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Genome editing: propelling the next generation of crop improvement

Jéssika Angelotti-Mendonça[1*], Alessandra Koltun[2*], Fernanda Freitas de Oliveira[2], Nathalia Volpi e Silva[1]

[1]Empresa Brasileira de Pesquisa Agropecuária - Embrapa Soja, Londrina, PR. [2]Universidade Estadual de Maringá – UEM, PR. E-mail: nathvolpi.lbi@gmail.com
* JA-M and AK contributed equally to this work.

Abstract
Climate change and population size records threaten food security. Therefore, the call for a more sustainable and efficient crop production has never been more urgent. Traditional plant breeding was one of the first successful approaches to expand cultivation areas and crop yield. Later, biotechnological tools and their products, such as genetically modified organisms containing exogenous DNA, further broadened the limits of agricultural results, yet bringing huge financial, bureaucratic, and public rejection hurdles. In the 90s, scientific advances brought the opportunity to drive mutations using engineered nucleases, and since 2013 CRISPR-Cas has emerged as the most practical toolkit to edit genomes. One of the most striking possibilities is to generate edited and non-transgenic plants. In this review, we present the working mechanism behind CRISPR-induced mutations and pinpoint the latest techniques developed, as well as its myriad of applications in agriculture. The enhancing scope of CRISPR ranges from introducing traits of agronomic interest – such as herbicide resistance, resistance/tolerance to biotic and abiotic stresses, and quality and durability of products – to accelerating plant breeding processes, including haploid induction, generating male-sterile lines, fixating hybrid vigor, and overcoming self-incompatibility. We also discuss regulatory issues surrounding edited plants and derived products around the world, challenges that must be overcome, and future prospects to harness all the potential of this amazing tool to guarantee the new crop production revolution.
Keywords: CRISPR-Cas; engineered nucleases; targeted mutations; precision plant breeding; agriculture.

Edição genômica: impulsionando a nova geração do melhoramento de plantas

Resumo
As mudanças climáticas e números recordes de população ameaçam a segurança alimentar. Portanto, o apelo por uma produção agrícola mais sustentável e eficiente nunca foi tão urgente. O melhoramento genético tradicional foi uma das primeiras abordagens bem-sucedidas para expandir as áreas de cultivo e o rendimento das safras. Posteriormente, ferramentas biotecnológicas e seus produtos, como organismos geneticamente modificados contendo DNA exógeno, ampliaram ainda mais os limites dos resultados agrícolas, apesar de ainda carregarem enormes obstáculos financeiros, burocráticos e de rejeição pública. Na década de 90, os avanços científicos trouxeram a oportunidade de conduzir mutações usando nucleases projetadas e, desde 2013, CRISPR-Cas surgiu como o kit de ferramentas mais prático para editar genomas. Uma das possibilidades mais marcantes é gerar plantas editadas e não transgênicas. Nesta revisão, apresentamos o mecanismo de ação por trás das mutações induzidas por CRISPR, identificando as últimas técnicas desenvolvidas, bem como sua miríade de aplicações na agricultura. O escopo de aprimoramento do CRISPR varia desde introduzir características de interesse agronômico – como resistência a herbicidas, resistência/tolerância a estresses bióticos e abióticos e qualidade e durabilidade de produtos – até acelerar processos de melhoramento genético de plantas, incluindo indução de haploidia, geração de linhagens macho-estéreis, fixação de vigor híbrido e superação da autoincompatibilidade. Também discutimos questões regulatórias em torno de plantas editadas e produtos derivados mundialmente, desafios que devem ser superados e perspectivas futuras para aproveitar todo o potencial desta ferramenta incrível para garantir a nova revolução na produção de culturas agrícolas.
Palavras-chave: CRISPR-Cas; nucleases projetadas; mutações direcionadas; melhoramento de plantas de precisão; agricultura.

1. Introduction

Since its discovery more than 10,000 years ago, agriculture has allowed civilization to exist and evolve. The need for improving the efficiency of agricultural systems to produce food and energy for an ever-growing population drove humans to create increasingly sophisticated techniques in vast areas. Among the strategies to push agriculture limits further, plant breeding has played a crucial role, setting new records regarding yield and tolerance to biotic and abiotic stressors. Crop improvement has a lively history and can be divided into 4 major benchmarks: traditional breeding, induced mutagenesis, genetically modified organisms (GMOs), and genome editing (GAO, 2021).

Traditional plant breeding aims to combine desirable traits through crossings. Although it has brought crucial advances for agriculture, it relies on and is limited to the genetic variability present in the species of interest and related crossable species, in addition to being laborious and time-consuming. Induced mutagenesis came to boost the naturally occurring mutation rates and create genetic variability that could occasionally result in a desired trait. However, the screening of large populations to find the plant harboring the trait of interest derived from the random mutation makes this strategy unfeasible for highly complex agronomic traits (HOLME *et al.*, 2019).

