Revista Brasileira de Milho e Sorgo
Brazilian Journal of Maize and Sorghum

ISSN 1980 - 6477
Journal homepage: www.abms.org.br/site/paginas

Kamila Ellen Souza de Oliveira[1], Camila Ribeiro[1], Andrea Almeida Carneiro[1] and Newton Portilho Carneiro[1](✉)

[1]Embrapa Milho e Sorgo
E-mail:kamila.ellen.s@gmail.com, cams.ribeiro@gmail.com, andrea.carneiro@embrapa.br, newton.carneiro@embrapa.br.

✉ Corresponding author

How to cite

OLIVEIRA, K. E. S.; RIBEIRO, C.; CARNEIRO, A. A.; CARNEIRO, N. P. Editing genomes via crispr/cas9 and applications in maize improvement. **Revista Brasileira de Milho e Sorgo**, v. 21, e1284, 2022.

EDITING GENOMES VIA CRISPR/CAS9 AND APPLICATIONS IN MAIZE IMPROVEMENT

Abstract – The CRISPR/Cas9 system is a gene editing method capable of accurately locating and altering a specific sequence of a genome. Currently, this technique stands out as a promising biotechnological tool for the improvement of different crops, overcoming the limitations of conventional breeding methods and accelerating the development of plants' agronomic characteristics. The basic principle of genome editing via CRISPR is to cause a double-stranded DNA break at a predetermined location by a sgRNA (single guide RNA) and through internal repair mechanisms cause mutations and, consequently, genetic variability. In prokaryotic organisms, it is an immune system that naturally protects against viral infection and has been modified to create different applications in higher organisms in the field of medicine and agriculture. In agriculture, it has been used in a large number of mono and dicotyledonous plants, including those of great commercial value such as soybean, cotton and maize. In maize, this technique has been used as a biotechnological tool for increasing production, quality, nutritional value, tolerance to biotic and abiotic stresses. CRISPR-Cas9 technology has enormous potential to contribute to increased food production with great benefit to the environment. This review explores the bases for the use of CRISPR/Cas9 technology to generate genetic variability and its use in the development of maize cultivars with better agronomic characteristics.

Keywords: *Zea mays*, mutations, gene editing, CRISPR, and transgene.

EDIÇÃO DE GENOMAS VIA CRISPR/CAS9 E APLICAÇÕES NO MELHORAMENTO DE MILHO

Resumo - O sistema CRISPR/Cas9 é um método de edição gênica capaz de localizar e alterar com precisão uma sequência específica de um genoma. Atualmente, essa técnica se destaca como uma ferramenta biotecnológica promissora para o melhoramento de diferentes culturas, superando as limitações dos métodos convencionais de melhoramento e acelerando o desenvolvimento de plantas com características agronômicas aprimoradas. O princípio básico da edição genômica via CRISPR é causar uma quebra da fita dupla de DNA em um local predeterminado por um sgRNA (single guide RNA) e através de mecanismos internos de reparo causar mutações e, consequentemente, variabilidade genética. Em organismos procariotos é um sistema imune que naturalmente protege contra infecção viral e tem sido modificado para criar diferentes aplicação em organismos superiores tanto no campo da medicina como agricultura. Na agricultura tem sido utilizado em grande número de plantas mono e dicotiledôneas incluído aquelas de grande valor comercial como soja, algodão e milho. Em milho essa técnica se destaca como uma promissora ferramenta biotecnológica para o aumento de produção, qualidade, valor nutricional e tolerâncias a estresses bióticos e abióticos. A tecnologia CRISPR-Cas9 tem um enorme potencial para contribuir com o aumento da produção de alimentos beneficiando o meio ambiente. Esta revisão busca explorar as bases do uso da tecnologia CRISPR/Cas9 para geração de variabilidade genética e sua utilização no desenvolvimento de cultivares de milho com melhores características agronômicas.

Palavras-chave: *Zea mays*, mutações, edição de genes, CRISPR e não-transgênico.

DOI: https://doi.org/10.18512/rbms2022v21e1284

Genetic variability is essential for developing new cultivars that are more productive, nutritious, and adapted to different biotic and abiotic stresses. Currently, targeted genome editing tools offer new opportunities to study gene function and develop new traits with a minor breeding cycle. Four genome-editing technologies developed in the last few decades have successfully generated targeted genetic modifications in plants. These technologies are known as (i) meganucleases; (ii) Zinc finger / ZFN nucleases; (iii) Transcription Activator-Like Effector Nucleases / TALEN; and (iv) Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR (Shukla et al., 2009; Gao et al., 2010; Li et al., 2013; Cermák et al., 2015). Gene-editing techniques using meganucleases, ZNFs, and TALENs have brought advances in the targeted manipulation of genomes. However, these approaches are complex and require elaborate protein engineering to establish the target sequence recognition site in the genome (Smith et al., 2006; Maeder et al., 2008). In contrast, the CRISPR-Cas9 system has revolutionized the field of genomic editing as it is simple, accurate, flexible, and low-cost (Doudna; Charpentier, 2014).

The CRISPR system is an RNA/protein-mediated adaptive immunity mechanism used by prokaryotes in defense against virus and plasmid attacks (Zhang et al., 2021b). Over the years, discoveries about this bacterial defense system have allowed its adaptation to be used in the laboratory, transforming the CRISPR system into an important and efficient tool for editing genomes in living organisms.

In summary, genomic editing mediated by the CRISPR/Cas9 system is based on Cas9 nuclease, an enzyme capable of promoting the double-strand break of DNA (Double-Strand Break / DSB), and on RNA-guide molecules (sgRNAs), which direct Cas9 activity to specific DNA sites, to edit genomic regions of interest (Lopes Filho et al., 2020). Different genomic regions can be edited depending on the different sgRNA molecules, which can also be designed to edit several genes simultaneously, facilitating the engineering of metabolic pathways of a multigene family or the editing of the same gene in plants with polyploid genomes (Mushtaq et al., 2021).

In this review, CRISPR technology will be described as a tool to generate genetically engineered germplasm for plant yield and stress tolerance.

CRISPR: Adaptive Defense System of Prokaryotic Organisms

The history of the CRISPR-Cas9 technology began when Ishino and collaborators in 1987 in Japan identified a region in the genome of the bacterium *Escherichia coli* with an unknown function containing a series of repeated regions interspersed with non-repeating or spacer regions (Ishino et al., 1987). This region was named by Jansen et al. (2002) from CRISPR (Grouped and Regularly Interspaced Short Palindromic Repeats or Clustered Regularly Interspaced Short Palindromic Repeats). Along with the CRISPR region, genes coding for polymerases, nucleases, and helicases were identified, and these genes became known as Cas or genes associated with the CRISPR region (Mojica et al., 2000) (Figure 1).

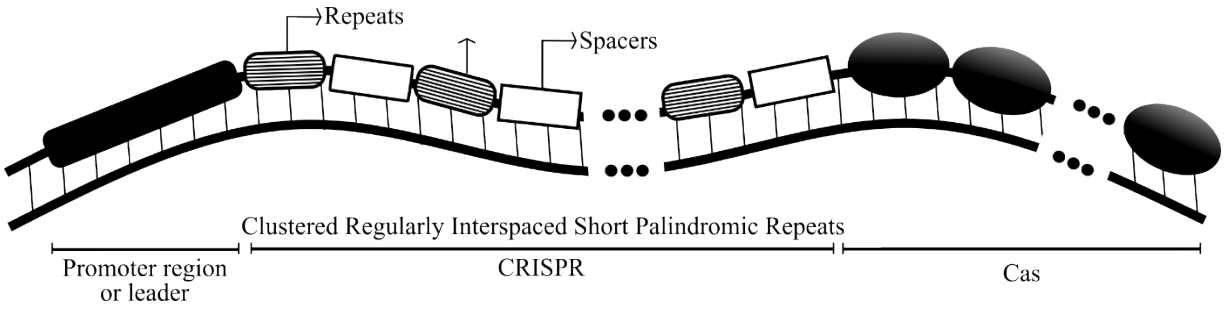

Figure 1: CRISPR - Grouped and Regularly Intercalated Short Palindromic Repeats. A region present in the bacterial genome is composed of a series of repeated regions interspersed with spacer regions. Next to the CRISPR region are genes that encode polymerases, nucleases, and helicases known as Cas or genes associated with the CRISPR region.

To understand the function of this region, research determined that the DNA fragments present between the repetitive regions were derived from invading phages, transposons, or plasmids. Furthermore, it was found that the bacteria that kept the memory of previous invasions in the spacer regions (DNA of the invading organism) were not infected a second time by the same phage (Bolotin et al., 2005). Then came the hypothesis, subsequently confirmed, that the CRISPR/Cas system functions as an adaptive defense mechanism of the bacterium against invasions by unwanted genetic elements (Mojica et al., 2005; Sampson & Weiss, 2014).

The study of the working mechanism of this DNA region showed that when the bacterium is infected by phages or other mobile genetic elements, such as plasmids and transposons, enzymes encoded by the Cas operon cleave the invading DNA into small fragments. These fragments are inserted into the bacterial genome between the repetitive sequences of the CRISPR locus, and as a result, the bacteria store the memory of previous infections and acquire protection against a subsequent attack by these invaders. This protective mechanism occurs because small RNAs, known as crRNAs (RNAs derived from the CRISPR region or CRISPR-derived RNA) or guide RNA molecules, corresponding to the invading DNA sequences, are produced by the bacteria (Figure 2) (Hille et al., 2018). Therefore, the entire CRISPR locus is transcribed from an AT (adenine, thymine) rich region known as the Leader Sequence (L), producing a pre-crRNA containing all the spacer and repeat sequences. Pre-crRNA is cleaved and gives rise to crRNAs in a step known as crRNA maturation. This process evolved differently among the different types of existing CRISPR-CAS systems (Deltcheva et al., 2011).

