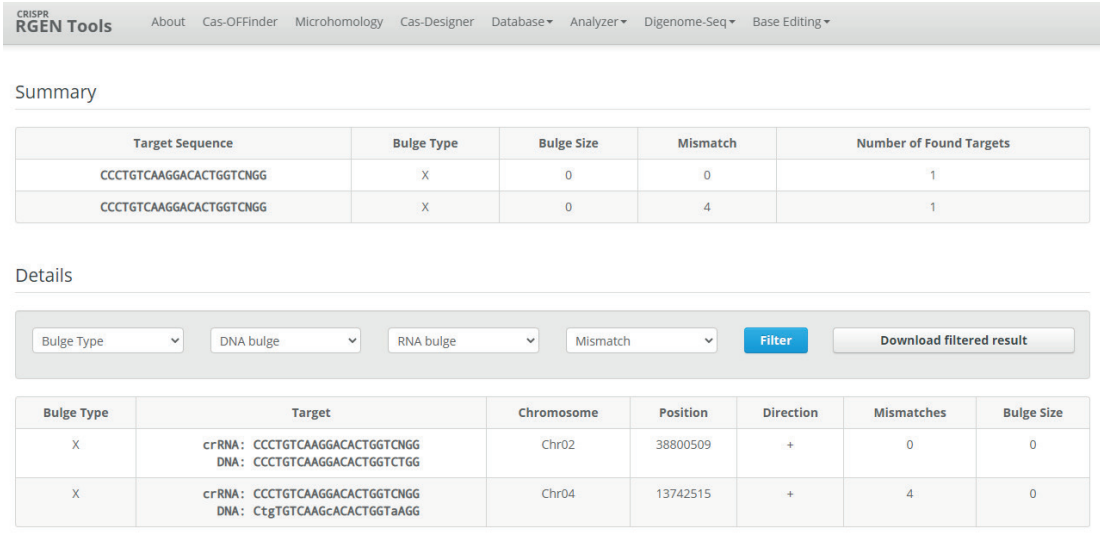


Figure 12. Result of the prediction of potential off-target sites in Cas-OFFinder. Output window of the gRNAs selected in the previous step, with the analysis of the cases of potential off-target sites found in the reference genome. The Summary section summarizes, for the same gRNA, on-target cases on one line (shown by 0 mismatches, being the target sequence itself in the genome) and off-target on another line (shown by 1 case with 4 mismatches, in this example). In the Details section, both cases are detailed regarding the pairing between the gRNA and the similar genomic sequence, but informing the chromosome location, in the Chromosome and Position columns.

Knockout simulation and homology prediction

Once verified and filtered only gRNAs with good specificity and without critical off-target activity, when there are potential off-target sites, it is important to simulate the knockout resulting from the editing, so that homology can be predicted with functional protein domains from the remaining polypeptide chain since it is expected to be truncated and its biological activity to be abolished. It is important to note that this type of analysis should be performed only when the objective is the knockout by editing, via indels, the coding sequences of target proteins. For this, the appropriate procedure must be performed as follows:

1) An in-silico translation should be conducted from the nucleotide residue where theoretically cleavage by the nuclease will occur so that a truncated polypeptide chain will be generated for later verification of homology. It is possible to conduct it in the online tool ExPASy Translate Tool[7]. From the cleavage point, select the upstream sequence and insert it into the nucleotide

[7] Available at: https://web.expasy.org/translate/

sequence bar (Figure 13). It is recommended, as an output format, to keep the Compact option selected, as well as the results shown on both tapes, enabling both options, without the need to select the specific genetic code. Finally, click on Translate!. It is important to remember that not every indel will cause an earlier stop codon to form along the target sequence or, more broadly, a frameshift that alters the polypeptide chain 3' from the cleavage point, modifying its secondary and/or tertiary three-dimensional structure and, thus, making native biological activity unfeasible. This is because insertions or deletions of nucleotide residues may occur in multiple numbers of 3, which keeps the rest of the sequence in the frame, not necessarily causing this deleterious effect due to the presence or absence of a few amino acid residues. However, due to the unpredictability of the type of indel to occur in each event, one must work on assuming the occurrence of non-multiple of 3 indels, enabling simulation.

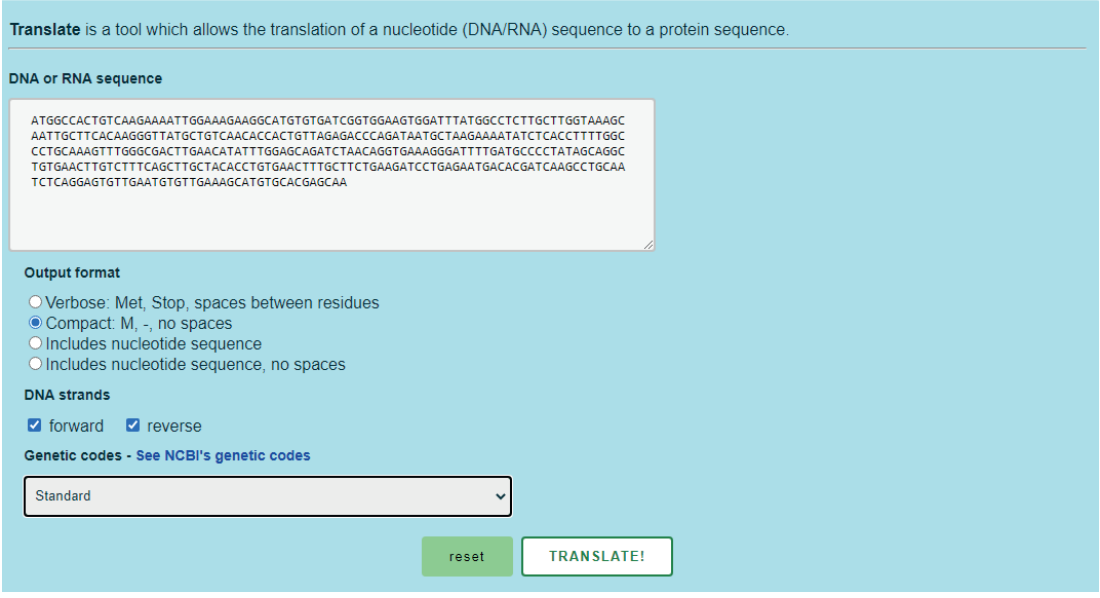


Figure 13. In silico translation of truncated sequences in the ExPASy Translate Tool. Input window of the simulated nucleotide sequence regarding truncation, showing different possible output formats and the possibility of representing 3 frames (Forward or Reverse only) or 6 frames (Forward and Reverse).

2) With the result of the analysis, a new page is opened (Figure 14), with the results of the *in-silico* translation, in all 6 possible ORFs (Open Reading Frames). Since the input sequence is usually inserted from the first residue of the start codon (ATG), all results in this way will be found in Frame 1 (5'-3'), with the truncated sequence marked continuously in red.

Figure 14. Result of in silico translation in ExPASy Translate Tool. Output window of the translated sequence, in 6 different ORFs (Open Reading Frames), with the sequence of the polypeptide chain, in this case, present in Frame 1 5'-3'.

3) Having the polypeptide sequence truncated, it is possible to copy and insert it in the online tool for predicting homology with functional protein domains, InterPro[8]. On this page (Figure 15), the sequence is entered in FASTA format, conducting the search and keeping the pre-established parameters. After that, a page with the analysis output (Figure 16) will be opened, informing about the presence or absence of protein families prediction referring to the remaining polypeptide sequence. If there is no prediction, it is likely that the resulting sequence does not form functional domains that perform a residual biological activity, which implies deleting the protein function associated with the gene subjected to the knockout.

[8] Available at: http://www.ebi.ac.uk/interpro/

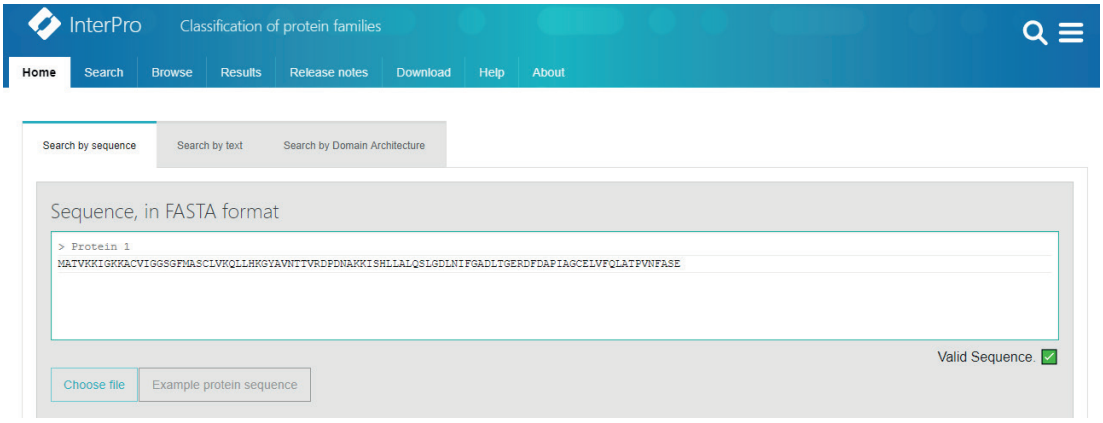


Figure 15. Search for protein homology in InterPro. Input window of the truncated polypeptide sequence to predict homology with functional protein domains by association with protein families.

Figure 16. Result of homology prediction with functional domains in InterPro. Output window of the truncated polypeptide sequence, showing the absence of homology with protein families, indicating that the remaining sequence has no known biological activity.

Once the absence of homology is confirmed, the gRNA meets all the requirements and properties of a useful candidate and is more likely to perform its function without the occurrence of undesirable effects and can be validated in vitro and

prepared for subcloning in expression vectors (approach: use of plasmids) or used alone (approach: IVT) or complexed to a nuclease (approach: RNP).

Part 2: in vitro

The second step to perform genome editing via CRISPR/Cas consists of in vitro validation of the isolated and mediating molecules in the editing process, which can be carried out using commercial kits. Because the genetic elements that make up an expression vector (in approaches to the use of plasmids) and that transfection methods vary greatly according to the target species and the explant used, the outline of these steps will not be detailed here, since this must be established in a particular way in each research project. However, the in vitro validation of the designed guides and associated nucleases is a more universal process for the different organisms to be edited.

Cleavage efficiency and validation of the nuclease-sgRNA complex

sgRNA must be able to guide the nuclease to the target site. Furthermore, the process will only be viable if, in addition to this capacity, the nuclease is efficient in promoting the cleavage of the sequence at the expected cleavage site. Thus, it is possible to verify the activity of both employing an in vitro cleavage efficiency test, in which the target sequence is amplified by Polymerase Chain Reaction (PCR) and the PCR product is cleaved by the complex. The formation of digestion fragments from the PCR product indicates that both the sgRNA and the nuclease perform their function normally, being able to proceed with genome editing in vivo. Otherwise, the absence of cleavage products indicates the lack of efficiency in the function of at least one of these components of the complex. For this, it is necessary to obtain the RNP corresponding to the complex that will be formed in vivo. In this case, obtaining the nuclease and sgRNA can occur as described in the topic RNPs: DNA-free systems, of this chapter. For works that use the RNP system for transfection, after validation, the complex can now be used directly to proceed with the editing. In the case of plasmid systems or transcripts, the corresponding nucleotide sequences of the validated molecules must be used.

A simple and succinct procedure (GenScript, 2018) for this validation, based on an in vitro test kit, can be followed as described below:

1) Perform the amplification of the target region by PCR (Note: it is possible to design pairs of primers for both the target region and for potential off-targets in CRISPOR, from the search output information table). The PCR product will

be used as a substrate for digestion with RNP nuclease. It is recommended that the product contains at least 200 bp flanking the sequence to which the gRNA is paired, both 5'and 3', being preferred that the PCR product has about 1 kb for better visualization of digestion.

2) Prepare 16 µL of RNP in a nuclease-free tube, according to the following reaction system, adapted from GenScript (2018):

Component	Volume/Concentration
sgRNA	3,6 pmol
Nuclease Cas (1 µg/µL)	1,5 pmol
Reaction buffer (Cas) (10X)	2 mL
H_2O nuclease-free	13,6 µL
Final volume	16 µL

3) Conduct the RNP assembly by incubating the reaction system tube at 37 °C for 10 min.

4) Add 450 ng of the PCR product to the RNP mix assembled in step 2, making up the volume with nuclease-free water to 20 µL and swirling slightly to promote mixing.

5) Incubate the product-RNP mixture for at least 30 min, at 37° C.

6) Check the occurrence or not of digestion through visualization based on agarose gel electrophoresis.

Part 3: in vivo

Finally, after the transfection and regeneration of candidate events for edited plants has been carried out, it is necessary to characterize the editings that occurred in vivo using different techniques, each presenting its advantages and disadvantages. Here, a step-by-step of each one will not be detailed, but the reader will be guided with literature about them, as well as with pertinent general information, to search on how to conduct them according to their preference, suitability to the design of their study, and available materials, reagents, and equipment in your laboratory.

Identification of edited plants

For the candidate events to be characterized in terms of altering the genomic sequence, it is of great interest to filter, when possible, plants that present phenotype, reducing the volume of evaluations, aiming at making the characterization more practical, simple, and less laborious.

When working with plasmid systems and genomic integration, it is possible, based on the expression vector used, and its genetic elements, to select explants that regenerate in a selective medium with resistance guaranteed by the marker with which they work, or that are visually observable as to the characteristic that the inserted reporter gene confers. However, when working with IVTs or RNPs, for example, selection based on these characteristics becomes impracticable, since it is generally not applicable. Thus, if it is not possible to conduct a screening based on a selective agent, or on the phenotype itself resulting from genome editing, the characterization must proceed for the selection of mutants. For this selection, the following methods are some of those that can be used in genotyping:

- Sequencing: very useful to detail the type and composition of the indel generated after cleavage and repair, informing on each of the strands the exact sequence of the nucleotide residues, both using the Sanger method, and NGS (Next-Generation Sequencing) approaches. In general, sequencing is usually a central or complementary approach to other less informative methods at the level of the genomic sequence, being used, when applied as a complementary method, to characterize only previously genotyped events.
- PCR-RE (PCR-restriction enzyme): based on an amplification step of the regions to be evaluated, which are usually the target sequences, and the regions of possible off-target. Then, a restriction enzyme digestion with a recognition site is carried out encompassing the cleavage site by the editing's nuclease. Thus, the digestion of the PCR product reveals that the restriction site remains intact and, therefore, there was no editing; the absence of digestion reveals that the restriction site was eliminated due to the indel resulting from genome editing. Although frequently used, the limitation of this technique lies in the need for the existence of such restriction sites, which is not always the case, given the reduced diversity of candidate gRNAs that are appropriate. For more information, read the work of Xie and Yang (2013).
- T7E1 / Surveyor ™ assay: based on the ability to recognize and cleave heteroduplex DNA molecules (derived from mismatches between strands) by T7 endonuclease and Surveyor enzymes. These enzymes recognize DNA molecules whose strands are mismatched. For this reason, they are useful in

detecting heteroduplexes, which correspond to heterozygous individuals for editing. These are simple and inexpensive techniques when compared to sequencing, but they do not reveal the nucleotide sequence of the regions evaluated. They also lack robust reproducibility and are susceptible to generating false-positive results when polymorphisms or random mutations occur in the evaluated sequences, although their use is commonly reported in the literature. Also, only heterozygous individuals for the editings can be detected. For more information, read the work by Vouillot et al. (2015).

- qPCR (PCR quantitative): there are countless variations of qPCR for genotyping event indels, both at the genomic level and at the transcriptomic level (via RT-qPCR), whose primers designed overlap with the target site to be edited, so that the basis of this technique lies in the fact that the occurrence or not of mutagenesis is indicated by the failure or success, respectively, of amplification. This is because editing tends to cancel the primer ringing in the edited region, interfering with the generation of the amplicon. However, smaller indels tend to interfere to a lesser extent in amplification. For this, the HRM (High-Resolution Melting) technique can be an alternative to identify smaller indels, as it consists of changing the fluorescence pattern in the analysis of the melting curve, which is generated during the formation of the DNA duplex. Thus, based on the profile of the curve, it is possible to clearly distinguish a wild individual (wild-type, WT) from a mutant. In general, qPCR/RT-qPCR is an accurate technique, but with a slightly higher relative cost than some other genotyping techniques. Thus, we recommend that the reader understand the details of the main methodological aspects from the work of Yu et al. (2014), Falabella et al. (2017), and Li et al. (2018).
- Western blot: a technique that consists of labeling a target protein using an antibody conjugated to a molecule that produces a visible signal after an enzymatic reaction, the antibody being directed to recognize a specific region of the target protein. The protein is detected on a membrane after transfer from an SDS-PAGE (polyacrylamide gel electrophoresis with sodium dodecyl sulfate). If the protein is present in its complete form, the region recognized by the antibody remains present, resulting in a colorimetric signal due to the catalyzed reaction; in the absence of the complete form, no signal is theoretically detected. It is a less used method for the characterization since, in addition to being more laborious and requiring more work time, it is possible to perform the characterization at the genomic or transcriptomic level, without the need for evaluation at the proteomic level. Besides, there is the possibility of generating false positives, since the antibody can cross-react with proteins structurally similar to the target protein, even though it

is already truncated due to editing. Normally, the detection of Cas protein is conducted, checking for events that express the nuclease and, therefore, are at first undergoing an editing process (Endo et al., 2019).

- Polyacrylamide gel (PAGE): the polyacrylamide gel forms a mesh with properties that generate a high-resolution capacity of DNA molecules, which allows detecting differences of up to about 2 bp between the strands and between different samples. Thus, it is a very accurate alternative for detecting all length ranges of the indels, despite being a little laborious, and not very fast. For more information, see the work of Zhu et al. (2014).
- Agarose gel (AGE) 4%-6%: although agarose gels, in their usual working concentrations (1% to 3%) are not useful for distinguishing indels by CRISPR/Cas, and therefore for genotyping events, Bhattacharya and Van Meir (2019) recently developed a simple method for genotyping using more concentrated gels, with 4% to 6% agarose. In this approach, indels of at least 3 bp can be detected visually based on the mobility property of heteroduplex molecules along with the gel mesh. Initially, heterozygotes can be differentiated from homozygotes after being subjected to 34 cycles of PCR. Subsequently, mutant homozygotes can be differentiated from wild homozygotes after an additional denaturation/renaturation cycle through hybridization with a wild-type control, which leads to the formation of homoduplexes in the case of wild homozygotes and heteroduplexes in the case of mutant homozygotes. The technique is quite advantageous in terms of practicality and simplicity compared to others such as qPCR, PAGE, Western blot, and T7E1/Surveyor tests, but so far it has been used only for animals, being promising to test its application in plants.

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CHAPTER 3

Genome editing by CRISPR/Cas via homologous recombination

Nathalia Volpi e Silva
Jéssika Angelotti-Mendonça
Elton Gargioni Grisoste Barbosa
Larissa Girotto
Mayla Daiane Correa Molinari
Liliane Marcia Mertz-Henning
Alexandre Lima Nepomuceno

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).

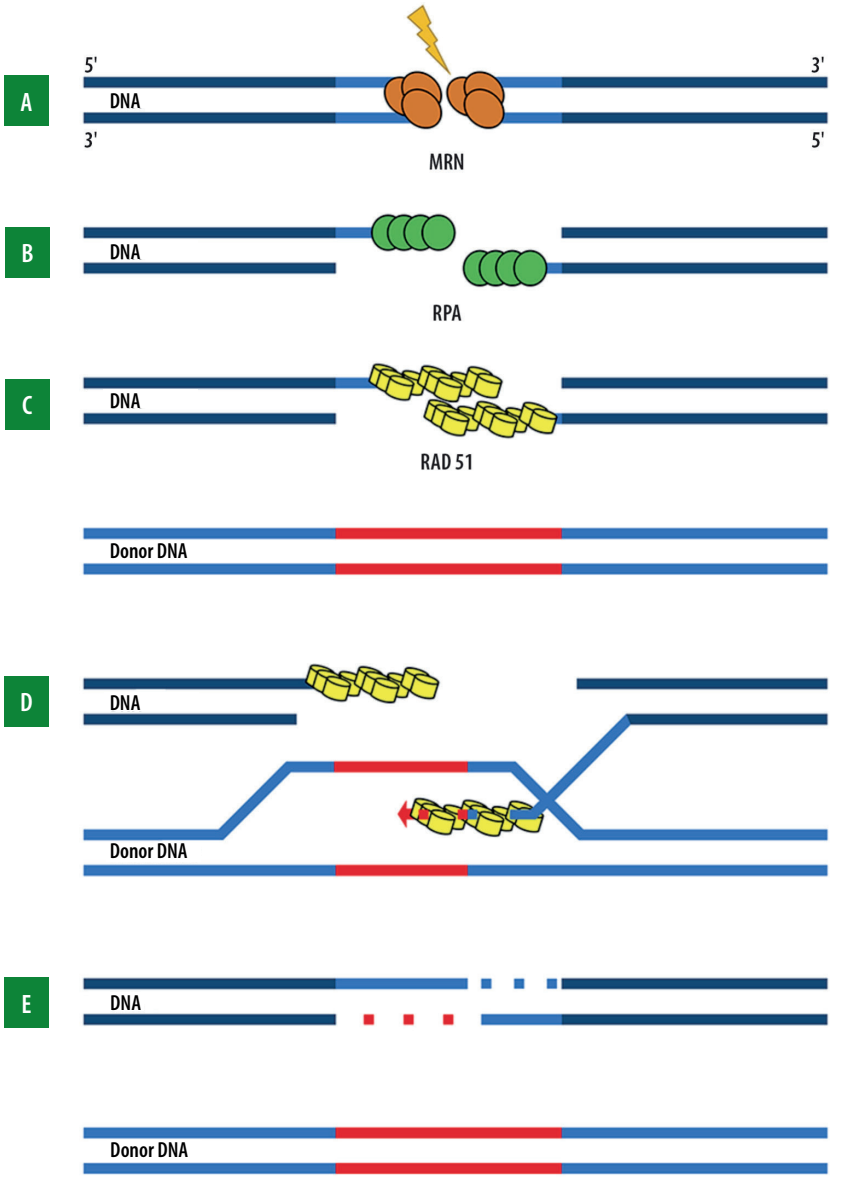

Figure 1. Repair mechanisms via HR by SDSA. A) HDR repair begins with a heterotrimeric complex (MRN) attaching to the damaged double-stranded DNA. B) Replication proteins (RPA) bind to the DNA after its resection and minimize the formation of secondary structures to facilitate the coupling of the RAD51 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.