The advent of recombinant DNA technology has made it possible to isolate and clone genes from the most diverse organisms, overcoming isolation barriers between species and even kingdoms. These exogenous DNA fragments can be introduced and expressed in plants, opening up a wide range of possibilities for the genetic improvement of crops (ANAMI *et al.*, 2013). GMOs have contributed to obtaining superior varieties, leading to higher yields, herbicide and pest resistance, and improved nutrition. Even though this technique broadened plant breeding, the organisms harboring randomly integrated foreign DNA are subjected to strict government regulations that are highly onerous to follow, strongly limiting the potential parties involved in GM crop creation and commercialization and the range of products available (RAMAN, 2017). The misled public opinion also hampers the full potential of GMOs (BRADFORD *et al.*, 2005).

Genome editing has emerged as the most recent breakthrough for basic and applied plant research with the rise of RNA-guided nucleases. The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR associated proteins) system represents its most advanced yet practical and affordable toolkit, allowing researchers to delete, replace, or insert specific DNA sequences in a predictable and targeted way (FENG *et al.*, 2013; SHAN *et al.*, 2013). It greatly expanded the initial genome engineering scope from GMO in terms of manipulating traits, and it offers DNA-free routes (WOO *et al.*, 2015) or that leaves no traces of foreign DNA (HE; ZHAO, 2020), possibly circumventing the burden of lengthy approval processes, depending on the path legislation takes.

New and genius spined-off techniques are being rapidly created and refined (ANZALONE *et al.*, 2019; KATREKAR *et al.*, 2019). These increasingly more effective and time-saving techniques have already been applied to upgrade a variety of crops (SHIMATANI *et al.*, 2017; ZONG *et al.*, 2017; CHEN *et al.*, 2019b; LIN *et al.*, 2020; ZHANG *et al.*, 2020b; ZHU *et al.*, 2020), unimaginably expanding the horizon of possibilities in this field and giving rise to the next generation of crop improvement.

Scientific breakthroughs and technological innovations in crop production, such as genome editing aligned with modern plant breeding techniques, are urgently needed to secure global food status. Our current system is collapsing and will not be able to feed ~10 billion people, a population number that will be reached in a not very far period – 2050 (FAO, 2017; SPRINGMANN *et al.*, 2018). Moreover, the global environmental situation leads to seeking more conscious, sustainable, and holistic approaches.

In this review, we will cover **1)** an introduction to the CRISPR genome-editing technique in plants, **2)** practical applications in crop improvement, **3)** regulatory issues, **4)** challenges and prospects of this outstanding novelty regarding the agricultural scenario.

2. The CRISPR-cas system and cas variations

As a biotechnological tool, the main used CRISPR-Cas system has two components: the CRISPR-associated (Cas) nuclease, with Cas9 from *Streptococcus pyogenes* being the most

commonly used, and the guide RNA (gRNA); both forming the Cas9:gRNA complex (JINEK *et al.* 2012; DOUDNA; CHARPENTIER 2014). Cas9 induces DNA double-strand breaks (DSBs) guided by a base-paired structure formed with a chimeric gRNA, resulting from the fusion of a trans-activating CRISPR RNA (tracrRNA), with structural purposes, and the CRISPR RNA (crRNA), containing a 20-nt complementary sequence to the target DNA (JINEK *et al.*, 2012; DOUDNA; CHARPENTIER, 2014). Thereby, Cas9 recognizes the target region based on the base-pairing complementarities with the gRNA. In addition, for the system to function, the target DNA must be near a protospacer adjacent motif (PAM) that varies according to the nuclease. For instance, Cas9 recognizes the PAM sequence NGG found directly downstream of the target DNA on the non-target strand (ANDERS *et al.*, 2014). The cleaving properties of the RNA-guided endonucleases lie on two catalytic domains: HNH and RuvC, each cutting a DNA strand (FURUHATA; KATO, 2021). The CRISPR-Cas system goes as far as creating the DSBs in a targeted and precise manner. Then, the cellular DNA repair pathways are accounted to try and repair the damaged DNA, eventually creating or introducing a mutation.

Plant cell machinery counts on two main pathways to repair DSBs in somatic cells: non-homologous end-joining (NHEJ) and homologous recombination (HR). DSBs created by the CRISPR-Cas system may be repaired by one of these mechanisms and when the route chosen is the error-prone system NHEJ, mutations may be generated in a specific site but in a random manner (PUCHTA *et al.*, 1993, 1996; GORBUNOVA; LEVY, 1997; SALOMON; PUCHTA, 1998). These mutations can be small deletions or insertions, also known as indels, and may change the gene reading frame, affecting the correct protein translation (VOYTAS; GAO, 2014). Usually, when aiming to knockout a gene or generate small mutations, without the need for specific changes in the sequence, the strategy is designed focusing on NHEJ. It represents an efficient approach considering that NHEJ is the preferred repair pathway in plant somatic cells, and it only requires the delivery of a nuclease and one or more gRNAs (PUCHTA, 2005; JINEK *et al.*, 2012). CRISPR-Cas can also be designed to make two DSBs simultaneously, leading to complete deletion of a target gene or fragment (VOYTAS; GAO, 2014), or even to target multiple sequences at the same time. For instance, several genes from a metabolic pathway may be mutated simultaneously (LI *et al.*, 2018; SÁNCHEZ-LEÓN *et al.*, 2018).