In some CRISPR systems, it is performed by the activity of endoribonucleases, such as CasE, Cas6, and Csy4, but in systems that do not have these enzymes, for example, in the CRISPR/Cas II system, crRNA maturation involves a trans-RNA or molecule of Activator RNA (tracrRNA),

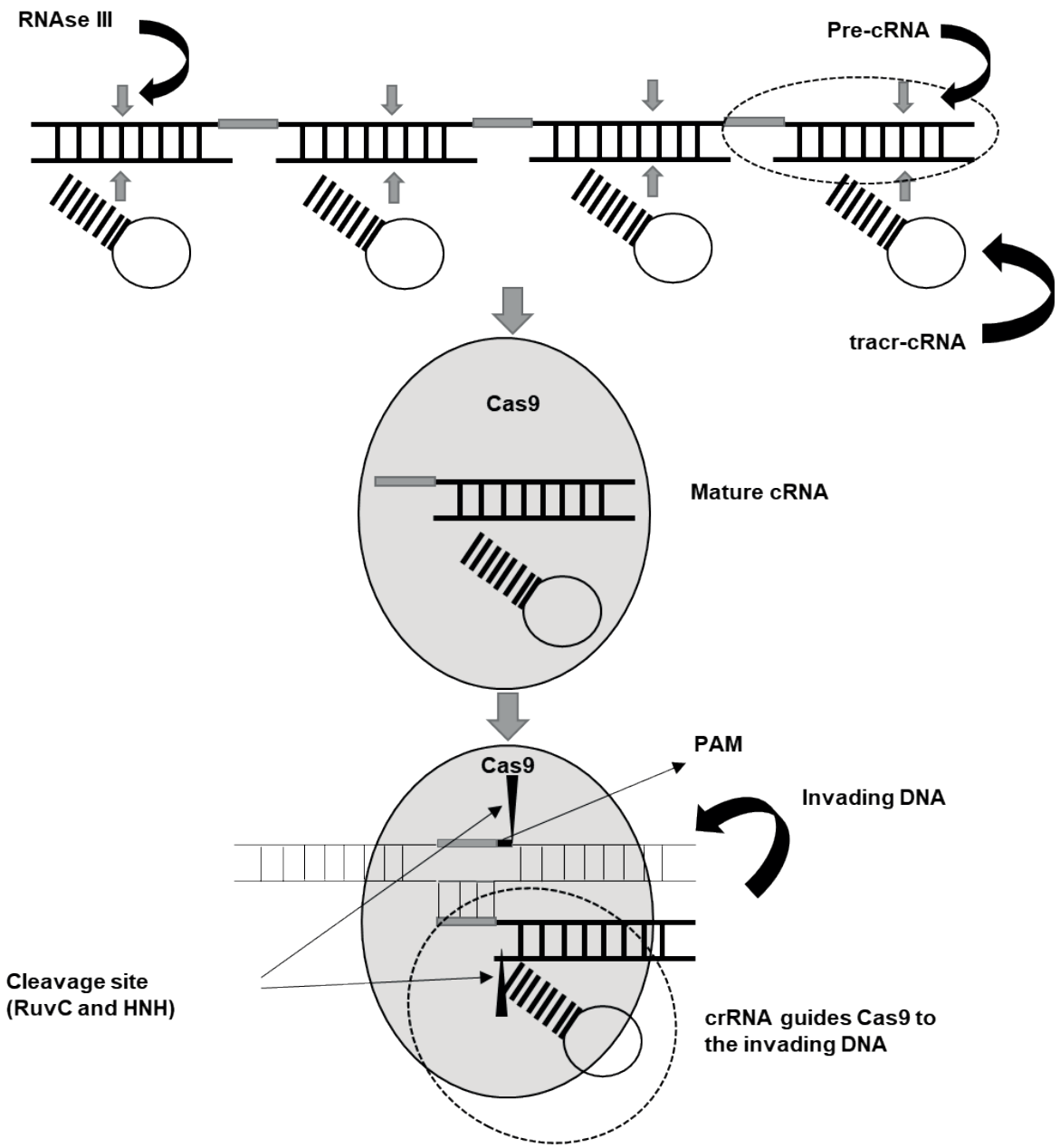

Figure 2: CRISPR-Cas9 acquired immunity system. Initially, a short sequence of the invading genetic material (spacer sequence) is integrated between repeat sequences from the CRISPR region. The memory recorded by the spacer is used to protect the bacteria against a new incursion by the same invader. The CRISPR region is fully transcribed from the leader/promoter region into a unique sequence. The pre-crRNAs are then processed into mature crRNA. The crRNA is then used as a lead molecule to specify the target of nuclease cleavage Cas (based on Jiang & Marraffini, 2015).

the Csn1 protein and an endogenous RNase III. TracrRNA is a 171 or 89 nt RNA molecule with a 25 nt region almost perfectly complementary (only one mismatched nucleotide) to the repeat regions of the CRISPR locus. This complementary region is used for the pairing between tracrRNA and the repetitive regions of the pre-crRNA. When these RNAs pair, the Cas9 enzyme binds to the duplex formed by tracrRNA and pre-crRNA, stabilizing the complex and recruiting RNAseIII that processes the pre-crRNA into several small crRNAs. After a second cleavage by a still unknown RNAse, the Cas9 tracrRNA and crRNA complex is ready to interfere with the invading DNAs. Although the function of the Csn1 protein is not fully understood, it is known to be essential during this process of crRNA maturation.

According to the hypothesis of Deltcheva et al. (2011), the Csn1 protein could function as an anchor molecule facilitating the pairing between tracrRNA and pre-crRNA. It could also work by making a second cut within the spacer region, trimming the crRNA sequences, or, finally, protecting the tracrRNA and pre-crRNA from degradation by other ribonucleases.

The formation of double-stranded RNA, resulting from the tracrRNA/crRNA interaction, is essential for the crRNA to recognize the target DNA and activate Cas9. The interaction of Cas9 nuclease with crRNA / tracrRNA results in a change in protein conformation, activating the interaction site with the PAM motif (Protospacer Adjacent Motif). PAMs are sequences of 2-5 nucleotides (5'NGG3' and 5'NNGRRT3') necessary for anchoring the nuclease Cas9 to the cleavage site. Since there are different nucleases, the PAM motifs can differ from each other. For example, NmCas9 from *Neisseria meningitidis* (Hou et al., 2013) recognizes the PAM sequence "NNNNGATT," while St1Cas9 from *Streptococcus thermophilus* (Deveau et al., 2008) recognizes the sequence "NNAGAAW."

The mature crRNA must associate with the complementary strand of the target DNA in the region adjacent to a PAM motif to proceed with the target DNA identification and cleavage process (Ran et al., 2013). Cleavage of the target sequence will only occur if a PAM motif is adjacent to it on the complementary strand of DNA. After the Cas9/crRNA/tracrRNA complex is associated with the target DNA through the PAM sequence, the DNA strands open immediately upstream of the PAM sequence, and the guide sequence (crRNA) interacts with the target sequence (Anders et al. al., 2014). Finally, the Cas9 nuclease can cleave the two strands of DNA generating blunt ends (Jinek et al., 2012).

When the same virus attacks the bacterium, the Cas9/crRNA/tracrRNA complex recognizes this virus and destroys it. The crRNAs, containing the specific sequences of the previous invading virus, act as a memory and guide the Cas enzymes to the invader that will be destroyed (Figure 2). Therefore, CRISPR is a bacterial locus that encodes small guide RNAs based on sequences from viruses, transposons, and plasmids that have previously invaded the bacteria. Together with Cas proteins, this region forms a natural system of acquired antiviral immunity present in several bacteria and Archaea (Mojica et al., 2000).

How to produce a genetically edited plant

Nuclease Cas, crRNA, and tracrRNA together make up the natural acquired immunity system of bacteria. Therefore, the "single guide RNA (sgRNA or gRNA) was developed, consisting of a fusion of the crRNA (CRISPR-derived RNA) and the tracrRNA (trans-activating RNA) by a chain of 4 nucleotides (GAAA) was developed to apply this system in research. Thus, the laboratory process comprises only two molecules, the nuclease Cas9 and the sgRNA (Jinek et al., 2012; Silva et al., 2016).

However, how can a double-strand DNA break generate genetic variability? When the DNA strand is broken, it needs to be repaired to maintain the genetic and physical integrity of the genome (Hiom, 2010). A double-strand break of DNA (DSB / Double-strand Break) can have deleterious effects on the viability of an organism and can cause serious injuries, capable of causing genetic instability (Chapman et al., 2012). Cells have developed two mechanisms for DNA damage repair to minimize and preserve genome integrity: NHEJ (*No Homologous End Joining*) and HDR (*Homologous-Directed Repair*) (Figure 3). The most direct way to repair a double-strand break is to join the two strands independently of the nucleotide sequence present in the region of the breakthrough NHEJ mechanism. The introduction or deletion of nucleotides can occur in this process, forming INDEL-type mutations (Hiom, 2010; Doudna; Charpentier, 2014) that can inactivate the target gene. Both repair pathways' ability to incorporate specific sequence changes and promote DSB in target regions are of great importance in genome engineering (Hilton and Gersbach, 2015).

The Cas9 nuclease must be stably in the cell nucleus, inserted into the plant genome of interest or transient, to generate a targeted break in a DNA double-strand and activate the NHEJ or HDR-mediated repair process, thereby creating a targeted mutation. It can be inserted in the form of DNA (expression vector containing Cas9), RNA (Cas9 mRNA), protein (Cas9 synthetic protein), or ribonucleoprotein (assembled protein/RNA complex). The Cas9 enzyme (SpCas9) from *Streptococcus pyogenes* is routinely used for genome editing (Silva et al., 2016).

In the NHEJ pathway, random insertion or deletion of DNA bases occurs to repair DSB. The most common resulting INDELS range between 5 and 9 nucleotides. It predominantly occurs in cells and is active throughout the cell cycle (Hilton and Gersbach, 2015), causing insertions, deletions, or substitutions type mutations. When DSB is repaired, the indels are formed to modify the original DNA sequence, leading to gene inactivation (Prado et al., 2020). Thus, the expression of genes present at the site where NHEJ repair was made can be, in general, completely interrupted, and the encoded proteins may not be functional (Cui et al., 2019).