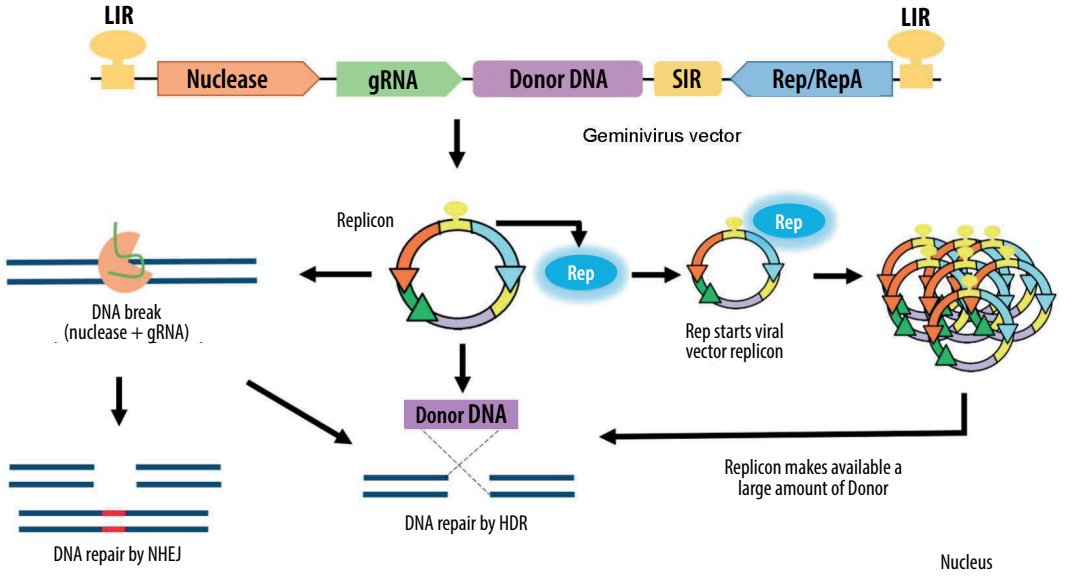

Figure 2. Mechanism of action of the geminivirus vector for genome editing in plants via CRISPR. Once in the cell nucleus, and after the translation of the Rep/RepA proteins, the entire sequence between the two LIRs will give rise to the geminivirus replicon through the circularization of DNA. The circular 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.

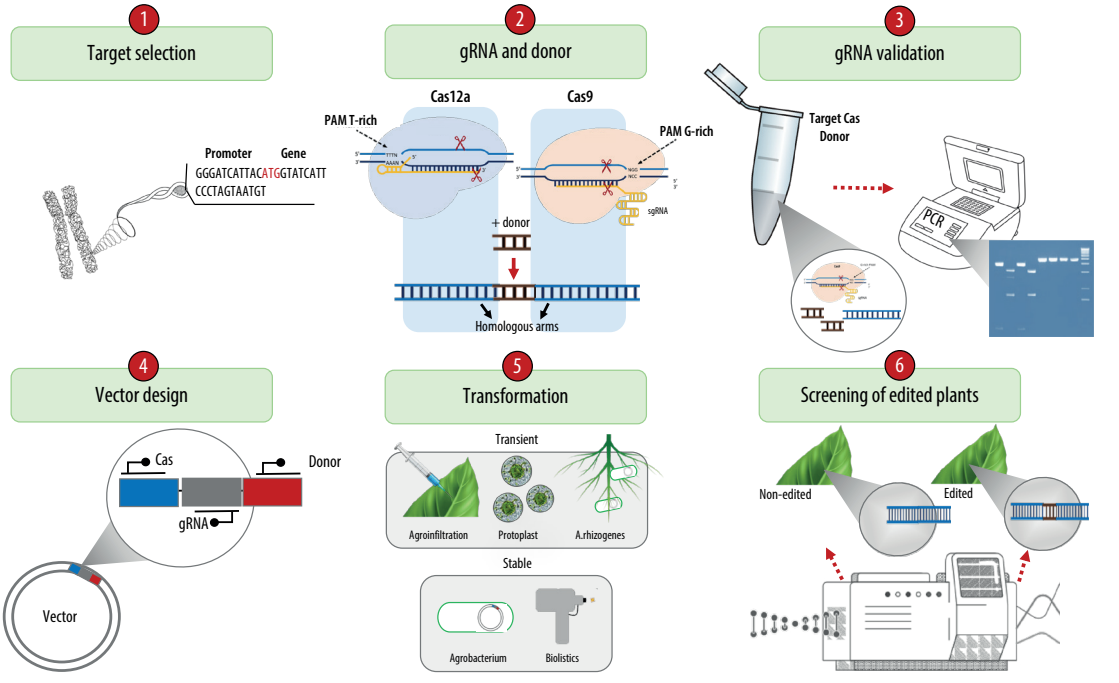

Figure 3. Pipeline of a gene-targeting strategy by CRISPR.

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

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GTATACATTTTCTAATCTTTATTTTCTATTTAATCCATGATTAAACCTTCCATAGTAATTAAACGACTCTAAATATAGGATAAATTTCCTTAATTTAGACTTGGAATGTGTCATTTTTTAATTATTACTTTGAAATT
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>Glyma.15G061400.2 C

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>Glyma.15G061400.3 D

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TTGCTTCTGACTTCTCTTCCAGGATATTAGCTTGATAGCTTCAGCTGTTTTTCATTGC

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.

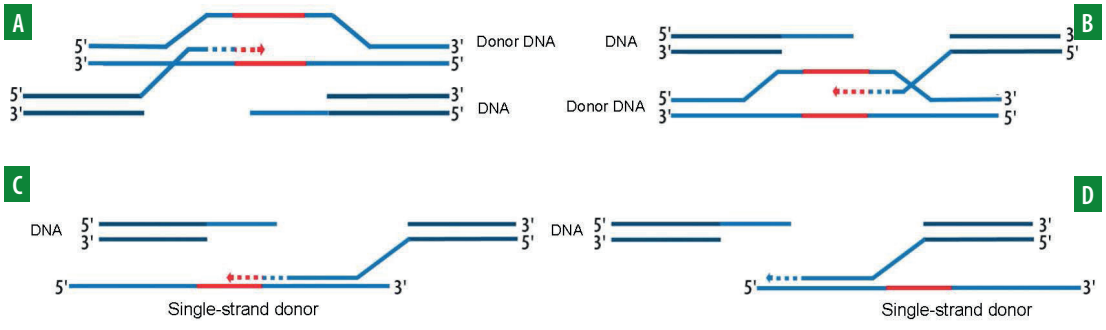

Figure 5. Difference between the use of double-stranded and single-stranded donors regarding the positioning of the edited sequence incorporated by the SDSA repair mechanism. A-B) Using a double-stranded donor, regardless of the location, the mutation will be incorporated. C) Incorporation of the edition 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).

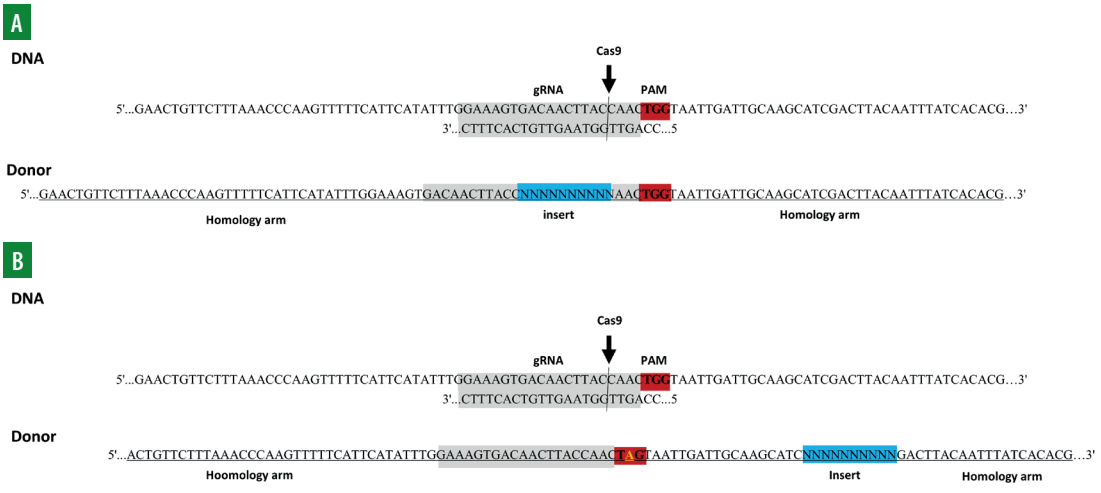

Figure 6. Donor template design strategies to avoid future DSBs after target editing by HR. A) Preventing the gRNA recognition site by editing. B) Silent mutation in the PAM region (cut by the Cas 9 nuclease). Legend: in gray, gRNA; in red, PAM; in yellow, silent mutation in PAM; in blue, edition (insertion).

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.

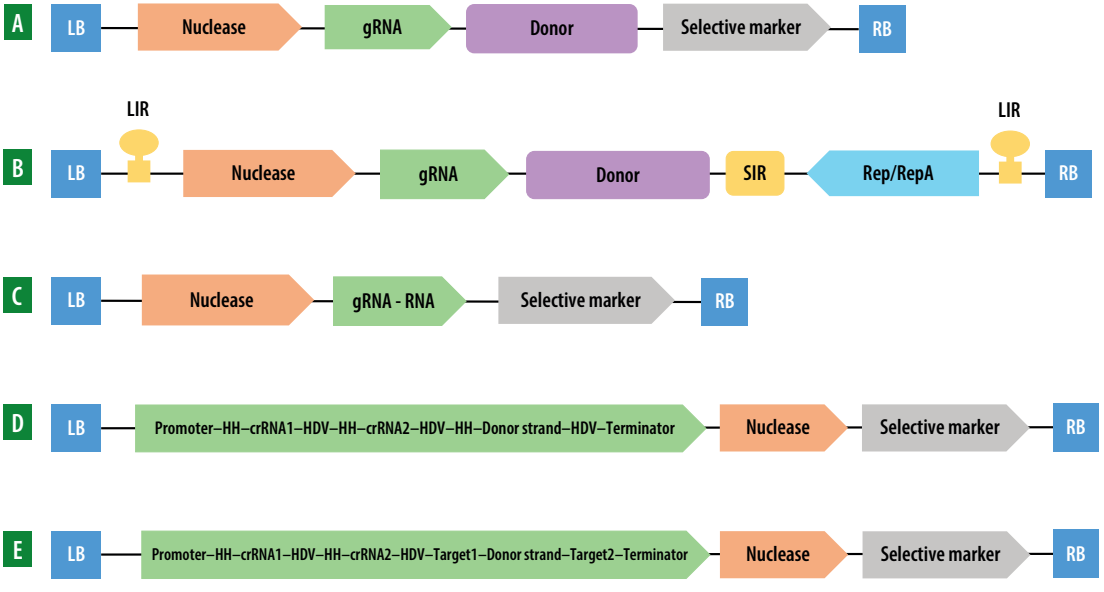

Figure 7. Examples of vector types used in plants. A) Vector with donor template being delivered as double-stranded DNA – a strategy used by Schiml et al. (2014). B) Vector based on Geminivirus viral replicon (GVR) – system proposed by Baltes et al. (2014). C) Binary Agrobacterium vector with RNA donor 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).

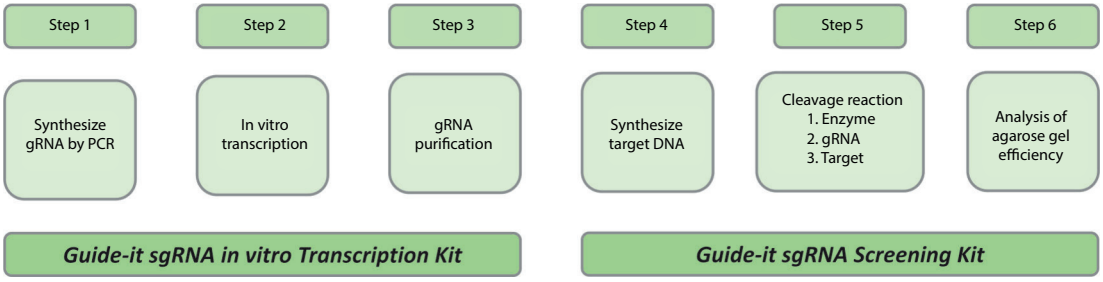

Figure 8. Scheme of steps for synthesis and validation of gRNA *in vitro* (Takara Bio, 2018).

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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CHAPTER 4

Modulation of gene expression in plants via CRISPR/dCas9 technology

Carolina Vianna Morgante
Fabricio Barbosa Monteiro Arraes
Clidia Eduarda Moreira-Pinto
Bruno Paes de Melo
Maria Fatima Grossi-de-Sa

Introduction

The regulation of gene expression includes a diversity of cellular processes that occur in a coordinated manner and in multiple stages to trigger the increase or reduction of a specific gene product. Gene expression can be induced by endogenous and environmental stimuli and regulated at different cellular levels, such as in the initiation of the transcription, RNA processing, and post-translational modification of the protein.

The manipulation of target genes is of paramount importance for understanding gene function and reprogramming of cellular activities. This allows the deepening of basic knowledge about biochemical and molecular processes and the intensification of characteristics of agronomic interest. At this point, precision is essential to obtain the success in applications of genetic engineering and synthetic biology.

In the last decades, technologies using site-specific nucleases for the precise manipulation of DNA have undergone a profound advance, emerging as promising alternatives for site-directed mutagenesis and fine control of gene expression. Among these technologies stand out those of genome editing, such as zinc finger nuclease (ZFN), Transcription Activator-Like Effector Nucleases (TALENs), and more recently, the CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats) associated with Cas nuclease. The latter has its revolutionary character, especially its specificity, universality, and relative simplicity (Pickar-Oliver; Gersbach, 2019). Also, CRISPR/Cas is a flexible tool that can be modified, which contributes to its continuous improvement and diverse applications in the study of cellular functions and biotechnology.

This chapter discusses CRISPR technology using the dead Cas9 variant (CRISPR/dCas9), emphasizing on its use for modulating gene expression in plants. The main strategies currently used are presented and discussed, for which a script was proposed, considering the main aspects for a good experimental design.

The dCas9 system

The CRISPR/Cas9 system was first identified in *Streptococcus pyogenes* and, subsequently, in other bacteria and most archaea, as a sophisticated adaptive immune system, guided by RNAs encoded by the CRISPR locus and Cas proteins (CRISPR associated protein), to provide acquired immunity mainly against bacteriophages (Hsu et al., 2014; Zhang et al., 2014). Its application in genetic engineering was made possible by the deep elucidation of the structures of Cas9 and guide RNA (gRNA).

The Cas9 from *S. pyogenes* is a large multifunctional protein (1.368 amino acid residues) composed of five domains and with nuclease activity (Jiang; Doudna, 2017). The structure of the apoenzyme Cas9 comprises the alpha-helical recognition lobe (REC) and the nucleic lobe (NUC). The latter contains the conserved nuclease domains HNH and tripartite RuvC, in addition to a more variable C-terminal domain (CTD) (Figure 1) (Jinek et al., 2014; Nishimasu et al., 2014).

In the native CRISPR/Cas9 system, mature gRNA is composed of two independent molecules of small non-coding RNAs, which interact with each other by base complementarity: (i) crRNA (CRISPR RNA), responsible for the recognition of the target DNA; and (ii) tracrRNA (transactivating crRNA or scaffold RNA), important for the anchoring of gRNA into Cas9 (Jinek et al., 2012). In vitro studies determined the minimum sequence of crRNA and tracrRNA required for its use in synthetic models. These two minimal sequences were joined, thus creating the sgRNA (single guide RNA) (Figure 1) (Anders et al., 2014; Dang et al., 2015; Hsu et al., 2013; Jinek et al., 2012; Nishimasu et al., 2014). After recognizing the target DNA sequence, through its complementarity with gRNA/sgRNA, Cas9 cleaves the double-stranded DNA (dsDNA) at three base pairs upstream from the PAM sequence (Protospacer Adjacent Motif), using its two distinct nuclease domains.

The HNH domain cleaves the DNA strand complementary to the sgRNA sequence (target strand), while the RuvC domain is responsible for cleaving the DNA strand opposite to the complementary strand (non-target strand) (Jiang; Doudna, 2017). Studies have shown that specific mutations in the domains HNH (for example, H840A) or RuvC (for example, D10A) can convert Cas9 into a nicking enzyme, which cleaves only one strand of dsDNA. Mutations in both domains abolish Cas9 endonuclease activity without interfering with its RNA-guided DNA-targeting ability, thus being designated as dead Cas9 or dCas9 (Table 1) (Jiang; Doudna, 2017; Jinek et al., 2014, 2012).

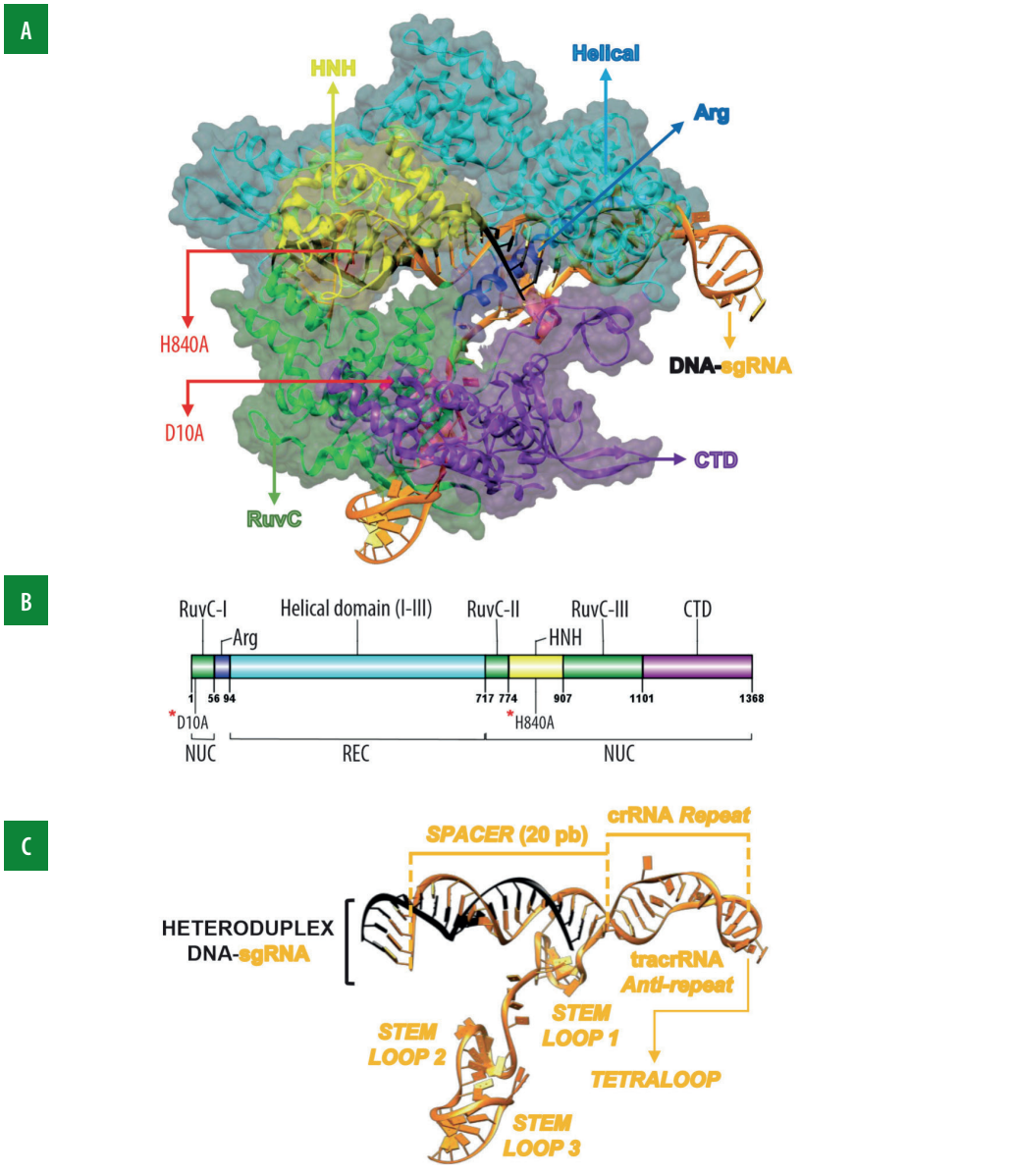


Figure 1. Structure of Cas9/dCas9 and sgRNA. Structural model (A) and diagrammatic representation (B) of the functional domains of Cas9/dCas9 associated with sgRNA, interacting with the target DNA strand (complementary strand represented in black). The NUC lobe comprises the C-terminal domain (CTD), RuvC-II and RuvC-III (tripartite) domains, and HNH nuclease domain. The dCas9 mutations in RuvC (D10A) and HNH (H840A) domains are represented in red (A) and with asterisks (B). Single mutation confers nicking activity to Cas9, while both together abolish its nuclease activity. The lobe REC is composed exclusively of the Helical domain. The structure of sgRNA (C) includes the crRNA (spacer + crRNA repeat) and scaffold RNA or tracrRNA (in this scheme, the anti-repeat tracrRNA is linked to crRNA repeat by a tetraloop, followed by three-stem loops). The structural representations are based on models deposited in a public database (PDB ID: 4008), available at https://www.rcsb.org/.

Table 1. Site directed mutagenesis on Cas9 nuclease of *Streptococcus pyogenes* (SpCas9) (UniProtKB - Q99ZW2) and its effects.