As for the HR repair pathway, it enables the insertion of large DNA sequences or even to perform specific changes in the target DNA. Although this system is less efficient than NHEJ, it brings the possibility to induce more accurate changes in the genome, magnifying the possibilities of manipulating plant metabolism, physiology, and products. The HR repair pathway uses homologous templates to repair the DSBs, usually the corresponding sister-chromatid. When manipulating this system to our favor, we provide a donor template to the cell containing the desired mutation and flanked by sequences that are similar to the DSBs surroundings, aiming to mimic a sister chromatid and trick the cell (HUANG; PUCHTA, 2019). Thereby, the HR pathway may use the provided donor instead of the sister-chromatid to repair the DSB, which usually happens more efficiently when the donor sequences provided are abundant. As the researcher can design different types of donor templates, HR offers several possibilities for manipulating plant genomes with a high level of accuracy, such as targeted gene knock-in, alterations of key amino acid residues within a gene's coding sequence, or specific changes in promoter elements or other cis-acting motifs that modulate gene expression (VOYTAS; GAO, 2014). Collectively, these findings make the CRISPR-Cas system a powerful tool for genome editing considering the high frequency of PAM in genomes, besides different PAMs required by other nucleases; theoretically, any gene could be engineered.

Even though Cas9 is the most used nuclease to date, other nucleases are available to be used as part of the CRISPR-Cas genome editing tool, such as Cas12a and Cas13. Cas12a is structurally similar to Cas9, also presenting a RuvC-like domain. However, instead of the HNH domain, it possesses a Nuc domain (SCHINDELE *et al.*, 2018). In addition, Cas12a does not require a tracrRNA in its gRNA structure to mediate its activity and it recognizes a T-rich PAM (5'-TTTN) (SCHINDELE *et al.*, 2018). In contrast to Cas9, which generates blunt-end DSBs proximal to the PAM, Cas12a generates overhanging-end DSBs distal from the PAM (SCHINDELE *et al.*, 2018) and, for this reason, it is usually preferred for CRISPR-Cas editing by HR (recently reviewed by VOLPI;

SILVA *et al.*, 2020). On the other hand, the CRISPR-Cas13a system acts exclusively on RNA (SHMAKOV *et al.*, 2015; SCHINDELE *et al.*, 2018), enabling new levels of control by operating at the RNA level. Cas13 can be used for targeting specific splicing isoforms (MAHAS *et al.*, 2017) and knockdown of noncoding RNAs (ncRNAs) (WANG *et al.*, 2016). Despite its importance, Cas13-containing systems do not allow transgene segregation since the cell must have the transgenic machinery to operate in the RNA.

Since CRISPR-Cas discovery and wide application in the last years, several new techniques have popped up. For instance, base and prime editors have been recently included in the CRISPR toolkit. Both are based on the use of a deactivated Cas (dCas), which is a catalytically impaired nuclease, whose purpose, along with the gRNA, is to solely guide another protein to the target, changing the DNA without creating DSBs (ANZALONE *et al.*, 2019). Base editors act in a single-base level. As for prime editing, it mediates targeted insertions (up to 44 bp), deletions (up to 80 bp), and point mutations including all 12 possible base-to-base conversions (C:T, G:A, A:G, T:C, C:A, C:G, G:C, G:T, A:C, A:T, T:A, and T:G) without requiring DSBs or donor DNA templates (ANZALONE *et al.*, 2019). These approaches not only represent the newest assets in the editing toolkit but also a step closer to the ideal goal of manipulating genomes, fast-forwarding evolution in our favor, with minimal unwanted genome perturbation (ANZALONE *et al.*, 2020).