For the induction of more precise mutations, the HDR repair pathway is more indicated. However, it is necessary to introduce a third molecule, the donor DNA, into the plant cell, in conjunction with the nuclease Cas9 and sgRNA. The donor DNA has the region that will be modified, for example, a sequence that encodes one or a few amino acids different from the original sequence - allelic substitution (Zhang et al., 2014) or an entirely new gene

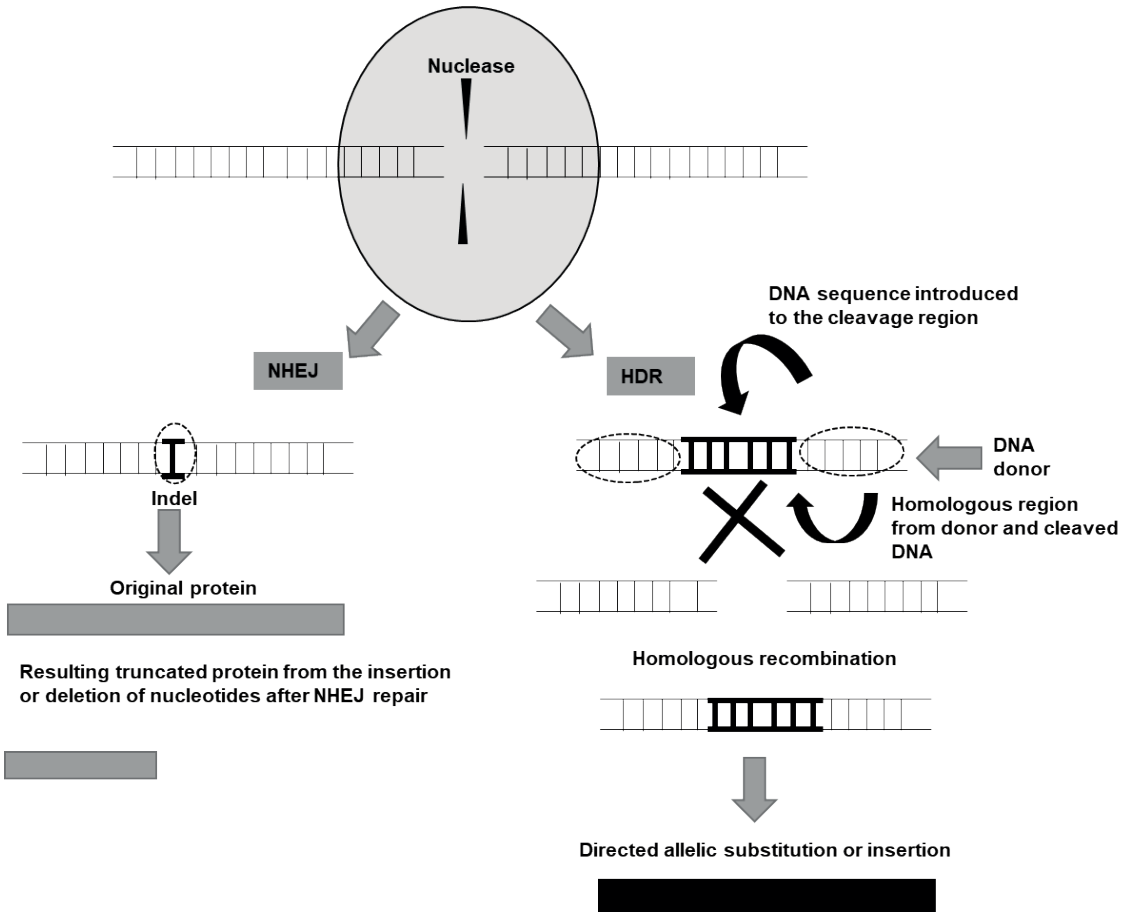

Figure 3: Repair of a DNA double-strand break by NHEJ (No Homology or Non-Homologous End Joining) or HDR (Homology-Directed Repair) processes.

that will be inserted into the genome - Knock-in (Fauser et al., 2012). The donor DNA is flanked by sequences homologous to the region of the genome where the gene-editing will be performed and will be inserted into the genome by homologous recombination. HDR repair is less efficient despite being more accurate since it only occurs in the early stages of the cell cycle (Hiom, 2010).

Despite being a tool to generate targeted mutations, the CRISPR/Cas system presents a limitation to the precise insertion of the gene or replacement of the DNA sequence. Recent research has shown that it is possible to target mutation to intragenomic genes free of selectable markers in maize plants (Barone et al., 2020). In the edited plants, DNA repair activated marker gene expression within the donor locus, generating a 5% targeted insertion of the donor sequence at the target locus (Barone et al., 2020).

The simplest and most easily manipulated method in the laboratory is insertion through the expression vector, where the nucleotide sequence of Cas9 is cloned under the control of specific

promoters and transcription terminators for the organism under study. For a more efficient expression of Cas9, it is necessary to adjust its codon to a nucleotide composition close to the GC content of the target species, changing the preferred amino acid codons (Silva et al., 2016). It is also important that the nucleotide sequence of Cas9 is flanked by a nuclear localization signal (NLS). This sequence of approximately 10 amino acids directs the protein to the nucleus. It can be positioned at the amino (Wu et al., 2014) and/or carboxy (Gao et al., 2015) ends, and when present at both ends, it has shown a more efficient result (Hou et al., 2013).

For the accuracy of the CRISPR/Cas system to be achieved, one of the most critical steps is the projection of the sgRNA, as it will guarantee the successful editing of the target genes (Fu et al., 2014). Design tools differ in design parameters and specifications, predominantly highlighting target effectiveness calculation models and off-target calculation algorithms to improve sgRNA specificity (Zhu, 2015; Young et al., 2019). This sequence can be designed with the help of online software, such as CRISPROR http://crispor.tefor.net/ (Concordet & Haeussler, 2018) and CHOPCHOP https://chopchop.cbu.uib.no/ (Labun et al., 2019), among others.

The gene encoding the guide RNA or sgRNA can also be cloned into an expression vector. In this case, an RNA polymerase III promoter (U3 or U6, a guide or target sequence (~20 nt), the universal sequence or scaffold (~80 nt), and the poly-T (TTTTT) terminator signal are used to construct a vector to express small RNAs.

The same vector can contain the region for the expression of Cas9 and the universal sequence, requiring only the cloning of the guide sequence for the gene of interest. The guide sequence must be located next to a PAM sequence (e.g., N20NGG), be unique in the genome, and contain approximately 20 nucleotides, with a GC content close to 50%. In maize, a study revealed that Cas9 expression using a promoter with high activity in calluses and reproductive tissues allowed the induction of effective mutations in 85% of target sites and allowed alteration in more than one region simultaneously (Feng et al., 2018). In addition, reporting or selection systems are also inserted to assist in the mutated genotyping process. In maize, plants edited by the CRISPR system were screened and selected through the expression of the DsRED protein in endosperm and embryos (Yan et al., 2021). Similar reviews identified plants that do not harbor the editing cassette but carry desired mutations in the target genes.

To increase editing precision, engineered nucleases were generated to target and digest DNA in the genome (Zhang et al., 2021a). For example, Cas12a or Cpf1 50, unlike Cas9, is a dual-function nuclease that recognizes 5' T-rich PAMs and self-processes its crRNA. The CRISPR/Cpf1 system allows gene insertion and deletion, as well as locus tagging in both monocots and dicots with a lower off-targets ratio (Alok et al., 2020). The MAD7 is an engineered nuclease of the class 2 type V-A CRISPR-Cas (Cas12a/Cpf1), with less homology to other Cas12a nucleases. For this reason, it was publicly released as a royalty-free nuclease to be democratically used in academia and industry. In addition, this nuclease can recognize T-rich PAM

sequences (YTTN) in plants (Lin et al., 2021).

Use of the CRISPR-Cas9 system in agriculture

With the development of the technique and the discovery of new tools that allowed its use in the laboratory, the CRISPR technology can precisely modify genes controlling agronomic traits of interest. Furthermore, only two basic elements, an endonuclease and a sgRNA complementary to the target gene sequence can allow the manipulation to generate the genetic modifications of characteristics for plant genetic improvement (Svitashev et al., 2016).

Among the main interesting traits for modification are those related to crop production, product quality improvement and genome editing for tolerance to biotic, and abiotic stresses (Bao et al., 2019; Chilcoat et al., 2017; Zaidi et al., 2020). Maize plants that had the edited *ZmLG1* gene had leaf angles 50% smaller than wild plants, which allows them to be cultivated at higher densities, consequently increasing the yield potential per cultivated area (Li et al., 2017).

In addition to accelerating maize genetic research by providing new possibilities for genetic improvement, one of the most immediate applications of CRISPR is the identification and functional analysis of genes. Research involving maize usually carries out this characterization in mutant populations, through the silencing of the gene of interest, with outstanding robustness and reliability (Nuccio et al., 2021). Examples of success include confirmation of the role of *ZmCCT9* in high altitude adaptation (Huang et al., 2018), characterization of the male sterility gene 33 (*ZmMs33*) (Xie et al., 2018), and confirmation of the role of *ZmCST1* on stomatal movement and plant carbon status (Wang et al., 2019).

Gene silencing has several other applications in agriculture. For example, Liang et al. (2014) used the CRISPR system to generate INDEL-like mutations and silence the *ZmIPK1A*, *ZmIPK,* and *ZmMRP4* genes of the phytic acid biosynthetic pathway in maize. Phytate represents about 75% of the total phosphorus in the seed and is an anti-nutritional compound. This study demonstrated that the CRISPR/Cas system could induce targeted mutagenesis in *Z. mays* with high efficiency and silence undesirable genes.

In another example, the CRISPR-Cas9 gene-editing system was used to generate a double-stranded DNA break in the gene encoding the enzyme NDP-glucose-starch-glucosyltransferase. This gene is part of the metabolic pathway of amylose production. The double-stranded repair caused INDEL-type mutations that resulted in the silencing of the enzyme NDP-glucose-starch-glucosyltransferase and obtaining a maize cultivar known as "waxy," which presents only amylopectin in the constitution of its starch. In unedited maize kernels, the ratio of amylose to amylopectin is approximately 1:3. Thus, Waxy maize has a high added value due to the chemical properties of amylopectin that are highly appreciated in food, adhesives, bioplastics, ethanol fermentation, and possibly silage industries (Cigan et al., 2017).