Position	Mutation[(1)]	Mutation effects	Reference
10	D → A	Non-complementary DNA strand to crRNA is not cleaved; nicking activity	Nishimasu et al. (2014)
15	S → A	Reduction of target DNA cleavage efficiency	Nishimasu et al. (2014)
66	R → A	Significant reduction of target DNA cleavage efficiency	Nishimasu et al. (2014)
70	R → A	Absence of target DNA cleavage	Nishimasu et al. (2014)
74	R → A	Significant reduction of target DNA cleavage efficiency	Nishimasu et al. (2014)
78	R → A	Moderate reduction of target DNA cleavage efficiency	Nishimasu et al. (2014)
97 – 150	Deletion	Absence of nuclease activity	Nishimasu et al. (2014)
165	R → A	Moderate reduction of target DNA cleavage efficiency	Nishimasu et al. (2014)
175 – 307	Deletion	50% loss of nuclease activity	Nishimasu et al. (2014)
302 – 409	Deletion	Absence of nuclease activity	Nishimasu et al. (2014)
475 – 477	PWN → AAA	Slight reduction in cleavage and interaction with target DNA	Jinek et al. (2014)
762	E → A	Cleavage of only one strand of the target DNA, probably the non-complementary to the crRNA	Nishimasu et al. (2014)
840	H → A	Non-complementary DNA strand to crRNA is not cleaved; nicking activity	Anders et al. (2014)
854	N → A	Reduction of target DNA cleavage efficiency	Nishimasu et al. (2014)
863	N → A	Cleavage of only one strand of the target DNA, probably the non-complementary to the crRNA	Nishimasu et al. (2014)
982 – 983	HH → AA	Correct sgRNA processing	Fonfara et al. (2014)
982	H → A	Reduction of target DNA cleavage efficiency	Nishimasu et al. (2014)
983	H → A	Cleavage of only one of the strands of the target DNA, probably not complementary to the crRNA	Nishimasu et al. (2014)
986	D → A	Cleavage of only one of the strands of the target DNA, probably complementary to the crRNA	Nishimasu et al. (2014)
1099 – 1368	Deletion	Nuclease activity is not detected	Nishimasu et al. (2014)
1125 – 1127	DWD → AAA	No change in cleavage efficiency, but a slight reduction in interaction with the target DNA	Jinek et al. (2014)
1132	G → C	Protein inactivation (probably)	Jiang et al. (2013)

To be continued...

Table 1. Continued.

Position	Mutation[(1)]	Mutation effects	Reference
1133 – 1135	RKR → AKA	Almost complete loss of nuclease activity	Anders et al. (2014)
1133	R → A	Dramatic reduction in the interaction with the target DNA, but a slight reduction in cleavage efficiency	Anders et al. (2014)
1135	R → A	Dramatic reduction in the interaction with the target DNA, but a slight reduction in cleavage efficiency	Anders et al. (2014)

[(1)] Abbreviation of amino acids: A - alanine; D - aspartic acid; E - glutamic acid; G - glycine; H - histidine; K - lysine; N - asparagine; P - proline; R - arginine; S - serine; W - tryptophan.

Applications of the dCas9 system in plants

The CRISPR system applications go beyond those related to site-directed mutagenesis by cleavage of dsDNA and activation of the repair system. The dCas9 protein offers a unique, multifunctional, and dynamic platform for the recruitment of proteins with different functions specific sites in the genome to promote, for example, the control of transcription regulation, epigenome editing, base editing, and genome imaging.

Base editing has become a powerful tool in plant biotechnology for site-directed mutagenesis. The development of herbicide-tolerant varieties for wheat, rice, corn, and watermelon crops, for example, was made possible by the introduction of point mutations into the genes encoding the enzymes acetolactate synthase and/or acetyl-coenzyme A, so that the final product is not transgenic (Kuang et al., 2020; Li et al., 2019; Tian et al., 2018; Zhang et al., 2019b). Base-editing systems require the fusion of Cas9, dCas9, or nCas9 variants, guided by a sgRNA, fused to a cytosine (C) deaminase, which induces the conversion of CG (Guanine) to TA (Thymine-Adenine) (CBE, cytosine base editor), or an A deaminase, which promotes the conversion of AT to GC (ABE, adenine base editor) (Gaudelli et al., 2017; Komor et al., 2016; Nishida et al., 2016). These systems have been successfully adapted for plants, including multiplex systems, in which more than one gene is simultaneously a target for mutations, with high efficiency, specificity, and without the occurrence of indels at the editing site. More satisfactory results were obtained with the use of nCas9 (Hua et al., 2018; Kang et al., 2018; Zong et al., 2017).

Another potent application of CRISPR/dCas9 is in genome imaging, used to visualize the spatial organization and temporal interactions of chromatin in real-time. For that, dCas9 is fused to fluorescent proteins, such as GFP (Green Fluorescent

Protein), resulting, for example, in a robust visualization of telomeric repetitions and their movements, multiple genomic loci in living cells, and protein-DNA interactions (Chen et al., 2013; Dreissig et al., 2017). Khosravi et al. (2020) optimized the method by inserting aptamers into the sgRNA scaffold able to recruit binding proteins fused to fluorescent proteins. Thus, a more significant number of reporter proteins is mobilized to the targeted sequence resulting in improved labeling.

The CRISPR/dCas9 system is a powerful tool to modulate the transcription of protein-coding and non-coding genes. It is a flexible and reversible tool since it can be used both for activation (CRISPRa, activator) and for repression (CRISPRi, interfering) of the transcription without permanently modifying the genome. Additionally, CRISPR/dCas9 can be used in multigene approaches to simultaneously modulate the expression of more than one gene, even in opposite directions. In this methodology, dCas9 and/or sgRNA are fused to transcriptional modulators (ModT), which act on activation or repression of the transcription. The ModT are proteins or protein domains that bind DNA to recruit key regulatory elements to control gene expression.

The fusion of ModT to dCas9 emerged as a strategy to enhance the effects of CRISPRi technology, initially developed based on the blockage of transcription, by physical interference on the binding of RNA polymerase and transcription factors to Transcription Start Site (TSS) or elongation process (Qi et al., 2013) (Figure 2A). The strategy of fusing ModT to dCas9 allowed the optimization of transcriptional repression and the development of a methodology for activation (Gilbert et al., 2013). Thus, CRISPR/dCas9 can be considered a generic and universal platform, since it is capable of promoting transcriptional activation and repression at different degrees in different species. In this way, the same transcriptional repressor or activator can be used to regulate transcription in cells of different species, with efficiencies varying according to the target genomic region, sgRNA design, delivery strategy (transformation method), and biological system in study.

The epigenetic regulation of chromatin through ModT, as acetyl and methyltransferases, is called epigenome editing (Hilton et al., 2015; Thakore et al., 2015). In plants, this method was used to induce drought tolerance in *Arabidopsis thaliana*, by the overexpression of *AtAREB1* gene (Abscisic Acid-Responsive Element Binding Protein 1), using dCas9 fused to an acetyltransferase 1, and also to alter the flowering time, through epigenetic regulation of the *AtFT* gene (Flowering Locus T) in *A. thaliana*, using the CRISPR/dCas9 system associated with an acetyltransferase or a methyltransferase (Lee et al., 2019; Roca-Paixão et al., 2019).

Next, the main ModTs, including transcriptional activators (AtvT) and transcriptional repressors (RepT), are presented, as well as the main strategies for modulating gene expression through the CRISPR/dCas9 system.

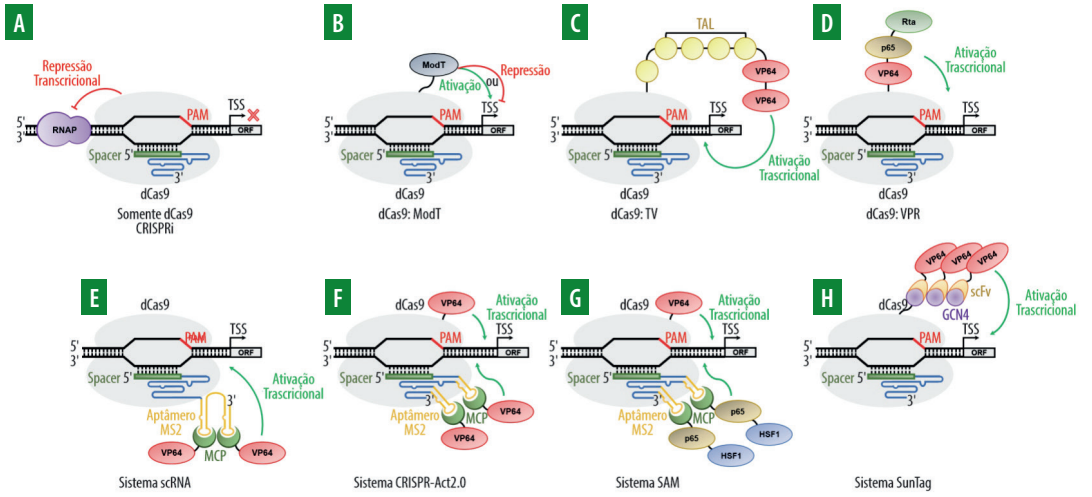


Figure 2. Strategies for the use of CRISPR/dCas9 technology in modulating gene expression. (A) Repression (CRISPRi, interfering) via RNA Polymerase (RNAP) blockage by the dCas9-sgRNA complex; (B) Activation or suppression by the fusion in tandem of transcriptional modulators (ModT) to dCas9; (C) Activation by the combination of ModTs (VP64 and TAD) fused to dCas9 (dCas9: VT); (D) Activation by the combination of ModTs (VP64: p65: Rta, VPR) fused to dCas9; (E) Scaffold RNA system (scRNA), in which the sgRNA containing an aptamer MS2 at its 3 'end recruits ModT (VP64) via fusion with MCP (MS2 Coat Protein); (F) CRISPR-Act2.0 system in which dCas9: VP64 is used in combination with a modified sgRNA with two aptamers MS2 that recruit ModT via MCP; (G) SAM system, optimization of the CRISPR-Act2.0 system, in which dCas9 is fused to a combination of ModTs (p65-HSF1); (H) SunTag system that consists of fusing dCas9 to tandem repeats of the GCN4 peptide that recruits ModT (VP64) via antibody (scFv) that binds to GCN4. TSS - transcription start site; ORF - open reading frame; PAM - protospacer adapter motif.

Strategies for modulating gene expression via CRISPR/dCas9

Increase of gene expression (CRISPRa)

Fusion of transcriptional activators to dCas9 protein (dCas9: AtvT)

This can be considered the pioneer strategy for activating transcription in plants by the CRISPR/dCas9 system. The sequence of a transcriptional activator (AtvT) is fused in tandem to the C-terminal region of the dCas9 protein. This complex is specifically targeted to the promoter region of a target gene by sgRNA (Figure 2B). Activators such as VP64, EDLL, TAD, and HAT (Table 2) have been used successfully to increase the expression of coding and non-coding genes (microRNAs) in plants, which can

be enhanced with the use of multiple sgRNAs complementary to sequences of the same promoter region (Lowder et al., 2015; Piatek et al., 2015; Roca Paixão et al., 2019; Vazquez-Vilar et al., 2016). However, the ideal number of sgRNAs varies depending on the dCas9:AtvT system used, and the excess may lead to an increase in non-specific targets (off-targets). Furthermore, the overabundance of sgRNAs and dCas9 can reach the saturation point and cause the attenuation of transcription due to the structural interference of physical binding of multiple dCas9-sgRNA complexes to the target DNA region (Piatek et al., 2015; Roca Paixão et al., 2019).

Table 2. Main transcriptional modulators used in the CRISPR / dCas system in plants.

Modulator	Type[(1)]	Source	Reference
VP64	AtvT	Artificial tetrameric repetition of the minimal activation domain of VP16 protein of Herpes Simplex Virus	Beerli et al. (1998)
EDLL	AtvT	Domain of the transcriptional activator AtERF98 (ethylene response factor) of *Arabidopsis thaliana*	Tiwari et al. (2012)
TAD	AtvT	*Xanthomonas campestris* Hax3 transcriptional activator DNA binding domain	Mahfouz et al. (2011)
TV	AtvT	6x TAD + 2x VP64	Li et al. (2017)
VPR	AtvT	Tripartite activator containing VP64, p65 (NF-kB trans-activator subunit) and Rta	Chavez et al. (2015)
HAT	AtvT	Catalytic nucleus of *A. thaliana* histone acetyltransferase 1	Roca Paixão et al. (2019)
p65-HSF	AtvT	NF-kB trans-activating subunit fused to the human heat-shock factor (HSP1) activation domain	Konermann et al. (2015)
SRDX	RepT	Domain of repression of the EAR motif (amphiphilic repression motif associated with ERF)	Ohta et al. (2001)
KYP	RepT	SET domain of the H3K9 KRYPTONITE (KYP) methyltransferase from *A. thaliana*	Jackson et al. (2002)
BRD	RepT	B3 repression domain of *A. thaliana* transcription factors	Ikeda e Ohme-Takagi (2009)
KRAB	RepT	Repressive domain of transcription factors that contain the Krüeppel zinc finger pattern of tetrapods	Mark et al. (1999)

[(1)] AtvT – Transcriptional Activator; RepT- Transcriptional Repressor

Another point to be considered is the location of sgRNAs. In general, sgRNAs targeting the region upstream of the TATA box and TSS correlate positively with

dCas9-mediated gene activation, probably due to the interference with the transcriptional initiation complex. When dCas9 is directed downstream or close to the TATA box, gene expression is negatively affected, probably through the physical blockage of transcription machinery by dCas9 (Deaner; Alper, 2017; Farzadfard et al., 2013). However, the optimal sgRNA distance upstream of the TSS for maximum gene activation can vary depending on the type of AtvT used (Piatek et al., 2015).

The choice of DNA strand (sense or antisense) also influences the efficiency of the method. Although there is no consensus, sgRNAs located on the sense strand and close to TSS are generally more efficient in activating transcription. Those located on the antisense strand can block RNA polymerase binding and initiation of transcription or lead to premature transcription termination (Howe et al., 2017; Piatek et al., 2015).

Thus, the increasing knowledge on the structure and functionality of the target promoter region (composition and role of cis-elements, recruitment of transcription factors, TSS position, among others) favors the success of dCas9 technology. Therefore, it is strongly recommended, as a first step, to test different sgRNAs, in different combinations, positions, and orientations in transient expression systems.

Another variation of this strategy is the fusion of multiple activators to the dCas9 sequence. In the dCas9: VP system, for example, the dCas9 protein was fused to six TAD modules followed by two VP64, which provided an expression increase of up to 190 times compared to the basal expression of the endogenous gene, both in monocotyledons and in dicotyledons (Figure 2C). The dCas9: VP system proved to be efficient in a multiplex system, promoting a simultaneous increase in the expression of three genes, and it was also effective in a DNA-free system, using complexes of ribonucleoproteins (RNP), but with a lower level of gene activation (Tables 2 and 3) (Li et al., 2017). However, the effects of fusing more than one activator to the dCas9 are not always additive. The dCas9: VP64: EDLL fusion, for example, resulted in a modest activation of endogenous genes, inferior to the classic dCas9: VP64 system, often considered weak and inefficient in plant cells (Table 3) (Li et al., 2017; Lowder et al., 2018). It must be taken into account that the fusion of an excessive number of activators can trigger the degradation of mRNAs due to the high number of repetitive sequences (Li et al., 2017).

An interesting observation is that there is a negative correlation between the endogenous basal expression of the gene and the magnitude of increased expression via dCas9: AtvT. Endogenous genes with lower basal expression are more likely to be strongly induced by this system, compared to those with higher basal transcription (Li et al., 2017; Lowder et al., 2018).

Table 3. Strategies for using the CRISPR/dCas9 system for the activation of gene expression in plants.

Plant species	Target-gene	CRISPR/dCas9 strategy	Transformation system	Activation level	Reference
A. thaliana	*AtPAP1*	dCas9:VP64 dCas9:VP64-EDLL dCas9:VP64-MS2-VP64 dCas9:VP64-MS2-EDLL dCas9:VP64- MS2-p65-HSF	Transgenic plants	7.0 x 4.0 x 45.0 x 30.0 x 3.0 x	Lowder et al. (2015) Lowder et al. (2018) Park et al. (2017)
	AtFIS2	dCas9:VP64 dCas9:VP64-EDLL dCas9:VP64-MS2-VP64 dCas9:VP64-MS2-EDLL	Transgenic plants	200.0 x 2.5 x 1500.0 x 30.0 x	Lowder et al. (2018)
	miR319	dCas9-VP64 dCas9:VP64-MS2-VP64 dCas9:VP64-MS2-VP64 + 3sgRNA	Transgenic plants	7.5 x 6.0 x 2.5 x	Lowder et al. (2015); Lowder et al. (2018)
	AtULC1	dCas9:VP64-MS2-VP64 + 3 sgRNAs	Transgenic plants	40.0 x	Lowder et al. (2018)
	AtWRKY	dCas9:TV dCas9:TV + RNP	Transgenic plants Protoplast/PEG	139.0 x 11.7 x	Li et al. (2017)
	pWRKY::luciferase	dCas9:TV dCas9:VP64-MS2-TV dCas9:VP64-	Protoplast/PEG	510.0 x 170.0 x 6.7 x	Li et al. (2017)
	AtRLP23	dCas9:TV dCas9:TV dCas9:TV + RNP	Transgenic plants Protoplast/PEG Protoplast/PEG	30.0 x 44.0 x 9.3 x	Li et al. (2017)

To be continued...

Table 3. Continued.

Plant species	Target-gene	CRISPR/dCas9 strategy	Transformation system	Activation level	Reference
A. thaliana	*AtWRKY* *AtRLP23* *AtCDG1*	dCas9:TV multiplex	Protoplast/PEG	80.0 x 37.0 x 192.0 x	Li et al. (2017)
	AtAREB1	dCas9:HAT	Transgenic plants	1.7 x	Roca Paixão et al. (2019)
	AtFWA *AtAPETALA3* *AtCLAVATA3* *Evadé transposon*	dCas9:SunTag-VP64	Transgenic plants	1.5 x 500.0 x 100.0 x 4000.0 x	Papikian et al. (2019)
	AtAVP1	dCas9:VP64-MS2-p65-HSF SAM	Transgenic plants	5.0 x	Park et al. (2017)
	AtFT	Cas9-MS2-VP64	Transgenic plants	30.0 x	Lee et al. (2019)
N. benthamiana	*NbPDS*	dCas9:EDLL + 3 sgRNAs dCas9:TAD + 3 sgRNAs	Agroinfiltration	3.4 x 4.0 x	Piatek et al. (2015)
	pNOS::luciferase	dCas9:VP64 dCas9:EDLL dCas9:EDLL-MS2-VP64	Agroinfiltration	3.0 x 3.0 x 4.0 x	Vazquez-Vilar et al. (2016)
		dCas9:SunTag-VP64 dCas9:SunTag -EDLL		3.5 x 3.0 x	Selma et al. (2019)

To be continued...

Table 3. Continued.

Plant species	Target-gene	CRISPR/dCas9 strategy	Transformation system	Activation level	Reference
O. sativa	*OsGW7*	dCas9:TV	Protoplast/PEG	79.0 x	Li et al. (2017)
	OsER1	dCas9:TV dCas9:TV + RNP	Protoplast/PEG	62.0 x 13.0 x	Li et al. (2017)
	Os03g01240	dCas9:VP64 dCas9:VP64-MS2-VP64 + 2 sgRNAs	Protoplast/PEG	2.0 x 3.0 x	Lowder et al. (2018)
	Os04g39780	dCas9:VP64 dCas9:VP64-MS2-VP64 + 2 sgRNAs	Protoplast/PEG	0.0 4.0 x	Lowder et al. (2018)
	Os03g01240 *Os04g39780* *Os11g35410*	dCas9:VP64 multiplex	Protoplast/PEG	2.0 x 2.0 x 2.0 x	Lowder et al. (2018)
	Os03g01240 *Os04g39780* *Os11g35410*	dCas9:VP64-MS2-VP64 multiplex	Protoplast/PEG	3.0 x 6.5 x 2.5 x	Lowder et al. (2018)

Modification of the sgRNA structure for activator recruitment

Even with all the improvements, the strategy of fusing ModT to dCas9 may not be able to modulate the expression levels of target genes satisfactorily. Also, the one-to-one ratio of dCas9-sgRNA in classical systems restricts the programming of multiple gene circuits to only one direction of regulation: repression or activation. This does not correspond to the complexity and sophistication underlying native transcription networks. However, similarly to ModTs and dCas9 fusions, the engineering of the sgRNA structure proved to be a modular and adjustable platform to diversify the positions to be recognized in the target genomes and the direction of transcriptional regulation mediated by the CRISPR system. This strategy is based on the modification of the sgRNA structure by the addition of RNA aptamers, making it capable of recruiting adapter proteins fused to ModTs to the target promoter region. Thus, a greater number of ModT in the target site can increase the magnitude of the activation levels (Konermann et al., 2015; Zalatan et al., 2015).

In the first systems using engineered sgRNAs, the sequence of RNA aptamers was inserted at its 3'end. These aptamers, such as MS2, fused to sgRNA scaffold, could interact with specific proteins such as MCP, fused with ModTs, allowing the specificity of the target in the genome, together with the modulating function. This system was called scaffold RNA (scRNA) (Figure 2E) (Jensen et al., 2017; Kiani et al., 2015; Zalatan et al., 2015).

Later, in the development of a second system, it was shown that the scaffold RNA region assumes a specific secondary structure, formed by a tetraloop and 3 stem-loops, important for the sgRNA anchoring in Cas9/dCas9 (Figure 1). It was evident that the tetraloop and stem-loop 2 protrude out of the dCas9-sgRNA complex, with the four distal base pairs of each stem completely free of interactions with the protein's amino acid side chains. Functionality studies have shown that substitutions and deletions in the tetraloop and stem-loop 2 regions do not affect the target DNA recognition function by dCas9 and that they can tolerate the addition of RNA aptamers (for example, MS2), which in turn, are recognized by specific proteins (for example, MCP). These specific proteins recruit a greater number of ModT to the target region in the genome, recognized by the dCas9-sgRNA complex. The technology of combining ModTs fused to dCas9, and the secondary structure of sgRNA was named as Synergistic Activation Mediator (SAM). A similar system adapted for plants using AtvT VP64 was called CRISPR-Act2.0 (Figure 2 F and G) (Konermann et al., 2015; Li et al., 2017; Lowder et al., 2018; Nishimasu et al., 2014).