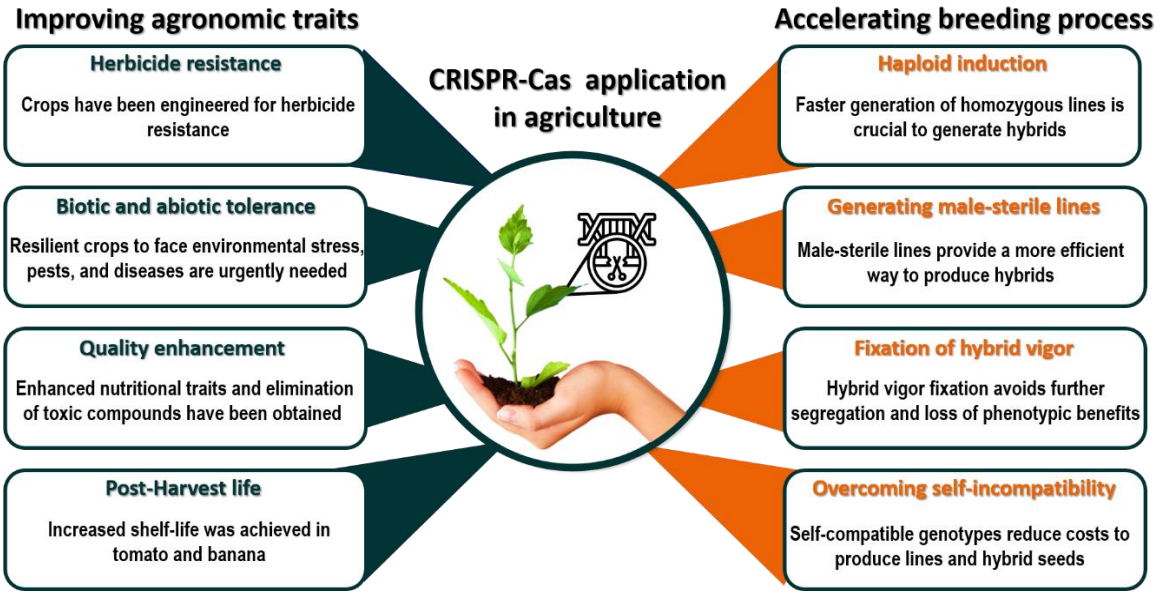
One of the biggest advantages of CRISPR-based systems is that targeted and precise mutations can be achieved and passed on to the next generation independently from the transgenic cassette since the mutation and the exogenous DNA are usually located in different chromosomes (WOO *et al.*, 2015; XU *et al.*, 2015; ZHOU *et al.*, 2015). The possibility of developing transgene-free plants carrying heritable mutations has a huge impact on cost and time length for generating biotechnology-mediated improved crops, besides broadening its accessibility by parties other than large companies (CHEN *et al.*, 2001; FENG *et al.*, 2014; SCHIML *et al.*, 2014; BELHAJ *et al.*, 2015; DING *et al.*, 2016; OSAKABE *et al.*, 2016).

Transgene-clean plants may also be generated by applying the CRISPR-Cas system as ribonucleoproteins (RNP) to act transiently in the cell, rather than being genetically integrated into the cell genome. In this strategy, nuclease mixed with gRNA is directly delivered to the plant cell, reducing the time spent to obtain non-GM plants (WOO *et al.*, 2015; SVITASHEV *et al.*, 2016; KIM *et al.*, 2017; LIANG *et al.*, 2017). These time-saving and non-GM advantages are especially valuable in perennial crops with a long generation time, clone-derived plants, in which segregation of the CRISPR-Cas locus is lengthy and impractical, respectively, and for directly edible crops such as horticultural species, due to public acceptance (KOLTUN *et al.*, 2018).

3. Next-generation plant breeding involving genome editing

CRISPR-Cas-mediated genome editing has the potential to enable certain achievements in crop improvement and fasten plant breeding programs. Some of these applications are summarized in Figure 1.

Figure 1. Practical applications of CRISPR-Cas in plant breeding.



CRISPR-Cas induced mutations in crop improvement

Herbicide resistance

Weeds drastically hinder crop productivity globally. Herbicide-resistant genotypes were generated as an alternative to maintain high yield. The transgenic approach has been used for this matter. However, currently, there seems to be a shift to the CRISPR-Cas technique aiming for non-GMOs. Genome editing tools have already been applied for this purpose in some highly important crops, such as rice (LI *et al.*, 2016; SUN *et al.*, 2016; ZHANG *et al.*, 2020a), soybean (LI *et al.*, 2015), maize (JIANG *et al.*, 2020), oilseed rape (WU *et al.*, 2020), tomato, and potato (VEILLET *et al.*, 2019).

The most common herbicide resistance pathway targeted by CRISPR-Cas has been the branched-chain amino acid biosynthesis, having acetolactate synthase (ALS) as the key enzyme. The mutation of ALS in specific amino acids results in resistance to herbicides such as sulfonylurea and imidazolinone. Herbicide-resistant rice was obtained using the HR pathway, either by the introduction of multiple discrete point mutations in *OsALS* gene (SUN *et al.*, 2016) or by prime editing, using a cytosine base editor to generate mutations in P171 and/or G628 codons of *OsALS* (ZHANG *et al.*, 2020a). The prime editing strategy was also used to generate sulfonylurea herbicide-resistant maize (LI *et al.*, 2020b), oilseed rape (WU *et al.*, 2020), tomato, and potato (VEILLET *et al.*, 2019).

Additionally, rice with resistance to auxinic herbicides, such as picloram 2,4-dichlorophenoxyacetic acid (2,4-D), was successfully generated by partial deletion of the *auxin signaling f-box OsAFB4* gene, which codes for a selective auxin receptor (GUO *et al.*, 2021). Also in rice, two amino acids of the *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) gene, involved in aromatic amino acid biosynthesis, were edited, leading to resistance to EPSPS-based herbicides, such as glyphosate. For that, two gRNAs were used to induce gene replacement by NHEJ, providing a DNA template containing the desired point mutations. Therefore, with directly joinable ends, NHEJ can accurately repair the CRISPR-Cas induced DSB. This strategy successfully generated rice plants resistant to EPSPS herbicides without using homologous recombination for gene swap (LI *et al.*, 2016). It is worth noting that these approaches require precise modifications for the target enzyme to no longer bind to the harmful herbicide molecules, yet still be functional.