The brachytic 2 (*br2*) gene from maize was edited after the repair via NHEJ, induced by the CRISPR-Cas9 system. This repair formed an INDEL that altered the amino acid reading frame resulting in a premature stop codon. As a result,

maize mutants for the br2 gene have reduced height because of the shortening of internodes, but the rest of the plant, such as leaves, flowers, and ears, maintained their standard size (Bage et al., 2020).

As an example of the use of the HDR repair mechanism, we can mention the generation of maize plants resistant to the herbicide chlorsulfuron after the targeted breakdown of the ALS2 gene that encodes the acetolactate synthase enzyme using the CRISPR-Cas9 system. An edited sequence of this gene, containing a replacement of the proline located at position 165 of the native ALS2 protein by a serine, was introduced into maize plants that already contained the Cas9 nuclease pre-integrated into the genome (Svitashev et al., 2015). The edited ALS2 gene, unlike the native gene, is not a target of the sulfonylurea group herbicides that inhibit the metabolic pathway of branched amino acid formation. Unlike transgenic plants, in this type of gene-editing, it is possible for future generations to segregate Cas9 from gene-editing, and consequently, no heterologous DNA fragment will be inserted into the genome.

Another modification performed in maize plants using the CRISPR/Cas9 system was the increased expression of the ARGOS8 gene, a negative regulator of the plant's response to ethylene. The phytohormone ethylene regulates the plant's response to various abiotic stresses, and it has been shown that when its expression is reduced, plants are more productive under water stress conditions. Usually, ARGOS8 gene expression is low in maize, and to modify this, Shi et al. (2017) used the CRISPR/Cas9 system to exchange the native ARGOS8 promoter, which provides a low-level of ARGOS8 protein, with the GOS2 promoter, which provides a moderate level of constitutive expression. The result obtained with this targeted genetic modification was an increase in the expression of the ARGOS8 gene, generating a decrease in the response of maize to ethylene and, consequently, an increase in productivity under conditions of water stress. In this example, a transgenic plant was created where the promoter of interest was inserted in a predetermined location.

Mutations in the SfABCC2 gene promote tolerance to *Bacillus thuringiensis* Cry1F toxin in *Spodoptera frugiperda* (Jin et al., 2021). Yield has also been demonstrated to be improved with a knock-down of ZmVLHP or ZmGW2 expression by significantly increasing total kernel number or kernel weight, respectively (Kelliher et al., 2019).

Insertion of the CRISPR/Cas9 system into the maize genome

It is necessary to transform the plant for the genome editing process via the CRISPR/Cas system or other traditional methods. For the CRISPR technology to work, it is necessary to deliver the Cas enzymes and the sgRNAs to the cells so that together they can edit the DNA sequences of interest. For this, methods already used to obtain transgenic plants can be applied (Zhang et al., 2021b).

Editing the genome mediated by CRISPR/Cas requires that only the molecular tool (sgRNAs and Cas) be delivered to the plant cell, and it is not necessary to leave the sgRNAs, and Cas stably expressed in plant genomes (Zhang et al., 2021b). Both Cas proteins and sgRNAs can

be transcribed and synthesized in vitro, where they can be delivered to plant cells using direct methods such as protoplast transformation and particle bombardment, or indirect methods such as Agrobacterium-mediated transformation (Woo et al., 2015; Zhang et al., 2016).

Agrobacterium tumefaciens (Frame et al., 2002; Vega et al., 2008) and biolistics (Sanford et al., 1987) are the most efficient methods to insert components of the CRISPR system into maize cells. In the context of genomic editing, *Agrobacterium* can insert only DNA molecules into the cell, whereas via biolistic, DNA, RNA, protein, or RNP molecules can be inserted. *A. tumefaciens* is a bacterium commonly found in soil and capable of causing plant tumors in the region of infection. Tumor formation is associated with the presence of a plasmid known as Ti (tumor-inducing) (Gelvin, 2010). Tumor induction occurs by transferring a segment of this plasmid, called T-DNA, to the host genome. The genes present in T-DNA are responsible for phytohormones production, promoting the disordered cell proliferation of plant cells. Several bacterial and plant genes are involved in transferring T-DNA from *Agrobacterium* to the plant cell (Gelvin, 2010).

It is possible to exchange the native genes present in the T-DNA for other genes of interest, for example, those encoding the nuclease Cas9 and the sgRNA of the CRISPR-Cas9 system. Once inside the plant cell nucleus, the genes present in the T-DNA can be expressed transiently or stably (Janssen and Gardner, 1990). T-DNA integration into the host genome does not occur in a transient expression, and gene expression is usually detected a few days after plant tissue infection, decreasing over time (Lacroix and Citovsky, 2013). The transient transformation can be used to express Cas9 nuclease and sgRNA for a short period. This period is enough to break the double-strand of DNA and its repair with or without homology. The transient expression has a lower efficiency in inducing mutations, but on the other hand, no foreign DNA is integrated into the genome (Andersson et al., 2017).

In stable expression, however, T-DNA is integrated into the host genome, producing a transgenic plant that continuously expresses the editing components of the genome. Consequently, the efficiency of mutations increases (Jansing et al., 2018), but there may be an increase in mutations at non-target sites (Chaparro-Garcia et al., 2015). It was also recently reported the possibility to use complex trait locus (CTL) to facilitate trait stacking in CRISPR constructs in maize. This can be utilized in sites found within a known chromosomal locus where multiple trait-related candidate genes are localized (Gao et al., 2020).

In the transformation via biolistics, metal microparticles coated with DNA, RNA, proteins, or RNPs can be accelerated towards the target cells with sufficient speeds to penetrate the cell wall without causing cell death by using a bomber known as a "gene gun" (Sanford et al., 1987). Compounds precipitated onto the microparticles are gradually released into the cell and can either be integrated (stable expression) into the genome or remain as an extra-chromosomal construct (transient expression) (Klein et al., 1987).

In maize, the transformation of embryonic cells via biolistic, using Cas-sgRNA ribonucleoproteins, has already been

successfully achieved (Svitashev et al., 2016). After the molecules were delivered to maize cells, their high regeneration power resulted in plants with the genome-edited via the CRISPR/Cas9 complex. By this delivery method, scientists have further demonstrated the mutagenesis of free DNA genes and selectable markers in maize and the recovery of plants with mutated alleles at high frequencies. The advantage of using biolistic in comparison to transformation via *Agrobacterium* is the independence of genotype. Thus, several genotypes or even different species can be used in the transformation process. However, the quality of integration events is lower, and the process usually results in plants with more than one copy of the gene of interest integrated into the genome.

Both *Agrobacterium* and biolistic can be used to insert the components of the gene-editing system together with a selection marker. However, the insertion site in the T-DNA genome, containing the nuclease Cas9 and sgRNA, will be random and different from where the DSB and its repair will occur. This procedure allows transgenes containing the components of the CRISPR system to be eliminated through Mendelian segregation, thus keeping only the mutated gene (Figure 4). Other methods are currently being implemented to optimize delivery in protoplasts, such as electroporation and PEG-mediated delivery, viral-based replicons, electroporation, and nanoparticles in intact tissues (Gordon-Kamm et al., 2021).

Also, when performing a CRISPR screen is important to generate many plants. To improve the number of plants in maize, Liu et al. (2020) generated over 4,000 independent transgenic events. In this study, they observed that only a few plants could have the capacity to generate a screening population of this size in recalcitrant species for transformation and tissue culture. However, recently it was demonstrated that overexpressing morphogenetic regulators such as *WUSCHEL2* and *BABY-BOOM* improves transgenic plants generation in several monocotyledon species through callus regeneration stimulation or by inducing somatic embryogenesis (Lowe et al., 2018, 2016). Thus, the improvement of plant regeneration capability might allow CRISPR screening to be scaled up. Interestingly, yield traits were also improved by editing the promoter from ZmCLAVATA3/EMBRYO SURROUNDING REGION-RELATED7 (CLE7), which increases meristem size regulated by CLE peptide signals in the CLAVATA-WUSCHEL pathway (Liu et al., 2021).

Non-transgenic versus transgenic edition

A genetically modified organism has a new combination of genetic material inserted into its genome (Cartagena Protocol / http://bch.cbd.int/protocol/text/). Genetically edited plants can generate three types of events, classified as NSD1, NSD2, or NSD3. NSD1 is random, site-specific mutations generated after joining the ends of the DNA that were cleaved by the Cas9-sgRNA system by the NHEJ repair mechanism. NSD2 are allelic substitutions induced after homologous recombination (HRD) repair of the cleaved DNA strand. In this process, homologous DNA is added and competes with the sister chromatids, replacing the original nucleotide sequence. Finally, NSD3 is inserted as a transgene through homologous recombination (EFSA, 2012; Wolt et al., 2016).