In plants, these systems have already been proven effective with the use of various combinations of AtvT (Table 2) (Lee et al., 2019; Lowder et al., 2018; Selma et al., 2019). The CRISPR-Act2.0 system showed superior ability to activate three

genes simultaneously in a multiplex scheme when compared to the classic dCas9: VP64 system (Table 3) (Lowder et al., 2018). However, the efficiency of the system can vary depending on the target gene, type of activators used, and the number of sgRNAs. For example, at the same time that the dCas9: VP64-MS2: VP64 system was able to activate the expression of the *A. thaliana AtFIS2* gene up to 1,500 times, a similar strategy, using dCas9: VP64-MS2: EDLL for activation of this same gene, presented a lower result than that obtained by the classic dCas9 system: VP64 (Table 3) (Lowder et al., 2018). These same authors launched the hypothesis that, for many genes, there must be a maximum limit for increasing its expression. Higher levels can trigger post-transcriptional gene silencing through the production of small interfering RNAs. Thus, strategies that should optimize the gene activation system via CRISPR/dCas9 do not always result in the desired effect, as exemplified in the activation of miR319 in *A. thaliana* (Table 3) (Lowder et al., 2018, 2015).

SUperNova Tag (SunTag) system for gene activation

This strategy was first applied in mammalian cells in an attempt to optimize the increase in gene expression by enhancing the number of activators in the target promoter region. In this method, dCas9 is fused with tandem repeats of the GCN4 peptide, which binds to an antibody (scFv) fused to ModT to (Figure 2H) (Tanenbaum et al., 2014). In plants, this strategy was adapted using the AtvT VP64 to increase in the transcription of endogenous genes and transposable elements up to 4,000 times (Table 3) (Papikian et al., 2019). A variation of this technique is the replacement of VP64 by the catalytic domain of methyltransferases to promote methylation of promoter regions and control of the transcription. Thus, the dCas9-SunTag system can be a potent tool for site-specific epigenome modulation. It is a highly specific system, capable of promoting transcriptional activation of genes, both in euchromatin and heterochromatin, and with heritable effects over multiple generations, even in the absence of the transgene (Papikian et al., 2019). In addition, the use of the dCas9-SunTag system associated with a catalytic domain of methyltransferases significantly minimizes generalized non-specific methylation, common when these domains are directly fused to dCas9 (Pflueger et al., 2018).

Reduction of gene expression (CRISPRi)

The dCas9, when directed by the sgRNA to a promoter region, in the vicinity of TATA-Box or TSS or a coding region of a gene, can physically block the assembly of the transcription complex and the initiation and elongation of transcription (Figure 2A). The CRISPRi is an efficient strategy of directed negative regulation

of gene expression, with reversible effects, low incidence of off-targets, and the possibility of being applied to multigene analyses (Qi et al., 2013). It is a functional platform in eukaryotes and an alternative strategy for modulating gene expression in prokaryotes, which does not have RNA interference (RNAi) machinery.

In plants, the efficiency of CRISPRi was demonstrated in transient transformation systems by the repression of a reporter gene in up to 80% (Table 4) (Piatek et al., 2015; Vazquez-Vilar et al., 2016). These latter authors still demonstrated the effects of dCas9-sgRNA complex location at promoter region on gene repression. They showed that gene expression was lower for sgRNAs located nearby TSS. They also observed a modest and non-significant additive effect of the simultaneous use of two or three sgRNAs on reducing gene expression (Table 4) (Vazquez-Vilar et al., 2016).

Fusion of transcriptional repressors to dCas9 (dCas9:RepT)

In this strategy, the dCas9 sequence is fused in tandem with one or more transcriptional repressors (RepTs) (Table 2), similarly to previously described for AtvT. In plants, the RepT mainly used so far is the SRDX, which is compatible with a multiplex system and reached maximum efficiency when three domains were fused to dCas9, with a reduction by up to 80% in gene expression (Lowder et al., 2018, 2015; Sarasua, 2020; Vazquez-Vilar et al., 2016). Although KRAB RepT is widely used in animal models, it was unstable and inefficient for gene expression modulation in plants, even when tested in different contexts, varying the type of Cas nuclease and the location of sgRNA in relation to the TSS (Table 4). KRAB is an exclusive motif of tetrapods, a fact that would limit its ability to recruit chromatin-modulation proteins in plant cells (Sarasua, 2020).

Interestingly, the dCas9: SRDX system was able to mask the effects on gene activation of dCas9: EDLL and dCas9: TAD so that gene expression remained unchanged when these ModTs were used jointly (Piatek et al., 2015). Although the dCas9: RepT strategy is a useful tool for modulating gene expression, in some cases, its efficiency is similar to or less than that obtained with the classical CRISPRi system without RepT (Table 4) (Sarasua, 2020; Vazquez-Vilar et al., 2016).

Another point to highlight is that the use of a different type of dCas protein can improve the efficiency of dCas: RepT systems. Tang et al. (2017) tested the dCas12a nuclease (also called dCpf1) fused with three domains of SRDX RepT [dCas12a: 3x (SRDX)] and observed a reduction of up to 90% in the transcription of the *miR159B* of *A. thaliana*. In a similar system, using dCas9: 3x (SRDX), this value was approximately 70% (Table 4) (Lowder et al., 2018). Sarasua (2020) also reported the superiority of dCas12a nuclease compared to dCas9 when both were

fused to the SRDX and BRD RepTs to reduce the expression of a reporter gene in a transient transformation system. Moreover, the use of two sgRNAs in these systems leads to a variable significant additive effect. Superior results were detected only for dCas9: SRDX combination, with no difference when one or two sgRNAs were used in dCas9: BRD and dCas12a: SRDX. For dCas12a: BRD, the use of two sgRNAs was less efficient (Table 4). The superior efficiency of dCas12a in suppressing gene transcription, compared to dCas9, may be related to the size of these nucleases. While dCas12a has approximately 1.250 amino acids, dCas9 has 1.368, which would facilitate dCas12a access to the target genomic region (Sarasua, 2020).

Modification of the sgRNAs structure for the recruitment of repressors

Despite being a widely used strategy for activating gene transcription, CRISPR-Act2.0 methodology has been used only once so far to suppress gene transcription in plants (Lee et al., 2019). The authors reported the failure of the method using SRDX RepT, but it was possible to achieve median gene repression levels with another RepT (KYP) (Table 4).

SUperNova Tag (SunTag) system for gene repression

This strategy was extensively explored in a detailed study by Sarasua (2020), who varied several parameters of the method, such as sgRNA location in relation to TSS, the number of sgRNAs (1 or 2), type of Cas nuclease (dCas9 or dCas12a), type of RepT (SRDX or BRD), and size of spacers (5 or 22 amino acids) between the GCN4 epitopes, using a transient system for monitoring the expression of the luciferase reporter gene guided by the nopaline promoter (*pNOS*). The system using dCas12a nuclease gave higher efficiency (Table 4), probably due to dCas12a smaller size, compared to dCas9, as previously discussed. The use of larger spacers of 22 amino acids between epitopes promoted better results since smaller spacers can offer steric impediment to antibody binding. In general, the SunTag system induced gene repression in a similar way as dCas: RepT strategy, showing to be slightly superior when two sgRNAs were used with dCas12a (dCas12a: Suntag - SRDX / BRD + 2 sgRNA). There was no significant difference between the two RepTs tested (SRDX and BRD) (Table 4).

Table 4. Strategies for using CRISPR / dCas9 system to suppress gene expression in plants.

Plant species	Target gene	CRISPR/dCas9 strategy	Repression level	Transformation system	Reference
A. thaliana	*AtCSTF64*	dCas9:3x (SRDX) + 3 sgRNAs	60	Transgenic plants	Lowder et al. (2015)
	miR159A *miR159B*	dCas9: 3x (SRDX) multiplex	80 70	Transgenic plants	Lowder et al. (2015)
	miR159B	dCas12a:3x (SRDX)	90	Transgenic plants	Tang et al. (2017)
	AtFT	dCas9-MS2-SRDX dCas9-MS2-KYP	0 40	Transgenic plants	Lee et al. (2019)
N. benthamiana	*NbPDS*	dCas9 dCas9:SRDX	20 50	Agroinfiltration	Piatek et al. (2015)
	pNOS: luciferase	dCas9 dCas9 + 2 sgRNA dCas9 + 3 sgRNAs dCas9:SRDX dCas9:SRDX + 3 sgRNAs dCas9:BRD dCas9:BRD + 3 sgRNAs	80 85 85 50 50 40 70	Agroinfiltration	VazquezVilar et al. (2016)
		dCas9:BRD dCas9:BRD + 2 sgRNAs dCas9:SunTag – BRD dCas9:SunTag – BRB + 2 sgRNA dCas9:SRDX dCas9:SRDX + 2sgRNAs dCas9:SunTag - SRDX dCas9:KRAB dCas12a:BRD dCas12a:BRD + 2sg RNA dCas12a:SunTag – BRD dCas12a:SunTag – BRD + 2sgRNAs dCas12a:SRDX dCas12a:SRDX + 2sg RNA dCas12a:SunTag – SRDX dCas12a:SunTag – SRDX+ 2 sgRNA dCas12a:KRAB	30 30 30 40 40 65 40 30 80 60 80 90 80 80 80 90 30	Agroinfiltration	Sasuna (2020)

Experimental design: important aspects to have in mind

As mentioned previously, gene expression modulation mediated by CRISPR technology contributes significantly to the understanding and orthogonal control of transcriptional and post-transcriptional regulations. Compared to other methods, such as RNAi technology, CRISPR technology is easy and offers RNA-mediated targeting of an individual or multiple genes, making it a powerful tool for the study of multifactorial native transcriptional regulation in diverse organisms, including plants (Deaner et al., 2017; Jensen et al., 2017; Zalatan et al., 2015). Thus, unique sets of multiplex experiments can be easily applied, and the use of more than one sgRNA allows rapid progression through interactive cycles of genetic engineering. By varying the expression of a single gene, it is possible to rapidly evaluate the combinatorial effects on a network of gene expressions to identify previously unknown targets.

There are many aspects regarding the experimental design that must be taken into account when using the CRISPR system for base editing or modulation of gene transcription. Thus, two important points are addressed in the following tutorials: (i) sgRNA design; and (ii) transformation cassette design (plasmid vectors).

sgRNAs design

Theoretical basis for choosing the best software/platform

Two main challenges for the biotechnological application of the CRISPR/Cas system are associated with the correct design of sgRNA: (i) reliable prediction of off-targets; and (ii) efficiency in recognition of on-targets (Xu et al., 2015; Zhang et al., 2015).

The parameters for a successful sgRNA design in CRISPR/dCas9 technology are the same as for native Cas proteins, even considering that DNA cleavage is not an important factor in CRISPR/dCas9. Thus, three main steps must be followed in sgRNA design: (i) identification of PAM sequences in the target region; (ii) selection of a 20 nucleotides sequence upstream from PAM, excluding it, named as spacer; and (iii) attachment of the spacer to the 5′ end of scaffold RNA, for the correct anchoring of sgRNA to Cas9/dCas9. A successful experimental design, especially of the sgRNA, includes the prediction of on-targets and off-targets (Doench et al., 2016; Tang et al., 2018).

For sgRNAs used with Cas9, guanines are preferred at positions 1 and 2 before the PAM sequence (NGG), while the presence of thymines at positions +4 and -4, flanking PAM, interferes negatively with nuclease cleavage efficiency (Wang et al., 2014; Wu et al., 2014). Also, the regions downstream from PAM can affect cleavage efficiency and sgRNA specificity, while the upstream sequences generally have no significant effect on these parameters (Doench et al., 2014). Cytosine is preferred at Cas9 cleavage site (position -3 next to PAM) (Cong et al., 2013; Xu et al., 2015), and a high GC content in the region comprising bases 4-13, downstream from PAM sequence, favors cleavage efficiency and sgRNA specificity. The sgRNA size is also a crucial factor for the specificity of the Cas9 target, with the minimum sgRNA size being 17 nucleotides for knockout gene experiments (Fu et al., 2014). For dCas9-mediated transcriptional and epigenetic modulation, the best sgRNA size is 20 nucleotides since a reduction in its size decreases the effectiveness of dCas9-mediated transcriptional regulation (Kiani et al., 2015; Smith et al., 2016). There is a certain degree of flexibility in the design of distal positions of sgRNAs used to recognize gene promoters in regions rich in nucleosomes or upstream activation sequences (UASs) (Kiani et al., 2015; Smith et al., 2016).

Powerful computational tools are continually developed and updated to facilitate in silico sgRNA design for biotechnological purposes (Chuai et al., 2017; Graham; Root, 2015; Wilson et al., 2018). However, how to choose the best software/platform for sgRNA design, given the availability of numerous options? There are several models for predicting sgRNA efficiency in silico, based on experimental evidence. To facilitate this choice, it is necessary to know what each software offers, highlighting its positive points and limitations.

Rule Set 1 is a predictive model based on a scoring system associated with an in silico learning method, which classifies the data in a linear and generalized way, in order to estimate the cutting efficiency of Cas9, with high correlations with experimental results (Doench et al., 2014; Liu et al., 2020). Independent studies contributed to improve the accuracy of this model, including more data sets to build a new model, called Rule Set 2, in which nucleotide analyses, regardless of the position and location of the sgRNA within the target sequence, can improve the prediction of off-targets and on-targets (Doench et al., 2016; Liu et al., 2020).

The software and algorithms developed to predict the specificity of sgRNAs are based on the determination of off-targets and have different types of data input (Table 5). All are built on two main methods: (i) alignment method, mainly used to predict all off-*targets in silico*; and (ii) a score method, in which sgRNAs are scored and classified according to off-targets identified through an alignment matrix.

In theory, putative off-targets can be identified by aligning the sgRNA sequences to a reference genome through sequence similarity. The tools Bowtie (Langmead et al., 2009) and BWA (Li and Durbin, 2009) allow the detection of off-targets but fail to identify small PAMs, since they were developed to align reads from Next Generation Sequencing (NSG) (Haeussler et al., 2016; Wilson et al., 2018). Another point is that these tools allow a limited number of mismatches, restricting the identification of putative off-targets (Langmead and Salzberg, 2012). However, not all nucleotide mismatches have the same effect on off-target cleavage, and the alignment-based prediction always identifies redundant off-targets, of which many are false positives. Considering these factors, CasOFFinder and FlashFry softwares, among those based exclusively on alignments, would be the best options to identify possible off-targets for any Cas nuclease (Liu et al., 2020). FlashFry software stands out by its high processing speed and easy-to-understand output.

Table 5. Most used online tools for sgRNA design [1]

Tool	Website	Species	Input data
Benchiling	https://www.benchling.com	Diverse	DNA sequence; gene ID
CCTop	https://crispr.cos.uni-heidelberg.de	Diverse	DNA sequence
CHOPCHOP	https://chopchop.cbu.uib.no	Diverse	DNA sequence; TSS; location or gene ID
CRISPETa	http://crispeta.crg.eu	Model plants	DNA sequence; location or gene ID
CRISPOR	http://crispor.tefor.net	Diverse	DNA sequence; location or gene ID
CRISPR-ERA	http://CRISPR-ERA.stanford.edu	Diverse	DNA sequence; TSS; ID do gene
CRISPR-GE	http://skl.scau.edu.cn	Plants	DNA sequence; location or gene ID
CRISPR-P 2.0	http://crispr.hzau.edu.cn/CRISPR2	Plants	DNA sequence; location or gene ID
CRISPR-PLANT	https://www.genome.arizona.edu/crispr2	Plants	DNA sequence; location or gene ID
CRISPR RGEN Tools	http://www.rgenome.net	Diverse	DNA sequence; location or gene ID sgRNA

To be continued...

Table 5. Continued.

Tool	Website	Species	Input data
CRISPRscan	https://www.crisprscan.org	Model plants	DNA sequence; location or gene ID
E-CRISP	http://www.e-crisp.org/E-CRISP	Diverse	DNA sequence; gene ID
EuPaGDT	http://grna.ctegd.uga.edu	Eukaryotic Pathogens	DNA sequence
FlyCRISPR	https://flycrispr.org/	Insects	DNA sequence
Yeastriction	http://yeastriction.tnw.tudelft.nl/#!/	*Saccharomyces cerevisiae*	Gene ID

(1) The information presented here is subject to website updates.

To improve off-target detection, the score-based method was developed using two approaches: (i) hypothesis-oriented, in which off-targets are scored based on the contributions of genome context specific factors to sgRNA specificity; and (ii) learning approach, in which sgRNAs are scored and predicted based on a training model, which considers the different factors that affect specificity.

In the hypothesis-guided approach, the MIT score (Hsu-Zhang) evaluates mismatches'contributions in different positions along sgRNA sequence using a weight matrix to identify possible off-targets. The MIT score is included in sgRNA design tools, such as CHOPCHOP (Labun et al., 2016) and CRISPOR (Haeussler et al., 2016). The Cutting Frequency Determination (CFD) is another popular score system for off-target prediction, which is taken into account the presence of non-canonical PAMs (NAG, NCG, and NGA), indels, and mismatches in the target sequences. CFD score shows a better performance than MIT score and is used by the softwares GUIDE-Seq (Tsai et al., 2015), CRISPRscan (Moreno-Mateos et al., 2015), GuideScan (Perez et al., 2017), CRISPOR (Haeussler et al., 2016), and Benchling (Doench et al., 2016).

The prediction of sgRNA specificity based on the structural characteristics of the Cas9-sgRNA complex proved to be superior to that based only on sequence characteristics. The CRISPRoff (Alkan et al., 2018) and uCRISPR (Zhang et al., 2019a) tools use energetic properties to predict off-targets and were more accurate in predicting off-targets when compared with tools that use other methods, such as MIT and CFD. However, none of them has been evaluated in large-scale experiments, and care should be taken when using them.

Empirical algorithms may fail in predicting off-targets since they consider few data sources. On the other hand, learning approaches build complex models using combinations of numerous resources. The CRISPR Target Assessment

(CRISTA) algorithm uses BWA as an off-target search tool and implements several parameters (PAM, nucleotide composition, GC content, chromatin structure, DNA methylation, secondary sgRNA structure, etc.) to predict cleavage (Abadi et al., 2017). CRISTA performs better than MIT and CFD. DeepCRISPR is a new innovative computational platform that unifies sgRNA on-target and off-target site prediction into one framework with deep learning (Chuai et al., 2018). This tool automates the identification of sequence and epigenetic features that may affect sgRNA efficacy. It was able to overcome other available tools for predicting off-targets (Doench et al., 2016; Hsu et al., 2013; Liu et al., 2020; Singh et al., 2015; Stemmer et al., 2015).

Many online tools combine some of the score methods mentioned above and even develop a unique evaluation system to provide alternative options for users, such as CHOPCHOP (Labun et al., 2019, 2016; Montague et al., 2014) and CRISPRscan (Moreno-Mateos et al., 2015) (Table 5). E-CRISP uses an exclusive score system denominated SAE (Specificity, Annotation, Efficacy) to determine the quality of sgRNAs. CCTop empirically assigns scores for the prediction of off-targets (Stemmer et al., 2015). CRISPOR (Haeussler et al., 2016) is a versatile platform that classifies sgRNAs according to different score systems to prediction-target activity and putative off-targets in the genome of interest. It also shows a list of PAM sequences to be selected by the user. The CRISPR RGEN platform (Sullenger, 2020) provides several computational tools, as well as information for sgRNA design for several Cas types. The platform has nine useful tools, including Cas-Designer (Park et al., 2015) and the BE-Designer (Hwang et al., 2018). Compared to other tools, Cas-Designer allows for mismatches when detecting DNA/RNA hybrids off-targets. This detection method is faster than others due to the association with the CasOFFinder algorithm. Sequences, genomic coordinates, and FASTA files are allowed as inputs. More than 350 genomes and 20 types of PAM sequences are available to users. The output results include on-targets, out-of-frame score, calculated by micro-homology, incompatibility number (0–2), and off-target sequences with mismatches of up to 1 base pair. On/off-targets are redirected to the Ensembl[1] genome browser for further evaluation. Unlike Cas-Designer, BE-Designer is used mainly for base editing. This tool offers four methods of base editing and the editing region is adjustable. The CRISPR-ERA (Liu et al., 2015) and CHOPCHOP v3 (Labun et al., 2019) permit sgRNA design for dCas9 system, and CHOPCHOP includes 30 different plant species. CRISPETa (Pulido-Quetglas et al., 2017) is used mainly for genome deletion using two sgRNAs.

The appropriate tool's choice should be made with caution since there are tools developed specifically for some organisms or cell types. For example, Yeastriction

[1] Available at: https://chopchop.cbu.uib.no/

(Mans et al., 2015) is developed for yeasts, FlyCRISPR (Gratz et al., 2014) for *Drosophila* and other insects, and EuPaGDT (Peng; Tarleton, 2015) for eukaryotic pathogens. CRISPR-P/CRISPR-P 2.0 (Lei et al., 2014; Liu et al., 2017), CRISPR-PLANT (Minkenberg et al., 2019), and CRISPR-GE (Xie et al., 2017) are applied for plant genome. CRISPR-P 2.0 is one of the most used platforms for genome editing in plants nowadays and allows sgRNA design and prediction of on-targets and off-targets. It supports the design of sgRNAs for 49 plant genomes, including species for which there is a well-assembled genome, and also permits sgRNA identification using user-supplied sequences as reference. It applies a modified score system to predict off-targets and sgRNA targeting efficiency using Cas9 and Cpf1 (Zetsche et al., 2015). This platform informs the GC's content, restriction endonuclease sites, microhomology of the sequence flanking the region to be edited, and the secondary structure of the sgRNA.

We chose CHOPCHOP[2] v3 (Labun et al., 2019) to present a tutorial on sgRNA design using dCas9, considering that this software contains the specific function for its nuclease, provides genomic data of more than 250 species, and undergoes constant updates. If you have previous in silico/ in vivo information about the promoter region of your interest, you can use other software, such as CRISPR RGEN or CRISPR-P 2.0.