Tolerance to abiotic and biotic stress

Another application of the CRISPR-Cas toolkit is to develop genotypes with resistance or tolerance to biotic and abiotic stresses. Due to climate change, resilient crops adapted to non-ideal environmental conditions are urgently

needed. In this context, drought-tolerant maize plants were developed through HR, introducing or swapping the promoter region of the *ARGOS8* gene by the *GOS2* promoter, containing drought-responsive cis-elements. The *ARGOS8* gene is a negative regulator of ethylene responses and its overexpression promotes cell expansion and/or division, mitigating yield loss by enhancing plant growth under drought stress (SHI *et al.*, 2017). In this study, a native and stronger promoter within the maize genome was used to enhance its expression.

Climate change also affects pathogen-host interaction and may lead to higher disease incidence, which threatens crop yield. Thus, CRISPR-Cas may be used to generate disease-resistant plants, mainly by silencing genes that negatively regulate defense pathways, shielding plants from pathogen attacks. Rice plants resistant to the fungus *Magnaporthe oryzae* were generated by a frameshift mutation in the gene *OsERF922*, which encodes an ethylene-responsive factor, a negative regulator of rice blast resistance (WANG *et al.*, 2016). Similarly, maize plants resistant to *Ustilago maydis* were obtained through knockout of *lipoxygenase3* (*LOX3*), another gene that negatively regulates fungal disease resistance (PATHI *et al.*, 2020). In wheat, genes related to susceptibility to powdery mildew (*Blumeriag aminis* f. sp. *tritici*) were knocked out, including three homoeoalleles of *mildew resistance locus* gene (*MLO*) (WANG *et al.*, 2014) and three homoeologous of *enhanced disease resistance1* (*TaEDR1*) (ZHANG *et al.*, 2017), enhancing wheat tolerance to the disease. Moreover, the CRISPR-Cas tool may also be used to create specific changes in genomes, such as single nucleotide polymorphism (SNP) replacement. Potato plants resistant to late blight, caused by *Phytophthora infestans*, were obtained by replacing a single base, A to C, in the second exon of *caffeoyl-CoA O-methyltransferase* (*StCCoAOMT*) gene by HR (HEGDE *et al.*, 2021).

In soybean, a CRISPR-Cas-based strategy was used to diversify plant innate immune system, enhancing disease resistance. This technique consisted of generating diversity on loci of the key defense family genes from the *nucleotide-binding-site-leucine-rich-repeat* (NBS-LRR) class, through gene rearrangements, resulting in new positive variants (NAGY *et al.*, 2021). Another new emerging approach is based on engineering plant-associated microbiome not only to improve crop growth but also to increase pathogen resistance. Plant and microbiome are so intrinsically intertwined that the related microbiome is considered the second genome of plants (KUMAR; DUBEY, 2020). Furthermore, plant pathogens change the population of antagonistic microbes and, consequently, the plant immune responses, leading to pathogen control (SHELAKE *et al.*, 2019). Thus the application of the CRISPR-Cas tool in synthetic biology has the potential to enhance plant resistance mechanisms against pathogens (CHAUDHARY *et al.*, 2021).

Quality and durability improvement

Beyond yield, other traits are relevant in crop production. For example, grains with low amylose content have been widely used in textile and adhesives industries, besides presenting better eating and cooking quality. The enzyme granule-bound starch synthase I (GBSS I), encoded by the *waxy* (*Wx*) locus, is required for amylose synthesis and determines the amylose content of the endosperm. In waxy maize, homozygous recessive *Wx* alleles result in maize grains mostly composed of amylopectin (QI *et al.*, 2020a). Thus, the deletion of 4- and 6-kb of the *waxy* gene using two gRNAs successfully generated maize mutants with reduced amylopectin in the endosperm, with content comparable to those genotypes with homozygous recessive *waxy* locus (GAO *et al.*, 2020). Similarly, the CRISPR-Cas targeted mutagenesis was used to generate waxy rice through the alteration of the Wx^b gene sequence using cytidine base editing (XU *et al.*, 2021b).

Grain components that can lead to allergic reactions may also be manipulated using CRISPR-Cas. Gluten proteins present in wheat can trigger coeliac disease in sensible humans. The alfa-gliadin protein family is the main group associated with coeliac disease, being encoded by around 100 genes. Thus conventional breeding methods cannot substantially reduce this allergenic compound content (OZUNA *et al.*, 2015). The CRISPR-Cas tool allowed to target conserved region of alpha-gliadin genes using two gRNAs to create indels that resulted in a reduction of 85% of gluten content in wheat grains (SÁNCHEZ-LEÓN *et al.*, 2018). Likewise, in soybean grains, the presence of raffinose oligosaccharides family (RFOs) hinders the soybean-based feed digestion by monogastric animals (HOU *et al.*, 2009). Le *et al.* (2020) used two gRNAs to knockout two soybean *galactinol*

synthase genes (*GOLS*), *GmGOLS1A*, and its homeolog *GmGOLS1B*, reducing the stachyose content by 35%, the most predominant RFO in soybean seeds. Furthermore, the vicilin-like glycoprotein Gly m Bd 28 K and the oil body-associated protein Gly m Bd 30 K are reported as major allergens in soybean. Therefore, in order to favor soybean consumption by humans, genes that encode both proteins were edited by CRISPR-Cas, generating grains free of these allergenic compounds (SUGANO *et al.*, 2020).