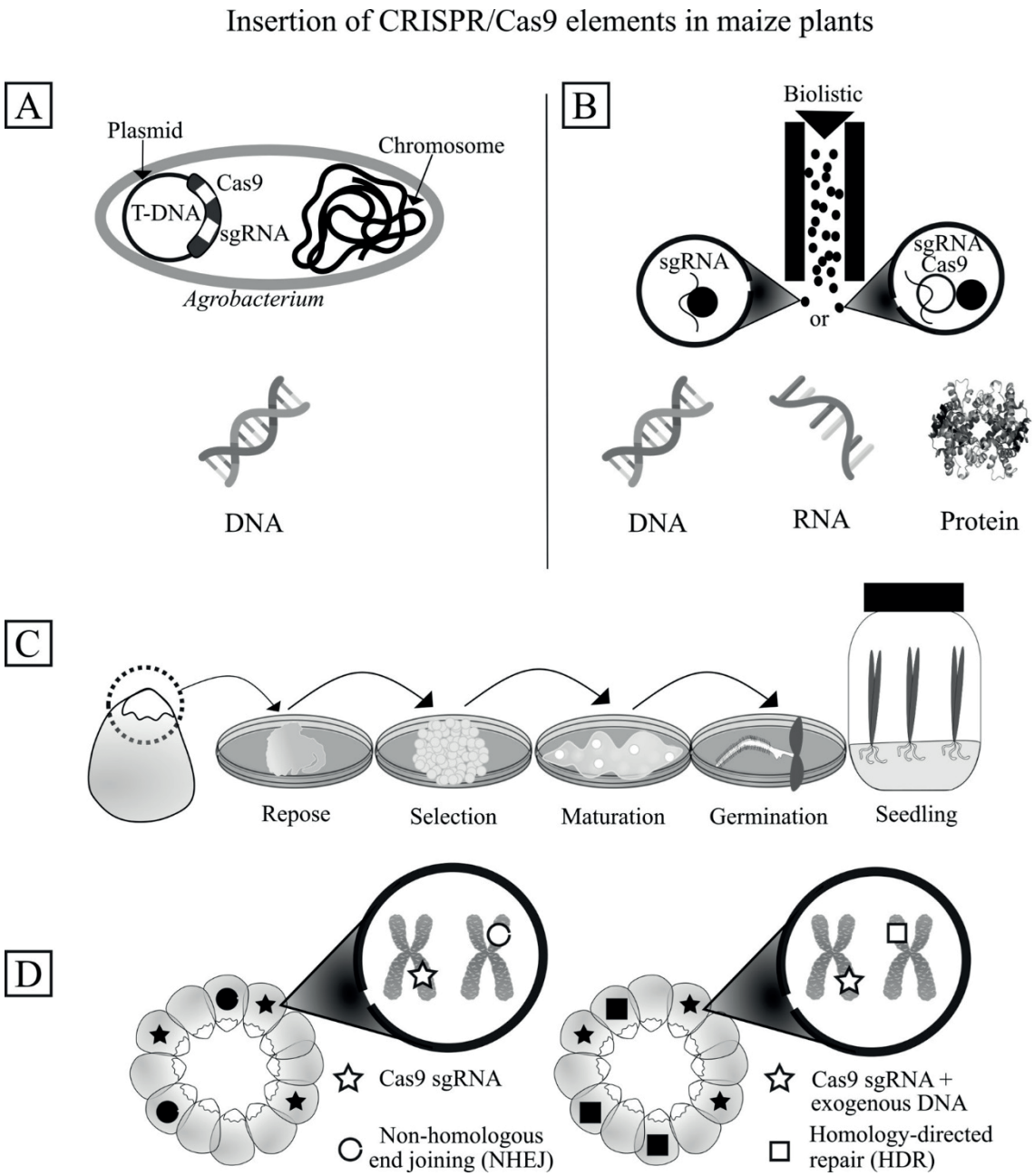

Figure 4: CRISPR/Cas9-mediated maize genome editing. Elements of the CRISPR-Cas9 system are introduced into maize plants as transgenes via Agrobacterium-mediated (A) or biolistic transformation (B). Regeneration of transformed maize events in tissue culture (C). The DNA cleavage occurs at a different location from the Cas9-sgRNA insertion (D). Due to the segregation of genes in the next generation, some seeds will contain the desired edition and no transgene (left and circle) or insertion of a transgene at a predetermined location (right and square).

In plants edited by the CRISPR-Cas9 system and classified as an NSD1 type event, the Cas/sgRNA complex can be removed using different molecular strategies (Curtin et al., 2012), which will produce a plant without heterologous genetic material (Curtin et al., 2012). This mutation is not different from one that could have occurred naturally, and, in general, regulatory bodies do not consider these organisms in the same context as GMOs (Wolt et al., 2016). The classification and regulation of biosafety of NSD2 type plants depend on the extent of the edition carried out, which may or may not be regulated as GMOs. Thus, each NSD2 edited plan will be analyzed case-by-case basis (Lusser and Davies, 2013). Plants classified as an NSD3-type event have transgenes inserted in the genome, and they will be regulated as GMOs. Depending on the country's regulatory framework, in the case of the site-directed insertion of the transgenethat does not affect any of the original plant genes, a smaller amount of data for the characterization of the risks may be required compared to a conventional transgenic (Wolt et al., 2016).

Therefore, the genome-editing technique will generate both plant varieties that are not considered GMOs and transgenic plants. This classification depends in part on the country's regulatory framework. For example, Brazil, Argentina, and the United States consider the presence of heterologous genetic material in the final product for it to be classified as transgenic. In contrast, for the European Union a transgenic organism is anything in which the genetic material was altered and did not occur naturally by crossing and/or recombination, regardless of the absence of heterologous DNA in the final product.

Genome editing is a powerful technique to increase genetic variability and generate more productive, nutritious, and tolerant plants to different biotic and abiotic stresses, thus contributing to food security worldwide. Nevertheless, for this to become a reality, a consensus must exist among different countries on genetic manipulation and commercialization of the generated products.

Final considerations

Research is increasingly advancing to improve the edition promoted by the CRISPR/Cas system, and this advance tends to benefit maize breeders a lot. Genome editing can accelerate the improvement of maize and other crops by making precise and predictable changes directly to the alleles of interest. Furthermore, CRISPR technology's modifications introduced into the genome are indistinguishable from those introduced through conventional breeding or chemical or random mutagenesis.

With genome editing, it is possible to manipulate the genetic material to induce mutations in regions of interest so that the organism presents a desirable phenotype. Genome editing technologies allow the manipulation of plant genomes of interest in a non-random manner and are therefore highly specific. Compared to other editing techniques, the CRISPR system has the advantages of being a simple, versatile, and precise technology that enables the improvement of multiple features simultaneously, which speeds up the development of commercial products (Cong et al., 2013; Prado et al., 2020).

Maize genetic improvement has

a great potential to benefit from CRISPR technologies. Compared to other crops, new genetic technologies have been more widely and intensely applied to maize. The rapid advances in maize genome editing could announce a new technological era with a potential trendsetter for all crops.

References

ALOK, A.; SANDHYA, D.; JOGAM, P.; RODRIGUES, V.; BHATI, K. K.; SHARMA, H.; KUMAR, J. The rise of the CRISPR/Cpf1 system for efficient genome editing in plants. **Frontiers in Plant Science**, v. 11, article 264, 2020. DOI: https://dx.doi.org/10.3389%2Ffpls.2020.00264.

ANDERS, C.; NIEWOEHNER, O.; DUERST, A.; JINEK, M. Structural basis of PAM-dependent target DNA recognition by Cas9 endonuclease. **Nature**, v. 513, p. 569-573, 2014. DOI: https://doi.org/10.1038/nature13579.

ANDERSSON, M.; TURESSON, H.; NICOLIA, A.; FALT, A. S.; SAMUELSSON, M.; HOFVANDER, P. Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. **Plant Cell Reports**, v. 36, n. 1, p. 117-128, 2017. DOI: https://doi.org/10.1007/s00299-016-2062-3.

ARPAIA, S.; BIRCH, A. N. E.; CHESSON, A.; DU JARDIN, P.; GATHMANN, A.; GROPP, J.; HERMAN, L.; HOEN-SORTEBERG, H.-G.; JONES, H.; KISS, J.; KLETER, G.; LAGIOU, P.; LOVIK, M.; MESSÉAN, A.; NAEGELI, H.; NIELSEN, K. M.; OVESNA, J.; PERRY, J.; ROSTOKS, N.; TEBBE, C. Scientific opinion addressing the safety assessment of plants developed using zinc finger nuclease 3 and other site-directed nucleases with similar function. **EFSA Journal**, v. 10, n. 10, article, 2943, 2012. Panel on Genetically Modified Organisms.

BAGE, S. A.; BARTEN, T. J.; BROWN, A. N.; CROWLEY, J. H.; DENG, M.; FOUQUET, R.; GOMEZ, J. R.; HATTON, T. W.; LAMB, J. C.; LEDEAUX, J. R.; LEMKE, B. M.; MANJUNATH, S.; MARENGO, M. S.; MORALES, E. Y.; GARCIA, M. O.; PEEVERS, J. M.; PELLET, J.; AVENDANO, A. R.; RYMARQUIS, L. A.; SRIDHARAN, K.; VALENTINE, M. F.; YANG, D. H.; CARGILL, E. J. Genetic characterization of novel and CRISPR-Cas9 gene edited maize *brachytic 2* alleles. **Plant Gene**, v. 21, 100198, 2020. DOI: https://doi.org/10.1016/j.plgene.2019.100198.

BAO, A.; BURRITT, D. J.; CHEN, H.; ZHOU, X.; CAO, D.; TRAN, L.-S. P. The CRISPR/Cas9 system and its applications in crop genome editing. **Critical Reviews in Biotechnology**, v. 39, n. 3, p. 321-336, 2019. DOI: https://doi.org/10.1080/07388551.2018.1554621.

BARONE, P.; WU. E.; LENDERTS, B.; ANAND, A.; GORDON-KAMM, W.; SVITASHEV, S.; KUMAR, S. Efficient gene targeting in maize using inducible CRISPR-Cas9 and marker-free donor template. **Molecular Plant**, v. 13, n. 8, p. 1219-1227, 2020. DOI: https://doi.org/10.1016/j.molp.2020.06.008.

BOLOTIN, A.; QUINQUIS, B.; SOROKIN, A.; EHRLICH, S. D. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extra-chromosomal origin. **Microbiology**, v. 151, n. 8, p. 2551-2561, 2005. DOI: https://doi.org/10.1099/mic.0.28048-0.

CARNEIRO, A. A.; GUIMARÃES, C. T.; VALICENTE, F. H.; WAQUIL, J. M.; VASCONCELOS, M. J. V.; CARNEIRO, N. P.; MENDES, S. M. **Milho Bt**: teoria e prática da produção de plantas transgênicas resistentes a insetos-praga. Sete Lagoas: Embrapa Milho e Sorgo, 2009. 25 p. (Embrapa Milho e Sorgo. Circular Técnica, 135).