Tutorial 1: sgRNA design for transcriptional modulation via dCas9

Step 1: Access URL https://chopchop.cbu.uib.no

Step 2: Choice of species of interest (Section *In*). The eukaryotic and prokaryotic species available so far are divided into the following categories: Arthropoda (37), Bacteria (58), Chordata (46), Cnidaria (1), Ctenophora (1), Echinodermata (2), Fungi (30), Mollusca (2), Nematoda (2), Others (12), Parasites (11), Plants (30), and Virus (20). In this tutorial, the plant species *Glycine max* (GCA_000004515.3) was chosen. You can request to the software developers the insertion of the genome sequence of a non-provided species. For that, you have to supply a FASTA file with the genome assembly, as well as a valid GFF3 file referring to its functional annotation (validated by gff3ToGenePred program, available on CHOPCHOP platform) (Figure 3). The ID assigned in the GFF3 file must match the identifier you want to use to search for the genes. Chromosome names must be identical in GFF3 and FASTA files, and gene coordinates must correspond to the FASTA file. Whether the requirements have been attended, a request must be sent by email (available in the "Add new species" section, below the place for selecting the species of interest). It is worth mentioning that the platform's maintainers perform the insertion of a new genome. The user is not allowed to insert the target genome manually.

[2] Available at: https://chopchop.cbu.uib.no/

Step 3: Choice of the target gene (section "*Target*"). The gene ID chosen for this tutorial is Glyma_09G153900. The target gene ID is inserted in this field and must correspond precisely with the genome deposed in the CHOPCHOP database. If there is no match, the platform itself will send the error message "Error status: 501" and the correct way to write the ID for the selected is given. This field acts synchronously with the gray "Paste Target" icon. This tool allows to find the gene according to the ID (RefSeq or ENSEMBL format), gene name, or gene coordinates in the genome (for example, chrX: 15,560,138-15,602,945). When clicking on the gray "Paste Target" icon, the user changes the icon's function to the "Gene Target" option, where it is possible to place the sequence of nucleotides to be analyzed. For dCas9-based strategy, sequence self-placement is not widely used since, in most cases, the genomic context of the promoter region of the target gene is lost, which is essential for transcriptional modulation via dCas9 (Figure 3).

Step 4: Choice of nuclease (section "*Using*"). In this field, the platform has so far made available five different types of nucleases for genome editing: (i) CRISPR/Cas9; (ii) CRISP/Cas9 nickase; (iii) CRISPR/Cpf1 (CasX); (iv) CRISPR/Cas13 (c2c2); and (v) TALEN. For this tutorial, the selected nuclease is "CRISPR/Cas9" (Figure 3).

Step 5: Choice of strategy (Section "*For*"). In this field, the approach or context in which the sgRNAs will be applied is selected (for this tutorial, the option "Activation" was selected (Figure 3):

a) *Knockout* (for mutations intending changes in the Open Reading Frame (ORF) of the target gene; available for almost all nucleases on the platform, exceptionally for CRISPR/Cas13): for applications with Cas9, it is possible to predict the rate of reading frame changes provided by each sgRNA. Some recommendations are suggested: (i) use the traditional Cas9 (20 nucleotides with PAM sequence 5'-NGG-3'); (ii) make sure that sgRNA has the fewest number of putative off-targets, preferably none; (iii) ensure that the selected sgRNA can recognize all isoforms of the target gene (not available for all genomes); and (iv) select sgRNAs downstream of any ATG in a frame within the ORF-structure (green boxes in the blue coding-region) to avoid the expression of truncated proteins.

b) *Knock*-down (for mRNA editing with CRISPR/Cas13; available only for humans and mice): in this mode, it is possible to search for off-targets in transcriptomes. It is possible to select the Cas13 system in "*Options*" (after selecting "CRISPR/Cas13" in the "*For*" section) and specify the protospacer flanking site (PFS) and the size of the sgRNA.

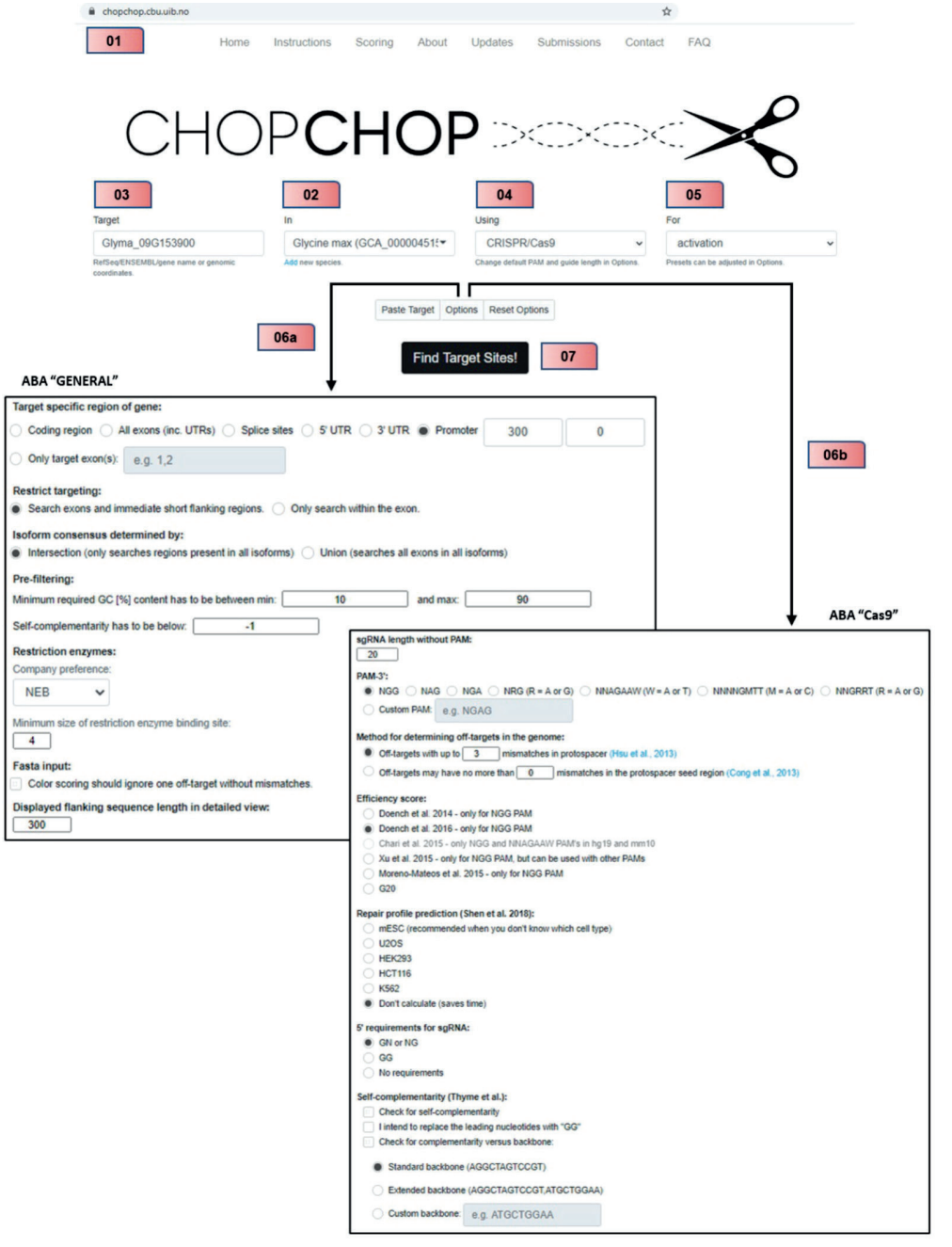


Figure 3. CHOPCHOP v3 – Parameters. Introduction of Tutorial 01 workflow for choosing the main parameters of sgRNAs design aiming at activation of a target gene from *G. max* (ID: Glyma_09G153900) via dCas9. The numbering shown in red rectangles corresponds to steps 1 to 7 of Tutorial 1.

c) *Knock-in* (for inserting DNA sequences in a region of interest; unavailable for CRISPR/Cpf1, CRISPR/Cas13, and TALEN): it is highly recommended to be familiarized with the different types of knock-ins available for choosing the most appropriate method (Nami et al., 2018). Knock-in by CRISPR technology allows site-specific genetic engineering through nuclease-induced double-stranded DNA (DSB) breakdown and, subsequently, DSB repair via cell repair pathways: non-homologous final junction (NHEJ) and homology-directed repair (HDR). An important aspect to consider when deciding the type of technique to use is whether the modification has a dividing- or non-dividing-cell as a target. HDR is restricted to the S and G2 phases of the cell cycle and is not suitable for non-dividing-cells. The sequences for the homology arms are provided on the sgRNA detailed results page. It is possible to adjust the position of the microhomology arms to the 5' end of the sgRNA (default: nucleotide -3 from the PAM sequence) and specify the size of the arm. It is recommended to check the complementarity between the inserted sequence and its microhomology arms. In the default settings for knock-in, homology arms up to 800 bp can be obtained. If a larger homology arm is desired (up to 2,000 base pairs), it is necessary to change the size of the flanking sequence shown on the home page ("*Options*" > "*General*" > "*Displayed flanking sequence length in detailed view*").

d) *Activation/Repression* (for sgRNAs targeting promoter regions; available for CRISPR/Cas9 and CRISPR/Cpf1 nucleases): in this option, the default for sgRNA design is: (i) activation mode: 300 bp upstream from the TSS; (ii) repression mode: 200 base pairs downstream and upstream from the TSS. It is recommended to use more than one sgRNA for these applications.

e) *Nanopore Enrichment*: used to enrich specific DNA fragments for sequencing through the Oxford Nanopore platform. It is only available for CRISPR/Cas9 and CRISPR/Cpf1 nucleases.

Step 6: Choice of advanced options (*Options section*). This section is found in the gray icon in the center of the home page and is extremely relevant to refine the results according to the characteristics of the target genome and sequence (Figure 3). When clicking on it, with the CRISPR/Cas9 nuclease previously selected, new options appear in three tabs: (i) "*General*", in which it is possible to adjust general parameters for the CRISPR technology; (ii) "*Cas9*" option, to adjust specific parameters for the Cas9 nuclease; and (iii) "*Primers*", to adjust parameters related to the design of primers that will be used in PCR experiments for mutations detection (basically used for knockout experiments). For dCas9 experiments, only parameters displayed in the "*General*" and "*Cas9*" tabs will be adjusted:

a) "*General*" tab:

- *Target a specific region of the gene:* it is possible to specify the region where the sgRNAs will be designed: (i) only the coding region (default); (ii) the entire exonic sequence (including 5'and 3'-UTR); (iii) splicing sites; (iv) only 5'-UTR; (v) only 3'-UTR; (vi) the promoter (it is possible to specify how many bp upstream and downstream from the TSS will be analyzed); or (vii) a specific exon or a specific subset of exons. If you want to target an intron, specify its genomic coordinates (maximum size, 40.000 base pairs).
- *Restrict targeting:* when searching for on-targets in a region of interest, in default mode, CHOPCHOP allows the sgRNA to link only outside that region, ensuring a cut on the target region. It is possible to disable this option in experiments with dCas9.
- *Isoform Consensus*: when analyzing genes with multiple isoforms, the "*Intersection*" or "*Union*" modes can be activated. In "*Intersection*" mode, CHOPCHOP searches only for sgRNAs present in all isoforms. In "*Union*" mode, CHOPCHOP looks for sgRNAs in all exons of all isoforms. Therefore, you can use this mode to target one or more specific isoforms. It is possible to disable this option in experiments with dCas9.
- *Pre-filtering:* it is possible to pre-filter sgRNAs based on GC content and self-complementarity score (set to -1 to disable, which is not recommended). sgRNAs that do not attend these requirements will not be reported in the final result table.
- *Restriction enzymes:* mutagenesis can be assessed by digestion with restriction enzymes. CHOPCHOP displays restriction sites at the target site and allows you to restrict your search to enzymes distributed by specific companies (NEB™, Fermentas™, Promega™, Roche™, Sigma™, and Stratagene™), as well as by the number of bases recognized by the enzyme. This parameter is not relevant in dCas9-mediated transcriptional modulation experiments.

b) "*Cas9*" tab:

- *sgRNA length without PAM:* since some studies suggest that truncated sgRNAs may show a greater cleavage efficiency, it is possible to design them shorter than 20 nucleotides (default), which is not recommended for experiments with dCas9.
- *PAM-3' sequence:* the default PAM sequence for Cas9 is NGG. Alternatively, it is possible to select a PAM from an orthologous system or insert a

personalized PAM. For *S. pyogenes* dCas9, it is recommended to use the default PAM.

- *Method for determining off-targets in the genome:* there are two options: (i) one-base mismatch from the first to the eleventh base from the 5' end of PAM sequence makes Cas9 cleavage unviable (Cong et al. 2013). However, mutations further upstream of PAM maintain cleavage activity. This method searches for mismatches only in the first nine nucleotides from the PAM sequence, considering that mismatches after PAM will not promote cleavage; and (ii) mismatches can be tolerated in any position, except in the PAM. This method looks for mismatches only in the 20 base pairs upstream from PAM (Hsu et al., 2013). This is the default method, and the most used for experiments with dCas9.
- *Efficiency score:* there are several methods to evaluate and calculate the cleavage efficiency by Cas9 nuclease. The simplest form of efficiency scoring is the "G20" (Doench et al., 2016) (default), in which guanine is prioritized at position 20, right after the PAM. This value is normalized on the CHOPCHOP platform ranging from 0 to 1. Since cleavage efficiency is not relevant in experiments with dCas9, it is recommended to use the default parameter.
- *Repair profile prediction:* according to specific models (Shen et al., 2018), it is possible to predict the DNA repair profile for a sgRNA. For this option, the cell type must be selected, but only animal cells are available (U2OS, HEK293, HCT116, K562), or the "*mESC*" mode, if the cell type to be edited is not known. For experiments with dCas9, the option "*Don't calculate (saves time)*" must be selected since it is time-consuming and is not applied to dCas9 approaches.
- *Requirements for the 5' end of the sgRNA:* depending on the polymerase used for the synthesis of sgRNA, it is possible to select its first two nucleotides aiming at the best transcription efficiency. For example, 5'-GN- (for the U6 promoter) or 5'-GG- (for T7 polymerase). Regarding the dCas9 system, if the purpose is base editing using RNP, in which sgRNA is transcribed *in vitro* by T7 polymerase, the option "GG" should be selected. In transcriptional modulation experiments, in which the sgRNA will be expressed *in vivo*, regulated by the U6 promoter, the option "GN or GG" must be selected.
- *Self-complementarity*: studies suggest that self-complementarity within the sgRNA or between the spacer and the scaffold RNA) can reduce the

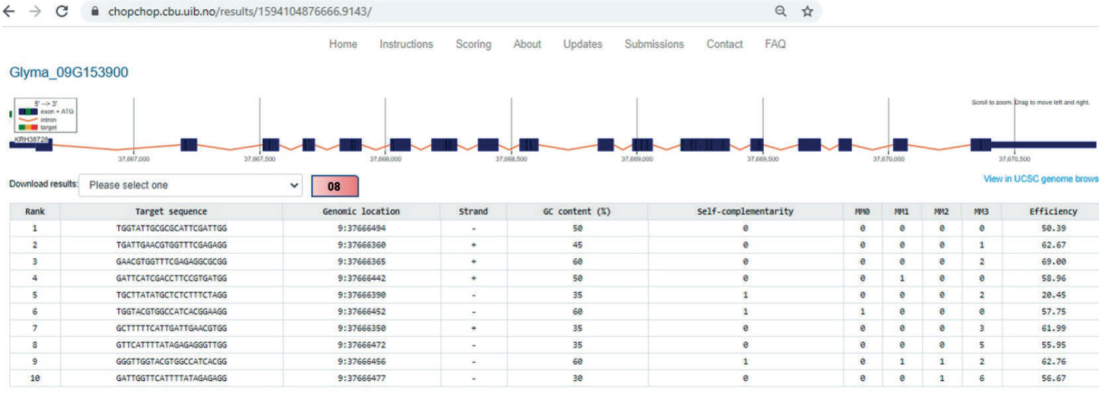
cleavage efficiency of sgRNA (Thyme et al., 2016). This option evaluates the complementarity within the sgRNA or between the spacer and a standard scaffold RNA (AGGCTAGTCCGT), an extended scaffold RNA (AGGCTAGTCCGT, ATGCTGGAA), or a custom scaffold RNA. Some users prefer to replace the first two nucleotides of sgRNA with "GG" for transcription via T7 polymerase. In this case, select the option "*I intend to replace the leading nucleotides with "GG"*. For experiments with dCas9, it is necessary to evaluate the options "*Check for self-complementarity*" and "*Check for complementarity versus backbone*". About the latter, if the experimental approach holds traditional RNA scaffold, you should select "*Standard backbone* (AGGCTAGTCCGT)", but if the approach uses RNA aptamers (for example, the SAM system), the "*Custom backbone*" option must be selected and the modified scaffold RNA sequence must be provided. Finally, the option "*I intend to replace the leading nucleotides with "GG"* should be selected exclusively for experiments with dCas9 aiming at base editing.

Step 7: Performing the analysis. After selecting all parameters, click on "*Find Target Sites!*" to submit the analysis. If everything is correct, the message "*Your job has been successfully submitted*" will be displayed. The analysis time may vary according to the selected parameters. CHOPCHOP displays the query results in a dynamic view and interactive table (Figure 4). The dynamic view displays all the on-target options for the region, color-coded according to a specific scoring system: green (best), amber (ok), and red (bad). In all cases, the gene is displayed in the 5'- 3' direction. All isoforms of the gene are displayed with their names and, at downstream, the ATG sites in the frame (green box). Simply click on a target in the visualization screen or on the options in the table and you will be redirected to a results page for each individual sgRNA, containing information about on-targets, off-targets, repair predictions (when applicable), micro-homology arms (knock-in mode), restriction sites, and primers designed for each region. In addition, CHOPCHOP lists the number of off-targets for each on-target with 0 (column "MM0"), 1 (column "MM1"), 2 (column "MM2"), or 3 (column "MM3") mismatches. Each on-target is ranked according to: (i) cleavage efficiency score ("Cas9" mode); (ii) number of off-targets and whether they have mismatches; (iii) presence of self-complementarity regions with more than three nucleotides. The number indicates the quantity of expected self-complementarity regions); (iv) GC content ("CRISPR/Cas9" mode): sgRNAs are more effective with a GC content between 40% and 70%; and (v) sgRNA location on the target gene (the closer to the 5' end, the better is the classification). Finally, each page of results provides (i) visualization of the target site of each sgRNA (with the cut site predicted in blue in the "CRISPR/Cas9" or "CRISPR/Cpf1" mode); (ii) primers

designed (in purple); (iii) restriction sites (in green unique sites in the amplicon, and in red non-exclusive sites); and (iv) details about the off-targets (genomic location, number of mismatches, and sequence).

Step 8: Download the results. The final result is available for download in four different formats: (i) BED (for viewing in the genome browser); (ii) GenBank (file with annotation and intron); (iii) TSV (results table; mostly used); and (iv) FASTA (Figure 4).

Other considerations: It is not wrong to suppose that the use of more than one sgRNA for different regions of the same promoter can, in association with the dCas9 complex and a ModT, amplify the desired levels of activation or repression (Deaner et al., 2017; Farzadfard et al., 2013; Gilbert et al., 2013). However, it is not valid in all cases, as discussed earlier. The presence/absence of nucleosomes close to the target gene can also interfere with the action of transcriptional regulators (Griesenbeck et al., 2003; Mao et al., 2011). In CRISPR-dCas9 system, in which ModTdepends on the interaction of the modulatory complex with the gene promoter region on DNA, the presence of nucleosomes is expected to be determinant to the efficiency of the technology in eukaryotes, considering that DNA is complexed with histones, making it more compact and less accessible (Horlbeck et al., 2016; Lee et al., 2007;



Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	TGGTATTGCGCGCATTCGATTGG	9:37666494	-	50	0	0	0	0	0	50.39
2	TGATTGAACGTGGTTTCGAGAGG	9:37666360	+	45	0	0	0	0	1	62.67
3	GAACGTGGTTTCGAGAGGCGCGG	9:37666365	+	60	0	0	0	0	2	69.00
4	GATTCATCGACCTTCCGTGATGG	9:37666442	+	50	0	0	1	0	0	58.96
5	TGCTTATATGCTCTCTTTCTAGG	9:37666390	-	35	1	0	0	0	2	20.45
6	TGGTACGTGGCCATCACGGAAGG	9:37666452	-	60	1	1	0	0	0	57.75
7	GCTTTTTCATTGATTGAACGTGG	9:37666350	+	35	0	0	0	0	3	61.99
8	GTTCATTTTATAGAGAGGGTTGG	9:37666472	-	35	0	0	0	0	5	55.95
9	GGGTTGGTACGTGGCCATCACGG	9:37666456	-	60	1	0	1	1	2	62.76
10	GATTGGTTCATTTTATAGAGAGG	9:37666477	-	30	0	0	0	1	6	56.67

Figure 4. CHOPCHOP v3 – Results. CHOPCHOP platform interface, in which the suggested sgRNA for dCas9-activation and a specific gene *of G. max* (ID: Glyma_09G153900) are presented. The ranking (column 1) is based on the number of off-targets presented in columns MM0, MM1, and MM2 (with one, two, or three mismatches, respectively). Even though it is not relevant for dCas9 technology, the platform provides cleavage efficiency values, in percentage, as shown in the last column. Other information is also presented, such as spacer sequence (column 2), position in the genome (column 3), DNA strand (sense or positive; antisense or negative; column 4), percentage of GC in sgRNA (column 5), and self-complementarity (column 6). The results can be downloaded (red square; step eight in Tutorial 1) according to the chosen format.

Radzisheuskaya et al., 2016; Rando; Chang, 2009; Rando; Winston, 2012; Smith et al., 2017, 2016). Thus, biochemical and *in vivo* pieces of evidence suggest that sgRNA design strategies should avoid regions close to the nucleosome core. Furthermore, the development of new tools for *in silico* design of specific and highly efficient sgRNAs should evaluate and include databases with DNA-accessibility maps and nucleosome positioning, becoming more reliable (Jiang; Pugh, 2009; Schep et al., 2015). Finally, as reported for native regulatory networks and some synthetic networks, the number of regulators associated with the regulon provides a fine-tuned adjustment of gene expression reprogramming mediated by CRISPR-dCas9. However, the use of several sgRNAs must be carefully analyzed, with a special focus on the position of regulatory elements and nucleosomes.