Additionally, CRISPR-Cas may be used to increase food quality. For instance, soybean and peanut grains with increased oleic acid content were generated via CRISPR-Cas-induced indels in the *fatty acid desaturase* (*FAD2*) gene, which encodes one of the enzymes responsible for converting oleic to linoleic acid (AL AMIN *et al.*, 2019; YUAN *et al.*, 2019). In tomato, lycopene content was enhanced using a multiplex system to knock down six genes involved in the carotenoid metabolic pathway, preventing the conversion of lycopene to beta and alpha-carotene (LI *et al.*, 2018). In rice, increased carotenoids levels were achieved by the introduction of a 5.2 kb cassette for carotenoids biosynthesis in two genomic safe harbors by HR, with no off-target effect in mutated plants (DONG *et al.*, 2020).

Food shelf-life and storage can also be enhanced by CRISPR-Cas. In tomato, several genes are well-known to have long-shelf-life properties; however, their introduction by traditional breeding led to loss in organoleptic quality (CASALS *et al.*, 2011). Therefore, the CRISPR-Cas system was used for targeted mutation without off-target effects. Yu *et al.* (2017) obtained tomato fruits with a longer shelf-life by introducing the known monogenic mutation *alcobaca* (*alc*), an allele of the non-ripening gene (NOR), using HR (YU *et al.*, 2017). Interestingly, tomato germplasm containing the natural alc mutation is correlated with small fruit size probably due to gene drag in the selection to extend shelf-life (CASALS *et al.*, 2012). Thereby, CRISPR-Cas is proven to be a precious asset in targeted mutation without some drawbacks of conventional breeding. CRISPR-Cas9 was also applied to edit *aminocyclopropane-1-carboxylate oxidase 1* gene (*MaACO1*), enhancing banana shelf-life (HU *et al.*, 2021). All of these examples are crucial improvements for the commercialization of such perishable products.

Application of CRISPR-Cas to accelerate breeding processes

Haploid induction

In crop breeding aiming to generate hybrids, obtaining homozygous lines is the first step. However, this may be a time-consuming and laborious process. Double haploid technology via *in vivo* haploid induction is generally applied to accelerate this step in maize and other crops. In this case, the gene-editing approach may be used to produce haploid inducer lines. In maize and rice, CRISPR-Cas induced mutations in a sperm-specific phospholipase, resulting in defective male gametophytes and triggering maternal haploid induction (LIU *et al.*, 2017; YAO *et al.*, 2018).

New emerging strategies for haploid induction, called haploid induction editing (HI-Edit) (KELLIHER *et al.*, 2019) and haploid inducer-mediated genome editing (IMGE) (WANG *et al.*, 2019a), consist in modifying an elite genotype via pollination of commercial backgrounds with a haploid-inducing line carrying a CRISPR-Cas cassette targeting a desired agronomic trait. Both HI-Edit and IMGE have the potential to be widely adopted to generate double haploids in several crops, replacing the burdensome and lengthy introgression process and generating double haploid lines with desired traits in only two generations (WANG *et al.*, 2019a).

Although double haploid lines generated by CRISPR-Cas represent valuable advantages, the identification of edited cells is still a challenge. To overcome this obstacle some techniques were developed. For instance, in ViMeBox, a visible marker driven by a tissue-specific promoter is introduced into CRISPR-Cas expression vectors. The ViMeBox tool may also be combined with HI-Edit (KELLIHER *et al.*, 2019) and IMGE (WANG *et al.*, 2019a), once ViMeBox allows the easy identification of diploids by exhibiting red visible embryos, increasing the efficiency of the process (XU *et al.*, 2021a).

Generating male-sterile lines

Hybrids are commonly used in agriculture due to heterosis or hybrid vigor associated with higher yield. Hybrid seeds are produced by crossing lines, wherein each parent donates either the male or female gamete. In crops such as wheat, rice, and maize, it is preferable to use male-sterile maternal lines to avoid contamination with female self-fertilized seeds.

Although male-sterile lines have been reported in several crops, generating them through conventional breeding requires a considerable amount of labor and time. Therefore, gene editing by CRISPR-Cas may be a faster alternative to obtain male-sterile lines.

For instance, in maize, CRISPR-Cas was used to delete an essential catalytic region of male sterility *26* (*MS26*) gene, translating a non-functional member of cytochrome P450 monooxygenase family and generating complete male-sterile plants (QI *et al.*, 2020b). Similarly, disruption of *TaNP1* genes, which encodes a putative glucose-methanol-choline oxidoreductase, resulted in complete male sterility in wheat (LI *et al.*, 2020a). Multiplex-directed mutations driven by CRISPR-Cas are especially valuable in polyploid crops, which present redundant alleles, being very difficult to be generated/or gathered by other processes from traditional crop improvement.