CERMÁK, T.; BALTES, N. J.; CEGAN, R.; ZHANG, Y.; VOYTAS, D. F. High frequency, precise modification of the tomato genome. **Genome Biology**, v. 16, article 232, 2015. DOI: https://doi.org/10.1186/s13059-015-0796-9.

CHAPARRO-GARCIA, A.; KAMOUN, S.; NEKRASOV, V. Boosting plant immunity with CRISPR/Cas. **Genome Biology**, v. 16, article 254, 2015. DOI: https://dx.doi.org/10.1186%2Fs13059-015-0829-4.

CHAPMAN, J. R.; TAYLOR, M. R. G.; BOULTON, S. J. Playing the end game: DNA double-strand break repair pathway choice. **Molecular Cell**, v. 47, n. 4, p. 497-510, 2012. DOI: https://doi.org/10.1016/j.molcel.2012.07.029.

CHEN, K.; WANG, Y.; ZHANG, R.; ZHANG, H.; GAO, C. CRISPR/Cas genome editing and precision plant breeding in agriculture. **Annual Review of Plant Biology**, v. 70, p. 667-697, 2019. DOI: https://doi.org/10.1146/annurev-arplant-050718-100049.

CHILCOAT, D.; LIU, Z.-B.; SANDER, J. Use of CRISPR/Cas9 for crop improvement in maize and soybean. In: WEEKS, D. P.; YANG, B. (ed.). **Gene editing in plants**. London: Academic Press, 2017. p. 27-46. (Progress in Molecular Biology and Translational Science, v. 149). DOI: https://doi.org/10.1016/bs.pmbts.2017.04.005.

CIGAN, A. M.; GADLAGE, M. J.; GAO, H.; MEELEY, R. B.; YOUNG, J. K. **Waxy corn**. WO2017132239A1, 3 August 2017. Patent. Available in: https://patents.google.com/patent/. Access in: 10 Oct. 2021.

CONCORDET, J. P.; HAEUSSLER, M. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. **Nucleic Acids Research**, v. 46, n. W1, p. W242-W245, 2018. DOI: https://doi.org/10.1093/nar/gky354.

CONG, L.; RAN, F. A.; COX, D.; LIN, S.; BARRETTO, R.; HSU, P. D.; WU, X.; JIANG, W.; MARRAFFINI, L. A. Multiplex genome engineering using CRISPR/Cas systems. **Science**, v. 339, n. 6121, p. 819-823, 2013. DOI: https://doi.org/10.1126/science.1231143.

CUI, Y.; DONG, H.; MA, Y.; ZHANG, D. Strategies for applying nonhomologous end joining-mediated genome editing in prokaryotes. **ACS Synthetic Biology**, v. 8, n. 10, p. 2194-2202, 2019. DOI: https://doi.org/10.1021/acssynbio.9b00179.

CURTIN, S. J.; VOYTAS, D. F.; STUPAR, R. M. Genome engineering of crops with designer nucleases. **Plant Genome**, v. 5, n. 2, p. 42-50, 2012. DOI: https://doi.org/10.3835/plantgenome2012.06.0008.

DELTCHEVA, E.; CHYLINSKI, K.; SHARMA, C. M.; GONZALES, K.; CHAO, Y.; PIRZADA, Z. A.; ECKERT, M. R.; VOGEL, J.; CHARPENTIER, E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. **Nature**, v. 471, n. 7340, p. 602-607, 2011. DOI: https://doi.org/10.1038/nature09886.

DEVEAU, H.; BARRANGOU, R.; GARNEAU, J. E.; LABONTÉ, J.; FREMAUX, C.; BOYAVAL, P.; ROMERO, D. A.; HORVATH, P.; MOINEAU, S. Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. **Journal of Bacteriology**, v. 190, n. 4, p. 1390-1400, 2008. DOI: https://doi.org/10.1128/jb.01412-07.

DOUDNA, J. A.; CHARPENTIER, E. The new frontier of genome engineering with CRISPR-Cas9. **Science**, v. 346, n. 6213, 1258096, 2014. DOI: https://doi.org/10.1126/science.1258096.

FAUSER, F.; ROTH. N.; PACHER, M.; ILG, G.; SÁNCHEZ-FERNANDEZ, R.; BIESGEN, C.; PUCHTA, H. In planta gene targeting. **Proceedings of the National Academy of Sciences of the United States of America**, v. 109, n. 19, p. 7535-7540, 2012. DOI: https://doi.org/10.1073/pnas.1202191109.

FENG, C.; SU, H.; BAI, H.; WANG, R.; LIU, Y.; GUO, X.; LIU, C.; ZHANG, J.; YUAN, J.; BIRCHLER, J. A.; HAN, F. High-efficiency genome editing using a dmc1 promoter-controlled CRISPR/Cas9 system in maize. **Plant Biotechnology Journal**, v. 16, n. 11, p. 1848-1857, 2018. DOI: https://dx.doi.org/10.1111%2Fpbi.12920.

FRAME, B. R.; SHOU, H.; CHIKWAMBA, R. K.; ZHANG, Z.; XIANG, C.; FONGER, T. M.; PEGG, E. K.; LI, B.; NETTLETON, D. S.; PEI, D.; WANG, K. *Agrobacterium tumefaciens*: mediated transformation of maize embryos using a standard binary vector system. **Plant Physiology**, v. 129, n. 1, p. 13-22, 2002. DOI: https://doi.org/10.1104/pp.000653.

FU, Y.; SANDER, J. D.; REYON, D.; CASCIO, V. M.; JOUNG, J. K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. **Nature Biotechnology,** v. 32, p. 279-284, 2014. DOI: https://doi.org/10.1038/nbt.2808.

GAO, H.; MUTTI, J.; YOUNG, J. K.; YANG, M.; SCHRODER, M.; LENDERTS, B.; WANG, L.; PETERSON, D.; ST. CLAIR, G.; JONES, S.; FEIGENBUTZ, L.; MARSH, W.; ZHENG, M.; WAGNER, S.; FARRELL, J.; SNOPEK, K.; SCELONGE, C.; SOPKO, X.; SANDER, J. D.; BETTS, S.; CIGAN, A. M.; CHILCOAT, N. D. Complex trait loci in maize enabled by CRISPR-

Cas9 mediated gene insertion. **Frontiers in Plant Science**, v. 11, article 535, 2020. DOI: https://dx.doi.org/10.3389%2Ffpls.2020.00535.

GAO, H.; SMITH, J.; YANG, M.; JONES, S.; DJUKANOVIC, V.; NICHOLSON, M. G.; WEST, A.; BIDNEY, D.; FALCO, S. C.; JANTZ, D.; LYZNIK, L. A. Heritable targeted mutagenesis in maize using a designed endonuclease. **Plant Journal**, v. 61, n. 1, p. 176-187, 2010. DOI: https://doi.org/10.1111/j.1365-313x.2009.04041.x.

GAO, Y.; ZHANG, Y.; ZHANG, D.; DAI, X.; ESTELLE, M.; ZHAO, Y. Auxin binding protein 1 (ABP1) is not required for either auxin signaling or *Arabidopsis* development. **Proceedings of the National Academy of Sciences of the United States of America**, v. 112, n. 7, p. 2275-2280, 2015. DOI: https://doi.org/10.1073/pnas.1500365112.

GARCIA, M. O.; PEEVERS, J. M.; PELLET, J.; AVENDANO, A. R.; RYMARQUIS, L. A.; SRIDHARAN, K.; VALENTINE, M. F.; YANG, D. H.; CARGILL, E. J. Genetic characterization of novel and CRISPR-Cas9 gene edited maize brachytic 2 alleles. **Plant Gene**, v. 21, 100198, 2020. DOI: https://doi.org/10.1016/j.plgene.2019.100198.

GELVIN, S. B. Plant proteins involved in Agrobacterium-mediated genetic transformation. **Annual Review of Phytopathology**, v. 48, p. 45-68, 2010. DOI: https://doi.org/10.1146/annurev-phyto-080508-081852.

GORDON-KAMM, W.; BARONE, P.; SVITASHEV, S.; SANDER, J. D.; KUMAR, S.; JONES, T. Strategies for CRISPR/Cas9-mediated genome editing: from delivery to production of modified plants. In: WILLMANN, M. R. (ed.). **Genome editing for precision crop breeding**. Cambridge: Burleigh Dodds Science Publishing, 2021. p. 195-230. DOI: http://dx.doi.org/10.19103/AS.2020.0082.12.

HILLE, F.; RICHTER, H.; WONG, S. P.; BRATOVIC, M.; RESSEL, S.; CHARPENTIER, E. The biology of CRISPR-Cas: backward and forward. **Cell**, v. 172, n. 6, p. 1239-1259, 2018. DOI: https://doi.org/10.1016/j.cell.2017.11.032.

HILTON, I. B.; GERSBACH, C. A. Enabling functional genomics with genome engineering. **Genome Research**, v. 25, n. 10, 1442-1455, 2015. DOI: https://doi.org/10.1101/gr.190124.115.

HIOM, K. Coping with DNA double strand breaks. **DNA Repair**, v. 9, n. 12, p. 1256-1263, 2010. DOI: https://doi.org/10.1016/j.dnarep.2010.09.018.

HOU, Z.; ZHANG, Y.; PROPSON, N. E.; HOWDEN, S. E.; CHU, L. F.; SONTHEIMER, E. J.; THOMSON, J. A. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. **Proceedings of the National Academy of Sciences of the United States of America**, v. 110, n. 39, p. 15644-15649, 2013. DOI: https://doi.org/10.1073/pnas.1313587110.

HUANG, C.; SUN, H.; XU, D.; CHEN, Q.; LIANG, Y.; WANG, X.; XU, G.; TIAN, J.; WANG, C.; LI, D.; WU, L.; YANG, X.; JIN, W.; DOEBLEY, J. F.; TIAN, F. ZmCCT9 enhances maize adaptation to higher latitudes. **Proceedings of the National Academy of Sciences of the United States of America**, v. 115, n. 2, p. E334-E341, 2018. DOI: https://doi.org/10.1073/pnas.1718058115.