Transformation cassette design

The use of CRISPR technology for transcriptional or epigenetic modulation has great potential for biotechnological application in plants, especially for the manipulation of the expression of endogenous genes that may be associated with desirable phenotypes. Specifically to dCas9, few approaches can be performed in a non-transgenic manner (DNA free via RNP). The transcriptional modulation in a dCa9 system requires the constitutive expression of this endonuclease, sgRNA, and ModTs. A critical step in the application of this technology is the design of the plant-transformation cassette, which includes the selection of genetic elements for optimal production of all components required in this modulation system.

Tutorial 2: Design of transformation cassette for transcriptional modulation via dCas9

For didactic purposes, a step-by-step tutorial is proposed to guide the design of a transformation cassette, which should be divided into four basic modules: (i) expression of dCas9; (ii) sgRNA expression; (iii) expression of Adapter:ModT fusion protein (optional, according to the selected strategy); and (iv) expression of the selection marker. Considerations about the subject are presented based on both laboratory experiments and recent literature (Figure 5).

Module 1: Expression of dCas9. The design of this module is an important factor to be considered in transcriptional modulation strategies. This module has three elements: (i) promoter; (ii) dCas9; and (iii) terminator (Figure 5).

a) **Promoter:** in most cases, the promoter chosen to drive dCas9 expression is recognized by RNA polymerase II (pol II).It needs to be strong (high level of

expression) and constitutive (expressed in all tissues and at all times). In plants, the most obvious choice would be the *CaMV35S* promoter, even though some aspects must be considered. Regarding it is of viral origin, *CaMV35S* promoter can be silenced in plants. Since the transcriptional modulation of a target gene requires constant dCas9 expression, the expected phenotype will be lost if its silencing occurs. The second aspect of being considered is the constitutive characteristic of this, and other promoters used, for example, *A. thaliana's AtACT2-5*). In plants, the constitutive expression of Cas9/dCas9 can provide undesirable pleiotropic effects due to its action on possible off-targets. Finally, the function of the gene whose expression will be modulated

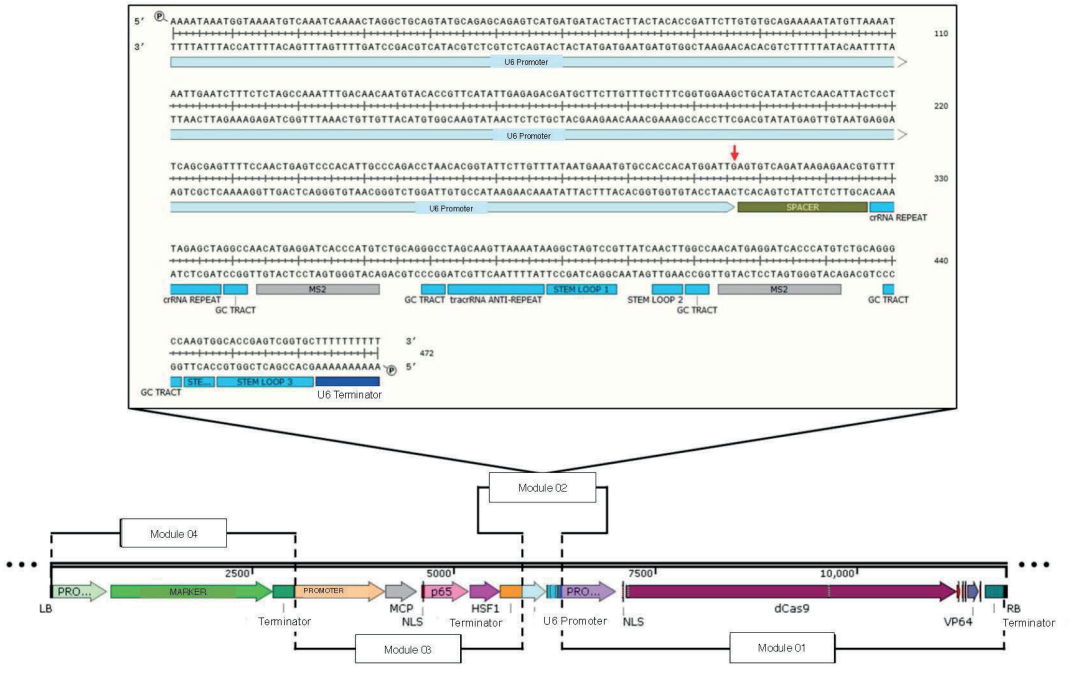


Figure 5. Design of transformation cassette – dCas9 technology (SAM system). As detailed in Tutorial 2, the transformation cassette is presented with the necessary elements for the activation of a specific gene, via dCas9 (SAM system), cloned into a binary vector. The cassette is didactically divided into four modules: (i) Module 1: Expression of dCas9; (ii) Module 2: Expression of sgRNA, with emphasis on main genetic elements (upper part of the figure). The scaffold RNA has the MS2 RNA aptamer sequence inserted in its structure both in the tetraloop and in the stem-loop 2. The red arrow highlights the importance of guanine (G) as the last nucleotide present in the 3' of the soybean *GmU6-10* promoter; (iii) Module 3: Expression of the Adapter:ModT fusion protein. In this module, the transcriptional modulators presented are protein domains from transcriptional activators p65 and HSF1, characteristic of the SAM system; and (iv) Module 04: Expression of the selection marker. *Acronyms:* LB - left border; MCP - MS2 Coat Protein; NLS - nuclear location site; RB - right border.

is the third aspect to be considered. Stress-related genes, when expressed constitutively, can lead to undesirable effects. In this way, if dCas9promoter is constitutive, genes with this characteristic would also be expressed at high levels constitutively. Thus, the choice of the promoter for the expression of dCas9 must be aligned with the function of the gene whose expression is intended to modulate. For example, if the desired phenotype is to improve plant resistance to a pathogen, an induced promoter-driven dCas9 expression can be considered.

b) **dCas9:** the nucleotide sequence of dCas9 must be carefully evaluated. The first aspect of being observed is whether the mutations necessary for the loss of catalytic activity are inserted in the sequence (D10A in the catalytic domain RuvC and H840A in the catalytic domain HNH). The second aspect is the presence of a nuclear localization signal (NLS) since the dCas9 activity will be performed exclusively in the eukaryotic nucleus. In plants, the main NLSs are (i) NLS from monkey virus SV40 (PKKKRKV); and (ii) Nucleoplasmin bipartite NLS (KRPAATKKAGQAKKKK). The number of repetitions and the region where they will be inserted is variable. Generally, the number of repetitions of each NLS range from 1 to 4, and it can be present in both amino and carboxyterminal of dCas9. Constructions with only one NLS are rare. NLSs can be inserted after ModT, with no loss of dCas9 activity. The third aspect of being considered is the fusion of dCas9 with ModT. Generally, this connection is mediated by a linker or bridge, corresponding to a sequence of 5 to 20 amino acids linking the dCas9 carboxy-terminal to ModT amino-terminal. These amino acids must provide flexibility and allow both proteins to show their correct activity. Some *in tandem* NLSs, spaced properly by at least five neutral amino acids, can be used as linkers between dCas9 and ModT. Moreover, the linker's length and amino acid composition between dCas9 and base editor domains can be crucial for the specificity of the edition. Short linkers will reduce the window of the nucleotides that can be edited. But be careful: exceedingly small linkers can interfere with the function of both proteins.

c) **Terminator:** the choice of the terminator is free. When using the *CaMV35S* promoter, it is suggested the use of 35S terminator (*t35S*), but any terminator recognized by an RNA pol II can be used, such as *tNOS* (NOS terminator of nopaline synthase gene from *Agrobacterium tumefaciens*). Experimental data showed that the presence of two different *in tandem* terminators (for example, *t35S* and *tNOS*) could increase transcription levels by improving the stability of transgene expression due to the reduction of post-transcriptional gene silencing (Basso et al., 2020).

Module 2: Expression of sgRNA. This module must be designed to efficiently delineate the expression of sgRNA and can be divided into four elements: (i) *U6* promoter; (ii) spacer; (iii) scaffold RNA; and (iv) *U6* terminator (*tU6*) (Figure 5).

a) **U6 Promoter:** the levels of sgRNA expression are directly correlated with the efficiency of CRISPR/Cas9-mediated genome engineering in eukaryotic cells. In general, RNA polymerase III (pol III) promoters are mostl used to drive sgRNA expression, since pol II promoters add extra nucleotides to the 5′ and 3′ ends (Hsu et al., 2013; Yoshioka et al., 2015). Originally, pol III *SNR52* and *RPR1* promoters were adopted for constitutive sgRNAs expression in yeasts (DiCarlo et al., 2013; Farzadfard et al., 2013; Gilbert et al., 2013). Then, to allow more flexibility in the design and higher expression levels of sgRNAs, two studies on constitutive delivery of sgRNAs tested the fusion of Delta Hepatitis Virus (HDV), and auto-cleavable ribozymes, allowing the expression of sgRNAs with pol II promoters (Gao; Zhao, 2014; Ryan et al., 2014). In addition to native pol III and inducible pol II promoters, different classes of promoters were used to regulate the expression of sgRNAs in eukaryotic systems (Farzadfard et al., 2013; Ferreira et al., 2018; Jensen et al., 2017; Nishimasu et al., 2014; Smith et al., 2016; Zhang et al., 2017). The *U6* stands out as pol III promoter that is naturally important in the endogenous expression of small non-coding nuclear RNAs, involved in intron splicing. Different *U6* promoters have been frequently used in plants and animals to express small RNAs at higher levels (Li et al., 2007; Miyagishi; Taira, 2002). Currently, this type of promoter is the preferred choice for regulating sgRNA expression in CRISPR/Cas9 (and variants) vectors (Friedland et al., 2013; Li et al., 2013). Moreover, the *U6* promoter has a highly conserved TSS, starting with a guanine nucleotide, which helps to improve the homogeneity of the transcribed sgRNA molecule and reduce the effects of off-targets (Li et al., 2007). CRISPR/Cas9 vectors with the *U6* promoter driven the sgRNA expression have been used successfully in several plant species. The *OsU6a*, *OsU6b,* and *OsU6c* promoters of rice are the most used for monocotyledons, and *AtU6-1* and *AtU6-29* from *Arabidopsis* are the preferred ones for dicotyledons (Jiang et al., 2013; Li et al., 2014; Mikami et al., 2015). However, there are limitations to its "universal" nature, since the *Arabidopsis U6* promoter was inefficient in wheat and rice (Shan et al., 2013). Thus, it is clear that the use of species-specific *U6* promoters can result in increased sgRNA expression and more efficient editing (Ng and Dean, 2017; Sun et al., 2015). In soybean, for example, the levels of sgRNA expressed by the endogenous promoter *GmU6* were twice higher than those obtained with the *AtU6-26* promoter, resulting in a considerable improvement in gene editing efficiency (14.7–20.2 % for *GmU*6 vs. 3.2–9.7% for *AtU6-26*) (Sun et al., 2015).

In this way, aiming at improving sgRNAs expression, at least one endogenous *U6* promoter has already been characterized for crops such as cotton, rice, barley, corn, sorghum, and wheat(Gasparis et al., 2018; Jiang et al., 2013; Long et al., 2018; Qi et al., 2016; Sun et al., 2015). It is also important to keep in mind that plant genomes contain several *U6* genes with different expression levels and not all *U6* promoters are equally efficient to drive gene expression (Domitrovich; Kunkel, 2003; Wang et al., 2008). For new plant species, it is important to characterize specific endogenous *U6* promoters to optimize its use and avoid waste of time. The main attention to be taken regarding the *U6* promoter experiments with Cas9 and dCas9 is the presence of a guanine (G) at the 3' end of the promoter (region closest to the first nucleotide of the sgRNA). If the available sequence of the *U6* promoter in question does not contain this nucleotide, the first nucleotide in the sgRNA must be a guanine. In some cases, some researchers add an extra guanine to the sgRNA sequence during design, to optimize its expression.

b) **Spacer :** this is the region of sgRNA that will effectively recognize the target sequence. The main aspects of its design have been thoroughly described previously. In the sgRNA expression cassette, the spacer comes immediately after the *U6* promoter and before the scaffold RNA.

c) **Scaffold RNA:** it is the constant sequence present in sgRNA, important in anchoring with Cas9/dCas9. As seen before, the structure of scaffold RNA from Cas9 and dCAS9 nucleases is formed by a tetraloop and three stem-loops. For dCas9 technology, the scaffold RNA can be presented in two ways: (i) classical form, with the standard sequence without any modification. In this approach, transcriptional or epigenetic modulation is mediated only by ModT fused with dCas9; (ii) modified form (SAM methodology), with RNA aptamers (for example, MS2, PP7, and COM) inserted in tetraloop and stem-loop 2 regions. The insertion of these RNA aptamers allows recognition by adapter protein, specific to each aptamer (for example, MCP, PCP, and COM) (Haimovich et al., 2016; Johansson et al., 1997; Lim and Peabody, 2002; Zhang et al., 2019c). These adapter proteins are fused with ModTs. Thus, the choice of the adapter protein needs to be in perfect agreement with the experimental strategy addressed. Regardless the strategy, the scaffold RNA comes immediately after the spacer and before the *tU6*.

d) **U6 terminator (tU6):** short and simple terminator (10 thymines added in 3' end of spacer) recognized by the most pol III (Gao et al., 2018). It should not be replaced by any other terminator used in the laboratory.

Module 3: Expression of Adapter:ModT fusion protein. This module is associated with SAM methodology (sgRNA with RNA aptamers). If using the classical approach (ModT fused only with dCas9 ,), this module must not be inserted in the vector design. Three main genetic elements can be highlighted: (i) promoter; (ii) fusion protein Adapter:ModT; and (iii) terminator.

a) **Promoter:** the choice of promoter for Adapter:ModT must follow the same criteria described in Module 1, for dCas9. If possible, both Modules should have the same promoter.

b) **Adapter**: ModT fusion protein: The adapter-aptamer coupling is specific. For example, if the MS2 aptamer is chosen, the adapter protein should be MCP. Due to its wide biotechnological application, the identification of new adapter-aptamer pairs is growing every day (Zhang et al., 2019c). Most of the constructions successfully used in dCas9 strategies presented ModTs fused *in tandem* with the carboxy-terminal MCP protein adapter, properly separated by linkers. There is no limit for the number of fused ModTs, but most constructions have only 1 or 2. Furthermore, the fusion protein needs to be transported to the nucleus to perform its activity. Thus, it is necessary to insert NLSs, following the same criteria presented in Module 1 for dCas9. Even in this case, NLS is commonly inserted between the adapter protein and the first ModT, also acting as a linker.

c) **Terminator:** similar to Module 1, the choice of the terminator is free. Any terminator that is recognized by a pol II can be used, for example, *tNOS*.

Module 04: Expression of Selection Marker. This module is specific to each plant species, considering the genetic transformation method that will be used. Similar to Modules 1 and 3, three elements are important: (i) promoter; (ii) selection marker, which in most cases is a gene that confers resistance to an antibiotic or an herbicide but can be any gene reporter, such as *gfp*, *uid*A, among others; and (iii) terminator. Since the design of this module can be variable, the promoters and/or terminators previously used in Modules 1 and 3 should not be repeated to avoid possible silencing in the plant genome (Figure 5).

Other considerations: Considering the vector design, some extra points need to be highlighted:

a) In several laboratories, the plasmid vectors are constructed by chemical synthesis. If this is the case, an important step to be considered is the codon usage optimization in Modules 1, 3, and 4. This care is extremely relevant considering the translation efficiency of some elements presented in these modules.

b) Keep all Modules in the same strand (direction), to avoid steric impediment during gene transcription.

c) It is suggested that unique sites for restriction enzymes be inserted between each module to facilitate *in vitro* manipulation, if necessary.

d) The sgRNAs can be expressed in simplex (1 sgRNA), or multiplex (2 or more sgRNAs). For multiplex systems, each sgRNA must have its *U6* promoter and terminator. In general, for the expression of different sgRNAs in the same plasmid vector, Module 2 must be repeated *in tandem* (preferably separated by unique restriction sites). Until now, there are no reports regarding the maximum number of sgRNAs that can be expressed in the same plasmid vector, but successful systems did not exceed four sgRNAs per vector.

e) The most used backbones are binary vectors based on the T-DNA from *A. tumefaciens*, but this can be replaced by another one, depending on the plant species and transformation method used. The cloning steps and subsequent amplification in bacteria can be hampered since the length of the final vector can reach 10.000 to 15.000 base pairs. If the chosen backbone is based on the T-DNA from *A. tumefaciens*, it is suggested to insert the selection marker closer to the left border (LB), to optimize the selection of transformed plants.

Final considerations

The CRISPR/dCas9 technology applied to the modulation of gene expression has as a main advantage the possibility of promoting transcriptional control of multiple gene targets simultaneously, simulating native networks of cellular metabolic pathways. It is a key tool for synthetic biology and deepening knowledge of plant pathways related to development, productivity, and response to biotic and abiotic stresses, which are frequent targets of genetic engineering for plant breeding. CRISPR/dCas9 is a functional technology and straightforward in transient transformation systems, allowing rapid simulation and visualization of the effects of gene expression modulation on cellular metabolic pathways. The different strategies available, varying elements such as ModT and nucleases, allow the adaptation and the use of this technique in different models.

An advantage of the CRISPR system for the transcriptional activation of an endogenous gene, compared to the overexpression strategy, using constitutive promoters to drive the complete coding sequence of a gene, is that this last strategy omits splicing patterns of endogenous genes and mask alternative transcripts. Therefore, the gene product may be unstable, interact with unconventional

partners, or have an incorrect subcellular localization (Park et al., 2017). However, when working with CRISPR/dCas9 technology, some topics deserve attention:

a) CRISPR/dCas9 technology does not induce definitive changes in the target genomic region, and the developed plants will always be transgenic. If the intention is to develop a biotechnological product, this can lead to a long and costly process for commercial release.

b) The effects on the modulation of target gene expression depend on transcriptional control's cellular processes and can vary between developed transgenic lines. Although inheritable throughout transgenic generations, gene modulation may also vary between mother and daughter plants or be influenced by environmental factors.

c) The technology requires minimal knowledge of the target promoter region sequence, which can be a limiting factor when working with species without detailed genomic information.

In this context, the constant improvements of CRISPR/dCas9 technology aiming at increasing its specificity and efficiency as, for example, with the use of other Cas orthologous, will increase the range of its use, especially in research with cultivated plants.

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CHAPTER 5

Regulatory framework of genome editing in Brazil and worldwide

Letícia Rios Vieira
Natália Chagas Freitas
Fernanda Justen
Vívian de Jesus Miranda
Bruno de Oliveira Garcia
Alexandre Lima Nepomuceno
Renata Fuganti-Pagliarini
Maria Sueli Soares Felipe
Hugo Bruno Correa Molinari
Edivaldo Domingues Velini
Eduardo Romano de Campos Pinto
Maria Lucia Zaidan Dagli
Galdino Andrade
Patricia Machado Bueno Fernandes
Liliane Marcia Mertz-Henning
Adilson Kenji Kobayashi

Introduction

The regulation of the use of products obtained through genome-editing techniques has been the subject of great debate worldwide. Currently, the discussions are mainly focused on whether products obtained by different strategies of site-directed nucleases (SDN) should or not be classified as Genetically Modified Organisms (GMOs).

In the SDN-1 application, the natural DNA cell repair pathway (Non-Homologous End-Joining - NHEJ) is explored to introduce simple random mutations (substitutions, insertions, and deletions) by systems such as CRISPR-Cas, TALENs, or Zinc Fingers Nucleases, which cause silencing of the gene product after breaking DNA (by Double-Strand Break - DSB).

In the SDN-2 approach, a template DNA is also used to introduce a change in the sequence of nitrogen bases (A, C, G, T) at the target site where the DSB occurred, exploring another natural repair system directed by a DNA fragment from the same species (Homology-Directed Repair - HDR).

In the SDN-3 approach, both NHEJ and HDR can be explored to insert one or more DNA fragments with sequences necessary for the expression of a gene (promoter, coding, and terminator region) at a specific location in the genome.

In the following topics, questions related to genome editing regulation in different countries are discussed in detail.

Regulation of gene editing in South America

Brazil

The total area in the world cultivated with genetically modified (GM) crops has increased a thousand times over the past two decades, from a few thousand hectares in 1996 to more than 190.4 Mha in 2019 (ISAAA, 2019). Among the countries producing transgenic crops, Brazil has the second-largest agricultural area, with more than 51.3 Mha, surpassed only by the USA, with 75 Mha. In the 2018/2019 crop season, over 95% of soybean fields were planted with GM cultivars; for corn, over 88% (first and second crop seasons), and for cotton, it reached over 84% of the total area (ISAAA, 2019).

Like many other plant breeding techniques, the use of GMOs in agriculture has become important for the production of food and plant by-products. However, unlike other technologies, the regulatory framework for GMOs is based on a broad list of requirements for risk assessment, which often differ from country to country. These requirements are primarily intended to protect human health, animal and environmental protection from possible adverse effects of the GMO. However, in many cases, such requirements are not proportional to the risks which results in a costly and time-consuming process. As an unintended outcome, due to the high costs of deregulation, only a few large multinational companies (currently BASF, Bayer, Corteva, and Syngenta) have adequate resources to deregulate new GM crops, whereas publicly funded research institutions, small and medium-sized companies, and universities are generally unable to develop a product that reaches the market. Even though they could benefit a broader range of stakeholders, particularly in poor regions, many socially beneficial technologies have been discontinued due to the regulatory limbo created by the GMO controversy.

However, after more than two decades of experience, without significant impact on human, animal, or environmental health, regulatory agencies are developing a more effective regulatory framework for emerging technologies, such as genome editing techniques and topical interfering RNA. Thus, allowing acceleration in the democratization of biotechnology in agriculture, making it more sustainable, guaranteeing food security, maintaining biosafety and economic, social, and environmental balance.