Fixation of hybrid vigor

Hybrids are widely used in crop breeding aiming for heterosis. However, the phenotypic superiority achieved in F_1 is lost in further crosses. Clonal propagation of hybrid F_1 seeds may keep heterosis benefits and facilitate hybrid seed production. This purpose can be achieved by apomixis, an asexual reproductive pathway that produces seeds without fertilization (WANG *et al.*, 2019b). In rice, F_1 heterozygosity was fixated via CRISPR-Cas by editing the meiotic genes *REC8*, *PAIR1,* and *OSD1* simultaneously, creating the so-called Mitosis instead of Meiosis (MiMe) genotype and enabling the production of diploid gametes and tetraploid seeds (WANG *et al.*, 2019b). In a further step, besides the multiplex approach with the three mentioned genes, Wang and collaborators simultaneously muted the haploid induction gene *matrilineal* gene (*MTL*). The MiMe plants with paternal genome eliminated by the mutated *MTL* produced self-fertilized F_1 hybrids and clonal seeds with the same ploidy and heterozygous genotype (WANG *et al.*, 2019b). Although using apomixis induced by CRISPR-Cas is a tremendously advantageous possibility to fix hybrid vigor, further studies are needed for this strategy to be widely applied in hybrid seed production.

Overcoming self-incompatibility

In flowering plants, self-incompatibility prevents self-fertilization by discriminating self and outcross pollen. This phenomenon occurs in several important crops, such as canola, potato, pome, coffee, olive, among others. This mechanism is often controlled by a single multiallelic locus, called S-locus (MUÑOZ-SANZ *et al.*, 2020). In *Solanaceae*, such as potato and tomato, the mutation of *S-RNase* gene, a co-dominant gene responsible for gametophytic self-incompatibility, resulted in self-compatible lines (QIN *et al.*, 2018; ENCISO-RODRIGUEZ *et al.*, 2019). In cabbage and oilseed rape, the sporophytic self-incompatibility was overcome by knockout of M-locus protein kinase and S-receptor kinase, respectively, also using CRISPR-Cas (CHEN *et al.*, 2019a; MA *et al.*, 2019). Creating self-compatible genotypes by CRISPR-Cas enables the generation of inbred lines, as well as hybrid seeds, in a cheaper and faster way. In addition, for fruit production, the generation of self-compatible genotypes may facilitate the pollination process by dispensing the cultivation of pollination plants in orchards (MA; QU, 2019).

4. Regulatory issues – raising hurdles for CRISPR crops

The regulations surrounding the new plant breeding technologies (NBTs), including CRISPR-Cas, are still being debated and established worldwide, due to their unanticipated possibilities (recently reviewed in TURNBULL *et al.*, 2021). Policymakers are defining, among other aspects, whether or not CRISPR-edited plants fall into special regulatory regimes as GMOs (PODEVIN *et al.*, 2012). Recently, many major countries have released updated regulatory frameworks, or are close to doing so. Whereas others are yet to release any formal decisions (SCHEBEN; EDWARDS, 2018; LASSOUED *et al.*, 2020). It seems that countries are fiercely debating the risks to health and the environment and cautiously considering all the implications of different policymaking options to their economy.

Law-making parties are taking into account either a process-based or a product-based approach to regulating genome-edited crops and derived products. For instance, the European Union and Russia assess the process, while the United States, Canada, Argentina, and Brazil analyze the product, irrespective of the technique used to obtain it (CTNBIO, 2018; KULUEV *et al.*, 2019; GAO, 2021). Although it is not unanimous, many researchers strongly argue that transgene-free edited crops do not pose

unreasonable risk, being indistinguishable from natural mutants; therefore, should not be subjected to any special safety assessment (KULUEV *et al.*, 2019; VAN EENENNAAM *et al.*, 2019; ZHANG *et al.*, 2020b). Strict regulation and social rejection – converging aspects – concerning genome-edited crops could undermine all major benefits brought by NBTs and hugely and negatively affect the evolution of the field (LASSOUED *et al.*, 2019).

The edition via CRISPR-Cas fits into the site-directed nucleases (SDN) framework. In Brazil, organisms containing mutations produced by SDN1 systems (rejoin of broken DNA ends by the error-prone NHEJ leading to small indels) do not qualify as GMOs since the Biosafety Law excludes products obtained by mutagenesis from the GMO scope, as they simulate natural mechanisms. SDN2 genome editing systems (repair via homologous recombination by adding a relatively short DNA fragment causing slight alteration), are analyzed on a case-by-case basis by the Brazilian legislation, mainly taking into account the presence of foreign DNA. Finally, SDN3 systems (repair via homologous recombination by introducing a large DNA fragment) normally fit as GMOs and can be evaluated individually depending on the origin of the DNA used (exogenous or not) (SPRINK *et al.*, 2016; CTNBIO, 2018).

In the USA, more than 100 genome-edited plant varieties have been designated as not regulated and have freely entered market channels, including oleic acid-enriched soybean (DO *et al.*, 2019), high-oil content camelina (WALTZ, 2018), and powdery mildew resistant wheat (WANG *et al.*, 2014). In 2018, the Brazilian legislation judged a high-amylopectin (waxy) maize as non-GMO. It is worth noting that new varieties undergo extensive field evaluations according to traditional breeding practices. On the other hand, the European Union has decided that organisms with an edited genome should be subjected to the same strict regulations as transgenics, even though there is no consensus among the countries that compose it.