ISHINO, Y.; SHINAGAWA, H.; MAKINO, K.; AMEMURA, M.; NAKATA, A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. **Journal of Bacteriology**, v. 169, n. 12, p. 5429-5433, 1987. DOI: https://doi.org/10.1128/jb.169.12.5429-5433.1987.

JANSEN, R.; VAN EMBDEN, J.; GAASTRA, W.; SCHOULS, L. Identification of genes that are associated with DNA repeats in prokaryotes. **Molecular Microbiology**, v. 43, n. 6, p. 1565-1575, 2002. DOI: https://doi.org/10.1046/j.1365-2958.2002.02839.x.

JANSING, J.; SACK, M.; AUGUSTINE, S. M.; FISCHER, R.; BORTESI, L. CRISPR/Cas9-mediated knockout of six glycosyltransferase genes in *Nicotiana benthamiana* for the production of recombinant proteins lacking beta-1,2-xylose and core alpha-1,3-fucose. **Plant Biotechnology Journal**, v. 17, n. 2, p. 350-361, 2018. DOI: https://doi.org/10.1111/pbi.12981.

JANSING, J.; SCHIERMEYER, A.; SCHILLBERG, S.; FISCHER, R.; BORTESI, L. Genome editing in agriculture: technical and practical considerations. **International Journal of Molecular Sciences**, v. 20, n. 12, article 2888, 2019. DOI: https://doi.org/10.3390/ijms20122888.

JANSSEN, B. J.; GARDNER, R. C. Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. **Plant Molecular Biology**, v. 14, p. 61-72, 1990. DOI: https://doi.org/10.1007/bf00015655.

JIANG, W.; MARRAFINI, L. A. CRISPR-Cas: new tools for genetic manipulations from bacterial immunity systems. **Annual Review of Microbiology**, v. 69, p. 209-228, 2015. DOI: https://doi.org/10.1146/annurev-micro-091014-104441.

JIN, M.-H.; TAO, J.-H.; LI, Q.; CHENG, Y.; SUN, X.-X.; WU, K.-M.; XIAO, Y.-T. Genome editing of the SfABCC2 gene confers resistance to Cry1F toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda*. **Journal of Integrative Agriculture**, v. 20, n. 3, p. 815-820, 2021. DOI: https://doi.org/10.1016/S2095-3119(19)62772-3.

JINEK, M.; CHYLINSKI, K.; FONFARA, I.; HAUER, M.; DOUDNA, J. A.; CHARPENTIER, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. **Science**, v. 337, n. 6096, p. 816-821, 2012. DOI: https://doi.org/10.1126/science.1225829.

KELLIHER, T.; STARR, D.; SU, X.; TANG, G.; CHEN, Z.; CARTER, J.; WITTICH, P.

E.; DONG, S.; GREEN, J.; BURCH, E.; McCUISTON, J.; GU, W.; SUN, Y.; STREBE, T.; ROBERTS, J.; BATE, N. J.; QUE, Q. One-step genome editing of elite crop germplasm during haploid induction. **Nature Biotechnology**, v. 37, p. 287-292, 2019. DOI: https://doi.org/10.1038/s41587-019-0038-x.

KLEIN, T. M.; ARENTZEN, R.; LEWIS, P.; MECELLIGOT, S. F. Transformation of microbes, plants, and animals by particle bombardment. **Nature Biotechnology**, v. 10, p. 286-291, 1992. DOI: https://doi.org/10.1038/nbt0392-286.

KLEIN, T. M.; WOLF, E. D.; WU, R.; SANFORD, J. C. High-velocity microprojectiles for delivering nucleic acids into living cells. **Nature**, v. 327, p. 70-73, 1987. DOI: https://doi.org/10.1038/327070a0.

LABUN, K.; MONTAGUE, T. G.; KRAUSE, M.; CLEUREN, Y. N. T.; TJELDNES, H.; VALEN, E. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. **Nucleic Acids Research**, v. 47, p. 171-174, 2019. DOI: http://dx.doi.org/10.1093/nar/gkz365.

LACROIX, B.; CITOVSKY, V. The roles of bacterial and host plant factors in *Agrobacterium* mediated genetic transformation. **International Journal of Developmental Biology**, v. 57, n. 6/8, p. 467-481, 2013. DOI: https://doi.org/10.1387/ijdb.130199bl.

LI, C.; LIU, C.; QI, X.; WU, Y.; FEI, X.; MAO, L.; CHENG, B.; LI, X.; XI, C. RNA-guided Cas9 as an *in vivo* desired-target mutator in maize. **Plant Biotechnology Journal**, v. 15, n. 12, p. 1566-1576, 2017. DOI: https://doi.org/10.1111/pbi.12739.

LI, J. F.; NORVILLE, J. E.; AACH, J.; MCCORMACK, M.; ZHANG, D.; BUSH, J.; CHURCH, G. M.; SHEEN, J. Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. **Nature Biotechnology**, v. 31, p. 688-691, 2013. DOI: https://doi.org/10.1038/nbt.2654.

LIANG, Z.; ZHANG, K.; CHEN, K.; GAO, C. Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. **Journal of Genetics and Genomics**, v. 41, n. 2, p. 63-68, 2014. DOI: https://doi.org/10.1016/j.jgg.2013.12.001.

LIN, Q.; ZHU, Z.; LIU, G.; SUN, C.; LIN, D.; XUE, C.; LI, S.; ZHANG, D.; GAO, C.; WANG, Y.; QIU, J.-L. Genome editing in plants with MAD7 nuclease. **Journal of Genetics and Genomics**, v. 48, n. 6, p. 444-451, 2021. DOI: https://doi.org/10.1016/j.jgg.2021.04.003.

LIU, H. J.; JIAN, L.; XU, J.; ZHANG, Q.; ZHANG, M.; JIN, M.; PENG, Y.; YAN, J.; HAN, B.; LIU, J. et al. High-throughput CRISPR/Cas9 mutagenesis streamlines trait gene identification in maize. **Plant Cell**, v. 32, n. 5, p. 1397-1413, 2020. DOI: https://doi.org/10.1105/tpc.19.00934.

LIU, L.; GALLAGHER, J.; AREVALO, E. D.; CHEN, R.; SKOPELITIS, T.; WU, Q.;

BARTLETT, M.; JACKSON, D. Enhancing grain-yield-related traits by CRISPR-Cas9 promoter editing of maize CLE genes. **Nature Plants**, v. 7, p. 287-294, 2021. DOI: https://doi.org/10.1038/s41477-021-00858-5.

LOPES FILHO, J. H.; SILVA, V. C. H. da; SANTOS, J. C. dos; DANTE, R. A.; GERHARDT, I. R.; YASSITEPE, J. E. C. T.; FERNANDES, F. R. Introdução à edição genômica em plantas. In: MOLINARI, H. B. C.; VIEIRA, L. R.; SILVA, N. V. e; PRADO, G. S.; LOPES FILHO, J. H. (ed.). **Tecnologia CRISPR na edição genômica de plantas**: biotecnologia aplicada à agricultura. Brasília, DF: Embrapa, 2020. p. 11-48.

LOWE, K.; LA ROTA, M.; HOERSTER, G.; HASTINGS, G.; WANG, N.; CHAMBERLIN, M.; WU, E.; JONES, T.; GORDON-KAMM, W. Rapid genotype "independent" *Zea mays* L. (maize) transformation via direct somatic embryogenesis. **In Vitro Cellular & Developmental Biology - Plant**, v. 54, p. 240-252, 2018. DOI: https://doi.org/10.1007/s11627-018-9905-2.

LOWE, K.; WU, E.; WANG, N.; HOERSTER, G.; HASTINGS, C.; CHO, M.-J.; SCELONGE, C.; LENDERTS, B.; CHAMBERLIN, M.; CUSHATT, J.; WANG, L.; RYAN, L.; KHAN, T.; CHOW-YIU, J.; HUA, W.; YU, M.; BANH, J.; BAO, Z.; BRINK, K.; IGO, E.; RUDRAPPA, B.; SHAMSEER, P. M.; BRUCE, W.; NEWMAN, L.; SHEN, B.; ZHENG, P.; BIDNEY, D.; FALCO, C.; REGISTER, J.; ZHAO, Z.-Y.; XU, D.; JONES, T.; GORDON-KAMM, W. Morphogenic regulators *baby boom* and *wuschel* improve monocot transformation. **The Plant Cell**, v. 28, n. 9, p. 1998-2015, 2016. DOI: https://doi.org/10.1105/tpc.16.00124.

LUSSER, M.; DAVIES, H. V. Comparative regulatory approaches for groups of new plant breeding techniques. **New Biotechnology**, v. 30, n. 5, p. 437-446, 2013. DOI: https://doi.org/10.1016/j.nbt.2013.02.004.

MAEDER, M. L.; THIBODEAU-BEGANNY, S.; OSIAK, A.; WRIGHT, D. A.; ANTHONY, R. M.; EICHTINGER, M.; JIANG, T.; FOLEY, J. E.; WINFREY, R. J.; TOWNSEND, J. A. et al. Rapid "open- -source" engineering of customized zinc-finger nucleases for highly efficient gene modification. **Molecular Cell**, v. 31, p. 294-301, 2008. DOI: https://dx.doi.org/10.1016%2Fj.molcel.2008.06.016.

MOJICA, F. J. M.; DÍEZ-VILLASEÑOR, C.; GARCÍA-MARTÍNEZ, J.; SORIA, E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. **Journal of Molecular Evolution**, v. 60, n. 2, p. 174-182, 2005. DOI: https://doi.org/10.1007/s00239-004-0046-3.

MOJICA, F. J.; DÍEZ-VILLASEÑOR, C.; SORIA, E.; JUEZ, G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. **Molecular Microbiology**, v. 36, n. 1, p. 244-246, 2000. DOI: https://doi.org/10.1046/j.1365-2958.2000.01838.x.