Brazilian legislation on GMOs

In Brazil, the Biosafety Law (Law No. 11,105, March 24, 2005) was an important regulatory mark establishing the safety norms and inspection mechanisms for activities involving GMOs and their by-products. This law, regulated by Decree No. 5.591, of November 22, 2005, was a comprehensive and complementary revision to a previous biosafety law (Law No. 8974, May 1, 1995), which was issued mainly to regulate the first commercial planting of glyphosate-resistant GM soybean in 1998. Besides determining the general rules for research and commercial activities with GMOs, the Biosafety Law regulates principles and establishes safety standards and mechanisms for monitoring activities involving GMOs and their by-products. The principles used to draft this law encouraged scientific advances in the areas of biosafety and biotechnology, life protection, human health, animal and plant health, as well as compliance with the precautionary principle for environmental protection. The Biosafety Law also established the National Biosafety Council (CNBS – in Portuguese *Conselho Nacional de Biossegurança*). In addition, as foreseen by the Biosafety Law, the National Technical Biosafety Commission (CTNBio – in Portuguese *Comissão Técnica National de Biossegurança*) was created to support the Federal Government in the establishment of the National Biosafety Policy. CTNBio is also responsible for issuing normative resolutions and instruction supporting the technological development of the sector with legal assurance and biosafety.

The objective and scope of the Biosafety Law is to provide safety standards and inspection mechanisms for construction, cultivation, production, handling, transportation, transfer, import, export, storage, research, environmental release, unloading, and commercialization of GMOs and their by-products. The law covers research activities and commercial uses of products developed for agriculture, human and animal health, the environment, and fishing. Anyone interested in carrying out one of these activities must request permission to CTNBio, which will respond within the deadline stipulated in the Normative Resolutions. All public and private organizations, national or foreign, that want to carry out activities or projects in Brazil, must request the Certificate of Quality in Biosafety (CQB - in Portuguese *Certificado de Qualidade em Biossegurança*) issued by CTNBio before starting any activity. CTNBio, through its Normative Resolutions, is responsible for establishing the biosafety guidelines for matters within its competence. Among its prerogatives, the law delegates to CTNBio the assessment of new technologies and their possible impacts on the environment, human and animal health in the country. If necessary, CTNBio may also propose regulations for these new technologies.

Regulation of new breeding technologies in Brazil

For any new technology, such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), it is essential to guarantee its safety. However, to allow technological advancement, all safety requirements must be proportional to the risk. When the Biosafety Law was issued, most of the Innovative Precision Improvement Techniques (TIMP - in Portuguese *Técnicas Inovadoras de Melhoramento de Precisão*), also known as New Breeding Technologies (NBTs) were still at an early stage and, at the time, were not considered.

Thus, in 2015, CTNBio established a task force of experts among its members to analyze the products of the new breeding techniques and define how these products should be included in the definitions of the Biosafety Law, and propose improvements in the current regulations. The techniques analyzed by the task force included gene editing, early flowering, reverse breeding, interfering RNA, oligonucleotide-directed mutagenesis, among others. For most products and technologies considered by the task force, the use of NBTs can accelerate the introduction of traits of interest in elite genotypes in breeding programs. In many situations, the final product could be classified as non-genetically modified (non-GM) for legislation purposes.

Several products obtained by editing genes result in genetic modifications that could be obtained by established mutation techniques, such as radiation and chemical mutagenesis. As the Brazilian Biosafety Law considers organisms obtained by mutagenesis methods as non-GM, the task force considered that, after a case-by-case analysis, some products could be excluded from the scope of the legislation on GMOs. CTNBio Normative Resolution No. 16 (RN16), proposing an updated NBTs regulation, was drafted based on the report of the task force and regulations and experiences of other countries. The RN16 draft was unanimously approved by the members of CTNBio and by the Legal Counsel of the Ministry of Science, Technology, and Innovations, and published in the Federal Official Gazette on January 15, 2018.

In general terms, the principle of RN16 is to determine, through a case-by-case consultation system, whether a product generated by NBTs should or not be classified as GMO by CTNBio (Figure 1). For this consultation, the developer institution must provide information about the original organism and the product, including the methods used to generate it, and its molecular analysis. The classification of a product as non-GMO (for legislation purposes) is based on the following criteria: (I) absence of recombinant DNA/RNA; (II) presence of genetic elements that could be obtained by crossing; (III) presence of induced mutations that could also be obtained by established techniques, such as exposure to radiation or chemicals; and (IV) the presence of mutations that could occur naturally.

In practical terms, products obtained by random mutation directed to the site that involves the junction of non-homologous ends (SDN1 mutation) or homologous repair directed to the site that involves few nucleotides (SDN2 mutation) and that meet the conditions established in RN16, could be designated as non-GMO, in a case-by-case analysis. In contrast, transgene inserts targeted to the site (SDN3 mutation) will normally be classified as GMO, in a case-by-case analysis, according to the RN16. If the product is designated as GMO, the developer must comply with all biosafety requirements and will be approved only after CTNBio's risk assessment. If the product is not classified as a GMO, it can be registered using existing procedures.

RN16 applies to all types of organisms, including plants, animals, and microorganisms, in the research and/or commercial release phase.

Brazilian regulatory framework

The Biosafety Law established a structure with four main organizations responsible for risk assessment, and management (Figure 1): (1) National Biosafety Council (CNBS), (2) National Technical Biosafety Commission (CTNBio), (3) Local Biosafety Committee (in Portuguese *CIBio – Comissão Interna de Biossegurança*) and (4) Registration and Inspection Organizations and Entities (in Portuguese *OERF - Órgãos e Entidades de Registro e Fiscalização*), which includes the Ministry of Agriculture, Livestock and Supply (MAPA); the Ministry of Health (MS), the Ministry of the Environment (MMA), and the Secretariat of Aquaculture and Fisheries.

The Institutions that intend to work with GMOs must establish a Local Biosafety Committee (CIBio) and request a Certificate of Quality in Biosafety (CQB) to CTNBio. The CQB is issued after analysis by CTNBio that authorizes the institution to carry out activities with GMOs in its facilities, considering that the required safety standards are met. After the approval of the CQB, any demand for commercial activity with GMOs must be submitted to CTNBio by the CIBio's president of the institution. For a commercial release request, a complete and detailed dossier with all biosafety risk assessments must also be included in the process. The guidelines for risk assessment are established in Normative Resolution No. 24, of January 7, 2020, which provides the regulation for the commercial release of GMOs and their derivatives. CTNBio evaluates the risk and prepares a technical report. If the GMO is approved for commercial release, it is forwarded to CNBS.

National Biosafety Council (CNBS – *Conselho Nacional de Biossegurança*)

CNBS is a collegiate body composed of eleven high representatives of the State, including the Chief of Staff of the Presidency, who presides over it; Minister of

Justice; Minister of Science, Technology and Innovations; Minister of Agricultural Development; Minister of Agriculture, Livestock and Supply; Minister of Health; Minister of the Environment; Minister of Development, Industry and Foreign Trade; Minister of Foreign Affairs; Minister of Defense, and the Secretary of Aquaculture and Fisheries.

CNBS provides advisory assistance to the President of the Republic in the formulation and implementation of the National Biosafety Policy, establishing principles and guidelines that consider socio-economic and political conveniences, and opportunities of national interest involved in the commercial use of GMOs and related products. The CNBS technical opinion on a final decision to release a GMO for commercial use will only be requested if socio-economic and/or strategic policy decisions are required. Technical judgment on the biosafety of a commercially used GMO is under CTNBio's responsibility. However, CNBS has 30 days to refute the commercial approval of a GMO after CTNBio has released its official position. If the refutation does not occur within 30 days, the product is automatically authorized for sale.

National Technical Biosafety Commission (*Comissão Técnica Nacional de Biossegurança* - CTNBio)

CTNBio, linked to the Ministry of Science, Technology, and Innovations (MCTI – in Portuguese *Ministério da Ciência, Tecnologia e Inovações*), is a multidisciplinary advisory and deliberative collegiate that provides assistance and technical support to the federal government to formulate, update, and implement the National Biosafety Policy for the development of GMO products or biotechnology products that at some stage could generate a GMO. CTNBio also establishes technical safety standards regarding the authorization of activities related to research, and the commercial release of GMOs. In addition, CTNBio is responsible for zoo-sanitary, phytosanitary, human health, and environmental risks assessment of GMOs, and also establishes risk management measures. Other competencies of CTNBio include authorizing the importation of GMOs for research, providing technical assistance to registration and inspection organizations, and monitoring the development and technical-scientific progress achieved in biosafety, biotechnology, bioethics, and related areas, with the aim to increase the capacity of protecting human, animal and plant health, and the environment.

CTNBio is organized into permanent sectoral sub-commissions in the areas of plant and environment, human, and animal health. The president of CTNBio is appointed by the MCTI Minister for a 2-year term, extendable for the same period. CTNBio has a

permanent executive secretariat that provides technical and administrative assistance and organizes monthly meetings (except in January and July).

CTNBio consists of 27 full members and their substitutes, who are also appointed by the MCTI minister after receiving nominations from other Ministries. All members have a two-year term, renewable for two consecutive terms. They must be Brazilian citizens with recognized technical competence and outstanding participation in the scientific community. All members must have a doctorate, and be professionally active in the areas of biosafety, biotechnology, biology, microbiology, health and environment, human/animal health, or related areas. Twelve members of the scientific community are directly appointed by the MCTI, while the others are appointed by one of the bodies of the CNBS: Ministry of Agriculture, Livestock, and Supply; Ministry of Health; Ministry of the Environment; Ministry of Agricultural Development; Ministry of Development, Industry and Foreign Trade; Defense Ministry; Secretariat of Aquaculture and Fisheries; Ministry of Foreign Affairs, and Ministry of Justice. The complete list of CTNBio's members can be found on its website.

CTNBio meetings can be held with the 14-member quorum (half plus one), including at least one representative from each of the four subcommittees. If necessary, representatives of the scientific community, the public sector, and civil society entities with experience in a specific field may be invited to attend meetings, but they are not entitled to vote. Any decision taken by CTNBio must be approved by a majority vote. To provide greater transparency to the process, all decisions are published in the official journal and open for public comment within 30 days, in the same way, that all meetings are open to citizens, who can consult the agendas, as well as all documents produced by the commission, which are available on the CTNBio's website[1].

Local Biosafety Committee (*Comissão Interna de Biossegurança* - CIBio)

Any public or private institution that uses genetic engineering techniques and methods to develop biotechnological products, which at some stage of development can generate a GMO, must have a CIBio, composed of individuals with adequate training and education in the areas of biotechnology, genetic engineering, biosafety or other related fields. The Biosafety Quality Certificate, a document necessary for CIBio to work under government control, is also issued by CTNBio to the institution in question.

[1] Available at http://ctnbio.mctic.gov.br

As a mandatory procedure, a researcher must be appointed as responsible for each project involving GMOs in the institution. Also, each CIBio is legally responsible for ensuring the biosafety conditions of the entity's facilities, conducting regular inspections, and sending an annual report of its activities and projects to CTNBio. Currently, CTNBio supervises 480 public and private institutions in Brazil.

Registration and Inspection Organizations and Entities (Órgãos e Entidades de Registro e Fiscalização - OERF)

The OERF include the Ministry of Agriculture, Livestock, and Supply; Ministry of Health; Ministry of Environment, and Secretariat of Aquaculture and Fisheries.

Per Law No. 11,105 and within its field of competence, in compliance with CTNBio's resolutions and technical opinions, OERF is responsible for monitoring GMOs and their by-products. Its responsibilities include: (1) inspecting research activities, (2) registering and inspecting the commercial use of GMOs, (3) authorization to import products for research and commercial use, (4) maintaining up-to-date information on institutions and researchers who carry out activities and projects; (5) assist CTNBio in defining parameters for assessing biosafety; (6) disclosing to the public, grant registrations and authorizations for commercial use of GMOs; and (7) enforce the law and apply the penalties established when a non-compliance is identified.

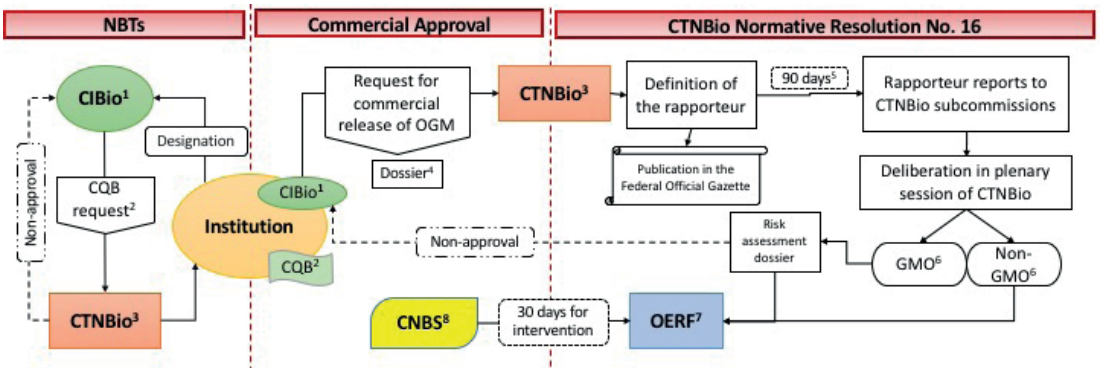

Figure 1. The workflow of the general process of approval and commercial release of products generated by NBT, according to the Brazilian Biosafety Law nº 11,105/2005 and Normative Resolution nº 16. Caption: 1) Local Biosafety Committee; 2) Biosafety Quality Certificate; 3) National Technical Commission on Biosafety; 4) Detailed dossier, with biosafety risk assessments; 5) Extendable for an equal period; 6) Genetically Modified Organism; 7) Registration and Inspection Organizations and Entities; 8) National Biosafety Council.

Overview of the status of biotechnology in Brazilian agriculture

Brazil is one of the main producers of agricultural goods in the world and one of the few countries that could considerably increase its production in the coming decades, without compromising environmentally protected areas, including the Amazon rainforest. In addition, Brazil also has great potential to become the main supplier of biofuels. Unlike most developed countries, where bioenergy production could compete with food production, Brazil could grow more than 30 Mha without destroying native and preserved environments or invading food production areas (Malingreau et al., 2012). Also, Brazil contains between 15% and 20% of global biodiversity, which has enormous potential as a source of new products for agriculture, medicine, and industry.

The Brazilian agriculture (from small to large farmers) and all the agribusiness related to it have all the conditions to increase their development at levels similar to those of other emerging economies and, consequently, help in the economic and social progress of the country contributing to feeding the growing world population. In the 1970s, the agricultural and livestock production flow to the Cerrado areas in the Midwest Region revealed how agribusiness can improve economic and social development. Some cities in the Midwest, for example, currently have the highest human development index in Brazil.

Many important achievements of Brazilian agriculture in recent decades resulted from the combined application of biotechnological and genetic improvement approaches. The combination of these methodologies is crucial to guarantee sustainable food production, in a scenario with multiple challenges resulting from climatic changes and a growing world population. Thus, to maintain productivity, it is essential to be alert, informed, and familiar with new technologies that could change concepts and paradigms of production and consumption.

In December 2018, CTNBio evaluated the first consultation on the commercial release of plants generated using NBTs in Brazil. A corn genotype in which the metabolic pathway for amylose production was knocked down by CRISPR/Cas9 was classified as non-GM. After analysis, CTNBio concluded that the mutation could have been obtained by conventional breeding methods or induced by other mutagens, such as ionizing radiation or by chemical agents. In this specific case, the reduction in amylose production resulted in almost 100% amylopectin content, which is interesting for some industrial uses of corn starch.

In another consultation, also in 2018, an edited yeast strain (*Saccharomyces cerevisiae*) called "Excellomol" with point polymorphisms introduced in specific genes was submitted. Excellomol increased the production of ethanol from

sugarcane. Such polymorphisms already occur naturally in the CBS 6412 strain of *S. cerevisiae*, originally identified in the production of sake. Since these mutations could have been introduced by other methods of mutagenesis the edited yeast was classified as non-GM.

Until March 2020, there were ten consultations since the approval of RN16. Five lines of microorganisms (*Saccharomyces cerevisiae*) for bioethanol production, hornless cow (for handling dairy cow), tilapia for improved fillet yield, waxy corn for starch quality, a vaccine for canine parvovirus control, and RNAi for the topical application to mosquito control. According to the provisions of the RN16, all these products, except the hornless cow, were considered by CTNBio as non-GMO. The development of NBTs evaluated by modern regulations that protect human and animal health and the environment will allow the democratization of biotechnology in Brazilian agribusiness. In this context, small, medium, and large national and international institutions could participate in the Brazilian and worldwide market, developing and introducing new solutions and products through a more sustainable approach without the controversy created about GMOs.

Argentina

Argentina was a pioneer country in the regulation of NBTs. In 2015, the Secretariat of Agriculture, Ganadería y Pesca (SAGyP) released the Resolution 173/15, which defines the assessment guidelines for NBT-derived crops. It is important to note that the resolution was drafted without the requirement of a list of specific technologies and is not restricted to the technical innovations available at the moment. As new breeding technologies are constantly published and patented, the inclusion of a specific list could compromise the speed of regulation of additional technological innovations.

The Comisión Nacional Asesora de Biotecnología Agropecuaria (Conabia) is the entity responsible for assessing, based on technical and scientific criteria, the potential environmental impact of the introduction of biotechnological crops in Argentine agriculture. The commission is recognized worldwide for its experience in the evaluation of dossiers, being considered as a reference center for the biosafety of GMOs by the Food and Agriculture Organization of the United Nations (FAO) (Ministerio de Agricultura, Ganadería y Pesca, 2019). Conabia has active participation in international debates related to biosafety and regulatory processes (USDA Foreign Agricultural Service, 2019). Therefore, Conabia is the body responsible for the evaluation and regulation of new breeding technologies, which guarantees compliance with the Resolution No. 173/15.

All products obtained by genome editing must be submitted to Conabia. The dossier can be submitted in two moments: after obtaining the final product or in the design phase of the creation process (project). In the design phase, inventors can consult Conabia to evaluate the expected product, determining whether the hypothetical product would be under GMO regulation or not. When the NBT-derived product is obtained, technical data on the genetic modification must be submitted to ascertain whether the expected regulatory status remains the same as preliminary assessment.

Under the regulatory framework, the evaluation time is 60 days, and electronic forms are available for speedy evaluation. The main criteria taken into account are (1) the techniques used in the process; (2) the new combination of the generated genetic material; and (3) the absence of a transgene in the final product. A genetic modification is considered a new combination of genetic material when a stable and permanent insertion of a gene(s) or DNA sequence(s) into the plant's genome is present. In such cases, the final product will be regulated as GMOs (Resolution 701/11 and 763/11). Also, even if a crop is exempt from GMO regulations but it has characteristics that pose a risk, it may undergo additional monitoring by the responsible authorities (Whelan; Lema, 2015).

Chile

In Chile, the *Servicio Agrícola y Ganadero* (SAG) is the entity responsible for regulating and monitoring the introduction, and propagation of genetically modified plants in the environment. An official SAG pronouncement in 2017 determined the regulatory procedure for crops obtained by NBTs, establishing a case-by-case approach, similar to Argentina. In general, crops developed using genome editing techniques that do not contain a new combination of genetic material are not subject to GMO regulations and are outside the scope of Resolution No. 1523/2001. For these purposes, a new combination of genetic material means a stable insertion of one or more genes or DNA sequences coding proteins, interference RNA, double-stranded RNA, signal peptides, or regulatory sequences (Whelan; Lema, 2019).

Individuals or legal entities, research centers, or universities interested in introducing a new crop obtained by NBTs into the Chilean territory must forward a request form to the SAG's Agricultural Protection and Forest Division. The assessment is carried out within 20 working days. The request form must contain technical information including the name of the species, the variety/lineage, the description of the phenotype obtained, the company or institution that developed the material, the methodology, and the characteristics of the biotechnological technique used

with the indication of the modified DNA sequences. Also, the applicant must inform whether the material has precedent for authorization in another country and if so, the official documentation must be presented. The SAG decision is valid for an indefinite period but can be canceled if new scientific discoveries are available.

Colombia

In Colombia, the technical control of production and commercialization of agricultural products is under responsibility of the *Instituto Colombiano Agropecuario* (ICA). In 2018, the Resolution 29299 was issued, which establishes the consultation guidelines for products obtained by NBTs, on a case-by-case basis. The procedure for determining whether a cultivar developed by NBT corresponds to a GMO or to a conventional organism takes into account the presence or absence of exogenous genetic material. According to the document text, a *cultivar* is designated as a generic name to refer to varieties, plant lines, hybrids, and clones used as planting materials. *Exogenous genetic material* corresponds to a gene, set of genes, DNA sequences, that are part of a genetic construction and were stably introduced into the genome, through modern biotechnology techniques, overcoming the natural physiological reproduction barriers. In this context, if a cultivar does not have exogenous DNA sequences, it is not classified as a GMO. and is free from the regulation proposed in Decree 4525/2005.

The request for evaluation of a product obtained by NBTs must be sent to ICA, which analyzes the documentation within 30 days. For this, the applicant must be registered with the ICA as a seed producer, seed importer, or plant breeding research unit. The documentation in the application covers the following technical-scientific information: (1) the taxonomic classification of the species; (2) methodology and genetic constructs used, including all genetic elements and, in the case of DNA-free editing, the protein and RNA sequences used; (3) the description of the generated phenotype; (4) alternative methodologies for generating the phenotype; (5) molecular characterization of the genetic modifications present in the improved cultivar compared to the original genotype and absence of exogenous material.

Paraguay

In Latin America, Paraguay was the fifth country to present its position and regulation about products obtained through NBTs. Resolution 565/2019 was sanctioned by the *Ministerio de Agricultura y Ganadería* (MAG), the competent

national authority in the agricultural and forestry sector. According to the resolution, the products are evaluated, on a case-by-case basis, by the *Comisión de Bioseguridad Agropecuaria y Forestal (Combio),* upon submission of the prior consultation form for products obtained by NBTs.