Despite some achievements, non-scientific political decisions have been made in the law-defining process regarding NBTs (URNOV *et al.*, 2018), which hampers the advancement of agriculture around the world. Deregulation of products generated via NBTs implies cheaper and accessible technology for public institutions and small companies, diversifying the participants in technological innovation (FRIEDRICHS *et al.*, 2019). However, if a restrictive regulatory approach is adopted and treats edited plants as GM crops, it would create huge financial burdens that only large multinational companies could afford, excluding universities, nonprofits, and other small companies.

Overall, the conflicting regulations on genome editing will have consequences for international trade that are yet to be determined, but that will probably lead to commercial impediments, decreased profits, and difficulty enforcing policies. Traceability is a major problem. Depending on the case, it is impossible to detect whether a DNA mutation resulted from natural evolution or CRISPR manipulation (GAO, 2018).

Another issue to be considered is product labeling. Some regulatory approaches have appeared regarding labeling the edited-plants-derived products as bioengineered (VAN EENENNAAM *et al.*, 2019). High caution should be taken in this matter to avoid past mistakes, such as the backlash once seen with GMOs, rising fake news and myths around these products. This would lead to even more delays to gene-edited food integration into the market, and be very detrimental to research and applications in this field.

Since genome editing is a highly complex molecular toolbox, a suitable science-based regulatory approach should be built to embrace its complexity and to cope with future advances, in order to decide whether some of them require special legal regulation (HUANG *et al.*, 2016; CHEN; GAO, 2020; LASSOUED *et al.*, 2020; MACNAGHTEN; HABETS, 2020). More effort is needed to promote knowledge-based discussions aiming to increase public and regulatory authorities' awareness and to move towards reasonable interpretations and decisions (reviewed in DUENSING *et al.*, 2018). A very well-thought-through approach balancing precaution and innovation will enable the use of CRISPR-Cas to bring innovative solutions to agriculture.

5. Challenges and future perspectives

Even though the CRISPR-Cas system is a highly promising approach at the forefront of scientific knowledge and applicability, some challenges still need to be addressed in the years to come. Since most genome editing techniques depend on an initial generation of transgenic plants, one of the major drawbacks is the tissue

culture step. Most crops still have low efficiency of transformation and regeneration and the protocols used are usually genotype-dependent. In most cases, the transformation-prone genotypes are not the elite ones, requiring an extra effort to later cross the plants (ATKINS; VOYTAS, 2020).

Several groups have been working on strategies to overcome bottlenecks in plant gene editing (reviewed in ATKINS; VOYTAS, 2020). A brilliant alternative to traditional regeneration techniques is somatic reprogramming, in which morphogenic regulators are expressed to determine cell fate. Embryo regeneration from somatic tissue has already been achieved with high efficiency using a developmental factor associated with somatic embryogenesis (*BABYBOOM; BBM*) and a meristem maintenance transcription factor (*WUSCHEL*; *WUS*) (LOWE *et al.*, 2016, 2018; MOOKKAN *et al.*, 2017). Other genes such as *growth-regulating factors* (*GRFs*), *grf-interacting factors* (*GIFs*), and *GRF-GIF* chimeras have also been used to improve the regeneration efficiency in monocot and dicot plants (DEBERNARDI *et al.*, 2020; KONG *et al.*, 2020).

Another challenge in genome editing is to improve HR efficiency. Some strategies have been developed, such as the use of geminivirus constructs (BALTES *et al.*, 2014), *in planta* gene targeting system (FAUSER *et al.*, 2012), cell synchronization in S/G2, and inhibition of cellular NHEJ repair (LIN *et al.*, 2014; GUTSCHNER *et al.*, 2016; HOWDEN *et al.*, 2016). Another work-in-progress technique to increase DNA insertion efficiency and size of donor DNA is the transposon-associated CRISPR-Cas system, which allows site-targeted DNA insertion without having to rely on host-cell repair machinery (MA *et al.*, 2021).

As for future prospects, besides overcoming the aforementioned obstacles, further enhancement of CRISPR-Cas efficiency is needed, as well as a massive effort to broaden its applicability to more crops. Excitingly, new approaches have arisen quite frequently. For instance, CRISPR-Cas genome editing tools now enable rearrangement of large chromosomal fragments, which can be used to break genetic linkages with deleterious genes or to generate linkages between favorable genes, and applied as a tool for synthetic plant chromosomes (RÖNSPIES; SCHINDELE; PUCHTA, 2021).

Here we have presented the CRISPR-Cas technique from its most basic technique to the most advanced and recent ones, along with several practical applications. It is undeniable that genome editing is a revolutionizing tool in many fields, including agriculture. It has the potential to accelerate the development of elite genotypes with urgently needed characteristics ranging from tolerance to stresses to enhanced nutritional value, besides opening up a new era in crop breeding.

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