MUSHTAQ, M.; DAR, A. A.; BASU, U.; BATH, B. A.; MIR, R. A.; VATS, S.; DAR, M. S.; TYAGI,

A.; ALI, S.; BANSAL, M.; RAI, G. K.; WANI, S. H. Integrating CRISPR-Cas and next generation sequencing in plant virology. **Frontiers in Genetics**, v. 12, article 735489, 2021. DOI: https://doi.org/10.3389/fgene.2021.735489.

NUCCIO, M. L.; CLAEYS, H.; HEYNDRICKX, K. S. CRISPR-Cas technology in corn: a new key to unlock genetic knowledge and create novel products. **Molecular Breeding**, v. 41, article 11, 2021. DOI: https://doi.org/10.1007/s11032-021-01200-9.

PRADO, G. S.; PINHEIRO, T. T.; FARIA, J. C. de; VIANELLO, R. P. Edição de genoma via non-homologous end joining (NHEJ) e ribonucleoproteínas (RNP). In: MOLINARI, H. B. C.; VIEIRA, L. R.; SILVA, N. V. e; PRADO, G. S.; LOPES FILHO, J. H. (ed.). **Tecnologia CRISPR na edição genômica de plantas**: biotecnologia aplicada à agricultura. Brasília, DF: Embrapa, 2020. p. 49-90.

RAN, F. A.; HSU, P. D.; LIN, C. Y.; GOOTENBERG, J. S.; KONERMANN, S.; TREVINO, A. E.; SCOTT, D. A.; INOUE, A.; MATOBA, S.; ZHANG, Y.; ZHANG, F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. **Cell**, v. 154, n. 6, p. 1380-1389, 2013. DOI: https://doi.org/10.1016/j.cell.2013.08.021.

SAMPSON, T. R.; WEISS, D. S. CRISPR-Cas systems:new players in gene regulation and bacterial physiology. **Frontiers in Cellular and Infection Microbiology**, v. 4, article 37, 2014. DOI: https://dx.doi.org/10.3389%2Ffcimb.2014.00037.

SANFORD, J. C.; KLEIN, T. M.; WOLF, E. D.; ALLEN, N. Delivery of substances into cells and tissue using a particle bombardment process. **Particulate Science and Technology**, v. 5, n. 1, p. 27-37, 1987. DOI: https://doi.org/10.1080/02726358708904533.

SHI, J.; GAO, H.; WANG, H.; LAFITTE, H. R.; ARCHIBALD, R. L.; YANG, M.; HAKIMI, S. M.; MO, H.; HABBEN, J. E. ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. **Plant Biotechnology Journal**, v. 15, n. 2, p. 207-216, 2017. DOI: https://doi.org/10.1111/pbi.12603.

SHUKLA, V. K.; DOYON, Y.; JEFFREY, C.; MILLER, J. C.; DEKELVER, R. C.; MOEHLE, E. A.; WORDEN, S. E.; MITCHELL, J. C.; ARNOLD, N. L.; GOPALAN, S.; MENG, X.; CHOI, V. M.; ROCK, J. M.; WU, Y. Y.; KATIBAH, G. E.; ZHIFANG, G.; MCCASKILL, D.; SIMPSON, M. A.; BLAKESLEE, B.; GREENWALT, S. A.; BUTLER, H. J.; HINKLEY, S. J.; ZHANG, L.; REBAR, E. J.; GREGORY, P. D.; URNOV, F. D. Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. **Nature**, v. 459, p. 437-441, 2009. DOI: https://doi.org/10.1038/nature07992.

SMITH, J.; GRIZOT, S.; ARNOULD, S.; DUCLERT, A.; EPINAT, J. C.; CHAMES, P.; PRIETO, J.; REDONDO, P.; BLANCO, F. J.; BRAVO, J.; MONTOYA, G.; PÂQUES, F.; DUCHATEAU, P. A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. **Nucleic Acids Research**, v. 34, n. 22, e149, 2006. DOI: https://dx.doi.org/10.1093%2Fnar%2Fgkl720.

SILVA, R. S.; KAGUE, E.; PEREIRA, T. C.; GONÇALVES, N. N.; AMBRÓSIO, C. E. Obtenção de Cas9 e gRNAs. In: PEREIRA, T. C. (org.). **Introdução à técnica de CRISPR**. Ribeirão Preto: Sociedade Brasileira de Genética, 2016. p. 109-133.

SVITASHEV, S.; SCHWARTZ, C.; LENDERS, B.; YOUNG, J. K.; CIGAN, A. M. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. **Nature Communications**, v. 7, article 13274, 2016. DOI: https://doi.org/10.1038/ncomms13274.

SVITASHEV, S.; YOUNG, J. K.; SCHWARTZ, C.; GAO, H.; FALCO, S. C.; CIGAN, A. M. Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. **Plant Physiology**, v. 169, n. 2, p. 931-945, 2015. DOI: https://doi.org/10.1104/pp.15.00793.

VEGA, J. M.; YU, W.; KENNON, A. R.; CHEN, X.; ZHANG, Z. J. Improvement of Agrobacterium-mediated transformation in Hi-II maize (*Zea mays*) using standard binary vectors. **Plant Cell Reports**, v. 27, n. 2, p. 297-305, 2008. DOI: https://doi.org/10.1007/s00299-007-0463-z.

WANG, H.; YAN, S.; XIN, H.; HUANG, W.; ZHANG, H.; TENG, S.; YU, Y.-C.; FERNIE, A. R.; LU, X.; LI, P.; LI, S.; ZHANG, C.; RUAN, Y.-L.; CHEN, L.-Q.; LANG, Z. A subsidiary cell-localized glucose transporter promotes stomatal conductance and photosynthesis. **Plant Cell**, v. 31, n. 6, p. 1328-1343, 2019. DOI: https://doi.org/10.1105/tpc.18.00736.

WOLT, J. D.; WANG, K.; YANG, B. The regulatory status of genome-edited crops. **Plant Biotechnology Journal**, v. 14, n. 2, p. 510-518, 2016. DOI: https://doi.org/10.1111/pbi.12444.

WOO, J. W.; KIM, J.; KWON, S. I.; CORVALÁN, C.; CHO, S. W.; KIM, H.; KIM, S.-G.; KIM, S.-T.; CHOE, S.; KIM, J.-S. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. **Nature Biotechnology**, v. 33, p. 1162-1164, 2015. DOI: https://doi.org/10.1038/nbt.3389.

WU, X.; SCOTT, D. A.; KRIZ, A. J.; CHIU, A. C.; HSU, P. D.; DADON, D. B.; CHENG, A. W.; TREVINO, A. E.; KPNERMANN, S.; CHEN, S.; JAENISCH, R.; ZHANG, F.; SHARP, P. A. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. **Nature Biotechnology**, v. 32, n. 7, p. 670-676, 2014.

XIE, K.; WU, S.; LI, Z.; ZHOU, Y.; ZHANG, D.; DONG, Z.; NA, X.; ZHU, T.; ZHANG, S.; LIU, S.; LI, J.; WAN, X. Map-based cloning and characterization of *Zea mays* male sterility33 (ZmMs33) gene, encoding a glycerol-3-phosphate acyltransferase. **Theoretical and Applied Genetics**, v. 131, n. 6, p. 1363-1378, 2018. DOI: https://doi.org/10.1007/s00122-018-3083-9.

YAN, Y.; ZHU, J.; QI, X.; CHENG, B.; LIU, C.; XIE, C. Establishment of an efficient seed fluorescence reporter-assisted CRISPR/Cas9 gene editing in maize. **Journal of Integrative Plant Biology**, v. 63, n. 9, p. 1671-1680, 2021. DOI: https://doi.org/10.1111/jipb.13086.

YOUNG, J.; ZASTROW-HAYES, G.; DESCHAMPS, S.; SVITASHEV, S.; ZAREMBA, M.; ACHARYA, A.; PAULRAJ, S.; PETERSON-BURCH, B.; SCHWARTZ, C.; DJUKANOVIC, V.; LENDERTS, B.; FEIGENBUTZ, L.; WANG, L.; ALARCON, C.; SIKSNYS, V.; MAY, G.; CHILCOAT, N. D.; KUMAR, S. CRISPR-Cas9 editing in maize: systematic evaluation of off-target activity and its relevance in crop improvement. **Scientific Reports**, v. 9, article 6729, 2019. DOI: https://doi.org/10.1038/s41598-019-43141-6.

ZAIDI, S. S.-e-A.; MAHAS, A.; VANDERSCHUREN, H.; MAHFOUZ, M. M. Engineering crops of the future: CRISPR approaches to develop climate-resilient and disease-resistant plants. **Genome Biology**, v. 21, article 289, 2020. DOI: https://doi.org/10.1186/s13059-020-02204-y.

ZHANG, Y.; IAFFALDANO, B.; QI, Y. CRISPR ribonucleoprotein-mediated genetic engineering in plants. **Plant Communications**, v. 2, n. 2, article 100168, 2021a. DOI: https://doi.org/10.1016/j.xplc.2021.100168.

ZHANG, D.; ZHANG, Z.; UNVER, T.; ZHANG, B. CRISPR/Cas: a powerful tool for gene function study and crop improvement. **Journal of Advanced Research**, v. 29, p. 207-221, 2021b. DOI: https://doi.org/10.1016/j.jare.2020.10.003.

ZHANG, H.; ZHANG, J.; WEI, P.; ZHANG, B.; GOU, F.; FENG, Z.; MAO, Y.; YANG, L.; ZHANG, H.; XU, N.; ZHU, J. K. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. **Plant Biotechnology Journal**, v. 12, n. 6, p. 797-807, 2014. DOI: https://doi.org/10.1111/pbi.12200.

ZHANG, Y.; LIANG, Z.; ZONG, Y.; WANG, Y.; LIU, J.; CHEN, K.; QIU, J.-L.; GAO, C. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. **Nature Communications**, v. 7, article 12617, 2016. DOI: https://doi.org/10.1038/ncomms12617.

ZHU, L. J. Overview of guide RNA design tools for CRISPR-Cas9 genome editing technology. **Frontiers in Biology**, v. 10, p. 289-296, 2015. DOI:https://doi.org/10.1007/s11515-015-1366-y.