The prior consultation form consists of six sections covering information of the applicant, the organism, molecular biology, the phenotype, authorizations, and references. In the Information section about the applicant, the legal representative and technical responsible for the application must also be presented. The Organism section includes the scientific name and a detailed taxonomic description of the species, including cultivar and lineage must be provided. The Molecular Biology section refers to a detailed description of the technique used, and the steps applied, a molecular description of the target nucleotide sequences and their functions in the organism before and after the application of the technique. If applicable, the genetic construction with the details of the genetic elements, the analysis of target sequences, and, in cases where an intermediate transgene was used, the evidence proving the absence of recombinant sequences must also be provided. In the Phenotype section, examples of products with a similar phenotype on the market, the analysis of the probability of occurrence of other effects besides the desired phenotype, the expected changes in the proposed uses of the organism, and changes in the management recommendations of the resulting organisms are requested. In the Authorizations section, indicate if the organism has already been authorized by the regulatory entity from another country and if so, provide the type of authorization. Finally, in the References section, copies of all publications mentioned in the form must be included. Based on the assessment of all these points, Combio will determine whether the organism is classified as genetically modified or not.

Regulation of genome editing in North America

Canada

Canada differs from other countries in approving a genetically modified organism (GMO), the regulatory approach is based exclusively on products, and not on the process or technique used to develop the new product (Ellens et al., 2019). The product or the plant with a new characteristic (PNT – a plant with a novel trait) needs to present modification that differs from the original variety to be analyzed by

regulatory agencies. A PNT is a plant in which a characteristic has been introduced intentionally, new to plants of the same species grown in Canada, with the potential to affect the usage and safety of the plant considering the environment and human health (Canada, 2019). This rule applies to both plants developed by classical breeding and genetically edited plants.

Products created or modified in Canada are regulated by several government agencies, including the Canadian Food Inspection Agency (CFIA), Health Canada, and Environment and Climate Change Canada (ECCC). The CFIA is responsible for plant regulation, animal feed, fertilizers, and veterinary products of biotechnological origin. The Plant Biosafety Office of CFIA is responsible for coordinating the safety assessment of new foods. Health Canada supervises food, medicine, and pest control products. The evaluations conducted by CFIA and Health Canada are based on scientific criteria and guidelines established by the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the Organization for Economic Cooperation and Development (OECD) (Dederer; Hamburger, 2019). ECCC operates on the regulation of all animal products of biotechnological origin not covered by another federal law and is based on the Canadian Environmental Protection Act, 1999 (CEPA).

In order to receive registration approval and be able to enter the Canadian market, a product with a new feature needs to undergo several tests carried out by technicians from CFIA and Health Canada. These tests aim to corroborate the results already provided by the applicant. After reviewing all the data provided by the applicant coupled with the new tests, if all conditions are met, the product is accepted.

In Canada, when GM crops were being developed in the early 1990s, regulatory systems were also in development. Thus, the laws were already available to the needs of these crops. There is no specific law for plants genetically modified by NBTs or conventional breeding. The regulation is based on the product, which is released for consumption based on its safety assessment.

Four legislation acts are involved in the regulation of agricultural products. The Seed Act regulates stability, and environmental risk, for example, plant potential to become a weed and the impact of a plant or its products on non-target species (Branch, 2019a). The Animal Feeding Act regulates risks related to toxicity, allergenicity, and digestibility (Branch, 2019b). The Food and Drug Act establishes the risk limits for toxicity, allergenicity, metabolism, and nutrition-related to human consumption (Branch, 2020). In addition, CFIA applies the following guidelines to analyze herbicide-resistant varieties, regardless of the technology used to create a variety:

- Directive 94-08: Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits.
- Directive 95-03: Guidelines for the Assessment of Novel Feeds: Plant Sources.
- Directive 96-13: Import Requirements for Plants with Novel Traits, including Transgenic Plants and their Viable Plant Parts.
- Directive 2000-07: Conducting Confined Research Field Trials of Plants with Novel Traits in Canada.

The International Service for the Acquisition of Agri-Biotech Applications (ISAAA) reported in 2017 that Canada planted an area of 13.12 million hectares with biotechnological crops, accounting for 7% of the world planted area. During the 21 years of commercialization of GM plants, Canada made a profit of US$8 billion (ISAAA, 2017), and with the new cultivars that are expected to be incorporated, increased profits in the coming years.

United States of America

In the United States of America, the regulation of crops produced through the use of genetic technologies is based on decades-old policies managed by various statutes and regulations, implemented by different federal government agencies (Dederer; Hamburger, 2019). American regulatory entities are the US Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA). These federal agencies aim to ensure that genetically modified crops and their products are safe for health, the environment, and agriculture.

The regulatory policy for biotechnological products was established with the Biotechnology Regulatory Framework, published in 1986 (OSTP, 1986), and later updated in 1992. This document allows three conclusions that influence American biotechnology policy to this day: products are not necessarily different from conventional products; regulations should not focus on the process but the product, and regulatory jurisdiction must be based on use.

The Animal and Plant Health Inspection Service (APHIS) and the USDA Biotechnology Regulatory Service (BRS) are responsible for releasing field tests, interstate movement, and import of GM plants that may present some risk (Dederer; Hamburger, 2019). APHIS has released a new regulation, the Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient Rule (SECURE Rule) (United States, 2020),

which aims to update and modernize the Plant Protection Act, removing outdated processes, and inserting biotechnological regulations (United States, 2020a).

The EPA regulates GM plants with pesticidal substances under the Federal Insecticides, Fungicides, and Rodenticides Act (FIFRA) (United States, 2020b) and pesticide residues in GE foods under the Food, Drug, and Cosmetics Act (FDCA) (United States, 1938).

The FDA regulates food safety under the FDCA. In the USA, new food developers are legally responsible for assessing food safety and complying with FDA regulations and statutes. And to ensure food safety, the FDA relies on legal provisions that prohibit adulteration, and incorrect food identification (Dederer; Hamburger, 2019). A food is considered adulterated if it has or contains one or more substances added that could be harmful to health or if the additive is not safe. Thus, if GM or processed foods do not show nutritional differences from conventional food, it is considered equivalent. According to the FDCA definition, substances that are "generally recognized as safe" (GRAS) are excluded from food additives. Due to new forms of breeding and gene editing, several discussions have pointed to possible changes in the regulations.

The American government has always encouraged the use of new technologies in agriculture as a way to increase its competitiveness (Bergeson, 2017) and, currently, the use of genome editing techniques in plants is on the rise. In 2019, the USA planted 75 Mha of transgenic crops, which corresponds to 40% of the world total, which was 190.4 Mha (ISAAA, 2019), and are the leaders in approving and cultivating genetically modified varieties.

Regulation of genome editing in the European Union

The European Union (EU) has the strictest legislation in the world regarding the cultivation and consumption of GMOs in its territory with less than 0.1% of the global area is grown with GM crops Davison; Ammann, 2017). Only a single transgenic *Bt* event (MON810) is currently authorized for commercial cultivation in Spain and Portugal. However, the EU is a major importer of transgenic soybean and corn for animal feed (Dederer; Hamburger, 2019).

The EU regulatory concept strictly follows the "Precautionary Principle", which considers that, if an action may cause irreversible public or environmental damage, in the absence of irrefutable scientific consensus, the burden of proof is on the side of those who intend to practice the act or action that may cause the damage. Different GM crops would be within this principle, since they were manipulated

in the laboratory, and would be different from the original crops. In the EU, the regulation banning GMOs is well established, however, with the advancement of genetics, especially the NBTs, the discussion on the use of biotechnology in agriculture is resuming.

The EU regulation on GMOs is based on Directive 2001/18/EC; Regulations (EC) No. 1829/2003 and No. 1830/2003; in Regulation (EC) No. 1946/2003; in Directive 2009/41/EC and Directive (EU) 2015/412 (European Commission, 2020).

Directive 2001/18/EC regulates the assessment of environmental risks and the release of GMOs, as well as their commercialization (import, processing, and transformation) within the European bloc. The concept of GMOs for EU, according to this legal classification, is: "'genetically modified organism (GMO)" means an organism, except for human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination". This concept does not apply to human beings. According to Annexes 1A and 1B of that directive, genetic modifications without the use of recombinant DNA, and obtained by in vitro fertilization techniques, natural processes such as conjugation, transduction, transformation, polyploidy induction, classical mutagenesis (chemical mutagenesis and radiation), and protoplast fusion are excluded from the classification of GMOs.

Gene editing via CRISPR does not normally involve transgenics - the transfer of "genes" between species. However, on July 25, 2018, the EU Court of Justice (EUCJ) determined that all plants obtained through gene editing must be considered GMOs and fall within the scope of Directive 2001/18/EC (Ruffell, 2018). Among the justifications presented by the court, is the fact that the mutations caused by these techniques constitute changes made to the genetic material of the organism in an "unnatural" way and that the process uses recombinant DNA techniques. This is an aspect of EU regulation that differs from US and Canadian standards, based on product safety, regardless of the process used to obtain it (Friedrichs et al., 2019; Leone, 2019).

The precautionary principle for new approaches was used as a justification for the prohibition, aiming to avoid possible harmful effects to human, animal health, and the environment. Most requests for GMOs in the EU have been denied, and those approved have limited consent for 10 years (renewable), with mandatory monitoring after placing on the market (Schulman et al., 2020). The average approval time for food for human and animal consumption (excluding cultivation purposes) could take around 5 years (Zimny et al., 2019).

Considering the European community's concern with GM food and animal feed, Regulation (EC) No. 1829/2003 restricts the unauthorized entry of GMOs into the EU and obliges suppliers of GM plants, and products to label food containing more than 0.9% of GMOs, informing the methods for its detection (Davison; Ammann, 2017). Regulation (EC) 1830/2003 of the European Parliament and the EU Council also regulates the traceability and labeling of GM foods, to ensure that consumers are informed about the presence of GMOs and their products, to allow an informed choice of the product (Davison; Ammann, 2017). Besides Directive 2001/18/EC, which requires mandatory monitoring after the commercial release of GM products, Directive 2009/41/EC complements and requires EU-Member to send a report every three years, describing their experiences with the released product, informing risk assessment, accidents, an inspection of compliance control, consultation and information to the public, and waste disposal (European Commission, 2012). Besides these measures, through regulation (EC) No. 1946/2003, about transboundary movements of GMOs, it became mandatory to introduce protective measures in the border areas of the territory, to avoid possible contamination between non-GM and GM crops neighboring countries (European Commission, 2020).

Even with all these regulations, there were cases of release of the entry of GMOs into the European bloc, contrary to the opinion of several EU-Member. Faced with this setback, Directive (EU) 2015/412 of March 11 (which amended Directive 2001/18/EC) emerged, which concerns the possibility for the Member States to restrict or prohibit the cultivation of GMOs in their territory using the "opt-out" clause, and the principle of subsidiarity (Davison; Ammann, 2017). Among the Member States that have chosen to completely restrict the entry of GMOs into their territory are Austria, the Walloon Region (Belgium), Bulgaria, Croatia, Cyprus, Denmark, France, Germany, Greece, Hungary, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Slovenia, Northern Ireland, Wales, and Scotland (United Kingdom) (Friedrichs et al., 2019). Plants edited by the new breeding techniques are considered GMOs by the European parliament and are therefore included in all EU GMO regulatory measures.

EU legislation that restricts GMOs and crops grown in Europe has significant economic impacts on the food sector, and agriculture. The EU seed market, for example, is estimated at €7 billion a year (Friedrichs et al., 2019). The impossibility of cultivating, and developing crops improved by modern biotechnology also causes the transfer of research investments outside the EU, impacting the research and innovation sector.

According to Brookes and Barfoott (2014), in the 17 years (before 2012) of the adoption of agricultural biotechnology by 17.3 million farmers, there was an economic benefit of US$ 116.6 billion, which increased to US$ 167.7 billion by

2015. GM crops generated a 37% reduction in the use of chemical pesticides, a 22% increase in agricultural production, and a 68% profit for farmers (Klümper; Qaim, 2014). Similar or greater benefits can be achieved in the EU, through adherence to edited crops, besides the benefits of using green technology, including the European bloc in the competitive global commodities market. Among the EU's agricultural challenges is the sustainable production of food with fewer crop protection products, irrigation, grown in a smaller area, under constant climate change. These requirements show the need for new improved cultivars, leaving genome editing as a promising solution for the European market. However, the EU needs to harmonize its biotechnology legislation with other countries, mainly with the main food producers in the world.

Regulation of genome editing in Asia

China strictly regulates the import and production of GMOs, according to the regulations issued by the Chinese Ministry of Agriculture and Rural Affairs (MARA) in 2001, which predicts the administration of the security of agricultural GMOs. In 2018, through the National Bio-Safety Committee (NBC), MARA amended the regulations on safety assessment, import approval, and GMO labeling. The revised rules impose additional tests and studies in the country on biotechnological products. The Chinese government, as of 2016, created a 5-year support plan for research initiatives aimed at gene editing (Cohen, 2019). The purchase of Syngenta by state-owned ChemChina in 2017 reinforces China's interest in the field of technology for food production. Despite being one of the countries with the largest number of publications related to gene editing, the legislation classifies edited organisms as GMOs. MARA indicated in 2019 that new regulations are under development, to align China with other countries, as these regulations may provide a simplified regulatory process for genetically edited products in the future (United States, 2019).

In Japan, products edited using NBTs are evaluated on a case-by-case basis and need to be notified to the government, which requires technical information about the technique used and the genes targeted for modification. Modified organisms that contain exogenous DNA in their construction or are under the regulations for GMOs, as well as edited cultivars that might cross with an unedited cultivar, must be notified (Sato, 2020). Regulation is carried out by four Ministries: Ministry of Agriculture, Forestry and Fisheries; Ministry of Environment; Ministry of Education, Culture, Sports, Science and Technology; Ministry of Health, Labor and Welfare. In

addition, an independent commission, the Food Safety Commission (FSC), carries out the risk assessment of food and animal feed.

Recently, India issued preliminary rules for the regulation of edited products, requiring safety tests that prove the effectiveness of gene editing. Like many other countries, India adopts a position that evaluates the process used for editing instead of evaluating the final product. For SDN-1 technique regulation, extensive data demonstrating that the gene edition was successful, is required. When using SDN-2 for editing organisms, besides proving the effectiveness of the edition, field tests are necessary to prove the efficiency of the transformation. When inserting an exogenous DNA using the SDN-3 technique, the organism follows the same process as GMOs, which include tests for human and animal food safety, and risks to the environment. The responsibility for carrying out the evaluations lies with the Institutional Biosafety Committee, the Genetic Engineering Evaluation Committee, and the Genetic Engineering Review Committee (India, 2020).

Indonesia does not have specific regulations for the production of edited cultivars. All products from gene editing are evaluated as GMOs. The assessments and regulations are carried out based on the Protocol on Biosafety of the Convention on Biological Diversity, based on the Government Regulation No. 21/2005. The responsibility for the initial assessment is under the responsibility of a non-departmental government agency. Subsequently, the analysis is sent to the Convention on Biological Diversity (CBD), a biosafety commission linked to the National Agency for Drug and Food Control (Badan POM). If the product meets the regulations stipulated by law, it is forwarded to the National Agency for the Control of Medicines and Food, and the notification is then reassessed by the biosafety committee, and released (Badan Pengawas Obat dan Makanan, 2012).

Regulation of genome editing in Oceania

Australia

Australia adopts a position that evaluates the process used for editing instead of evaluating the final product. Organisms resulting from a gene-editing process are regulated by the Gene Technology Regulator (GTR). GTR is responsible for regulating the production and release of GMOs, based on the standards described in the Gene Technology Regulations 2001, made under the Genetic Technology Act 2000.

With the NBTs, such as CRISPR, transcription activator-like effector nucleases (TALENs), and Zinc-finger nucleases (ZFNs), the regulatory process needed to be adapted. In 2019, the Gene Technology Amendment (2019 Measures No. 1) was created. The NBT-derived product is systematically reviewed to determine whether it should or not be classified as a low-risk transformation or transformation exempt from notification. The regulations are described in sections 140 and 141 of the amendment. Item 4 of Annex 1 of the amendment states that organisms modified with NBTs are not considered GMOs since no nucleic acid is present. SDN-1 is not regulated due to similarity to traditional mutagenesis techniques. SDN-2 and SDN-3 may or may not insert an exogenous DNA into the organism's genome with stable or transient character (Eckerstorfer et al., 2019).

As the regulation of organisms modified by NBTs is the responsibility of the GTR, in Australia and New Zealand food is regulated by a joint system, the Food Standards Australia New Zealand (FSANZ). FSANZ is responsible to develop and define pre-market regulations, such as food labeling from gene editing (Food Standards Australia New Zealand, 2019).

New Zealand

New Zealand, unlike Australia, considers all gene-editing techniques as GMOs. The country adopts a position of caution and observance of the regulations stipulated globally, to adapt its system over time, according to international developments (Fritsche et al., 2018).

Research involving genetically edited plants is supervised by the Environmental Protection Authority (EPA), which is responsible for supervising the development, and release of GMOs under the Hazardous Substances and New Organisms (HSNO) Act 1996. All gene-editing techniques are regulated, even when exogenous genes are not incorporated (New Zealand, 2019).

Harmonization of global legislation on genome editing in plants

In the Brazilian evaluation (similar to what to other countries in the Americas, Japan, Australia, Israel, among others), mutations produced by SDN-1 are not classified as GMO in the light of Biosafety Law. The same occurs with products obtained by classical improvement, or by mutations induced by various external

factors, such as exposure to UV light, ionizing radiation, chemical substances, or even errors during DNA replication. The accumulated knowledge on the genome of different species has allowed a more precise modification when compared to traditional mutation systems as radiation or chemical agents used in the development of commercial varieties for decades.

Genome editing systems type SDN-2 may or may not be classified as GMOs under the Brazilian legislation and most countries in the Americas, in case-by-case analyses. SDN-2 is similar to natural mutagenesis, altering small portions of genomic DNA, as occurs in genetic improvement programs, or changes caused by chemicals/radiation, or even in the natural differentiation of germplasm from a species collected in different locations. The main differentiating factor in considering products obtained by the SDN-2 system as GMOs has been the presence of DNA of another species in the final product.

SDN-3 system, on the other hand, due to the complexity of the introduced genetic elements, normally is classified as GMOs, always depending on a case-by-case analysis, and the origin of the DNA used.

Unlike the Brazilian Biosafety Law, which excludes mutagenesis from the scope of GMOs, the decision of the Court of Justice of the European Union on the subject (case C-528/16, of July 25, 2018) established that Directive 2001/18/CE, on GMO risk analysis, applies to products obtained by "new mutagenesis techniques", that is, SDN systems.

The European scientific community, as well as companies that develop products with biotechnological techniques, has provoked a discussion with European regulatory agencies, aiming to review this decision and align European legislation with the rest of the world. In this sense, the European Food Safety Authority (EFSA), in a public consultation carried out in May 2020, evaluated the possibility of products obtained by SDN-1 and SDN-2 systems having a different risk analysis than what currently occurs in the European Union in the relation to GMOs. In a first discussion panel, EFSA had concluded that risk assessment methodologies on SDN-3 system could be simplified compared to what is done with GMOs, since in the SDN-3 system the introgression in the of gene sequences into the genome occurs in a targeted manner and defined place, unlike processes with traditional transgenics, in which insertions in the genome are random.

In a second discussion panel, the EFSA also decided that the conclusions of the first panel would be partially applicable to the SDN-1 and SDN-2 systems. Since SDN-1/SDN-2 approaches aim to modify an endogenous DNA sequence whose

final product does not contain exogenous DNA. Therefore, these products would not present any of the potential risks related to the insertion of a transgene.

Several countries, including Brazil, understand that the introduction of variability in species of economic importance could help achieve important sustainability goals, healthier foods, less use of chemical pesticides, contributing to a healthier environment, and mitigation of problems caused by global climate change, among other possibilities. However, innovations in genetics must take into account the basic principles of biosafety.

Legislation should not stop technological development and the possibility of generating biotechnology-based should not be restricted to a few institutions and companies, as occurred in the case of transgenics, considering the expensive and time-consuming approval processes created by each country. If products resulting from targeted mutagenesis, mainly SDN-1 and SDN-2 systems, are subject to the same risk assessment requirements as traditional GMOs/SDN-3, technological development may be restricted to few large companies, limiting competition and market share.

Brazil, Argentina, Canada, Chile, Colombia, USA are among the first to have legislation that regulates the use of gene editing techniques. In these countries, the increase in number, type, and size of institutions/companies developing products of interest to society, is clear. Moreover, there is a significant increase in the number of species worked with NBTs. In GMOs, investment was only viable for major commodities, such as soybeans, corn, and cotton, among the main ones.

As SDN-1 and SDN-2 systems simulate/imitate mechanisms of genetic variability induction that occur constantly and frequently in nature, their detection in genome-edited products is practically impossible. The European Union, in the report "Detection of food and plant foods obtained by new mutagenesis techniques" (European Commission, 2019), recognizes that products whose genome has been edited may be indistinguishable from products altered by natural processes or by conventional reproductive techniques.

Brazil, in alignment with the national and international scientific community, and with the legislation of several countries, many of which are commercial partners, understands the importance of harmonizing the biosafety laws of food exporting and importing countries. Biosafety laws should reflect and welcome technological progress, maintaining food quality and safety but also allowing diversification of participants in the production chain. Products generated by genome editing, mainly by the SDN-1 and SDN-2 systems, should not be subject to risk analysis

requirements like GMOs if they could also be obtained by conventional methods or spontaneous processes in nature.

Harmonization of regulatory rules also allows creating legal assurance for developers in each country, avoiding individual national/regional rules for products resulting from conventional random mutagenesis or the use of SDN systems. Also, it prevents two indistinguishable products from being regulated in two different ways.

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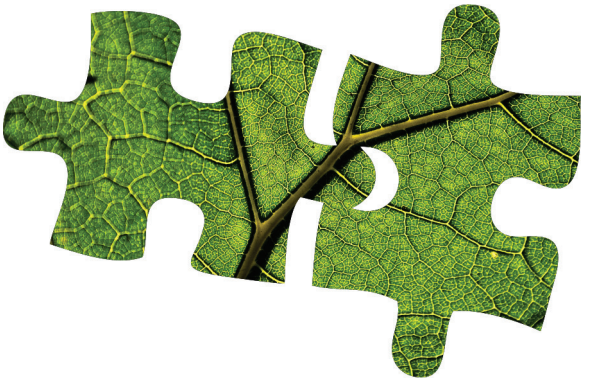
Embrapa

Agroenergy



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