CHAPTER 1

Introduction to genome editing in plants

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Present and future challenges of modern agriculture

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

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

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

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

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

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

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

Gene editing tools for precision crop breeding

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

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

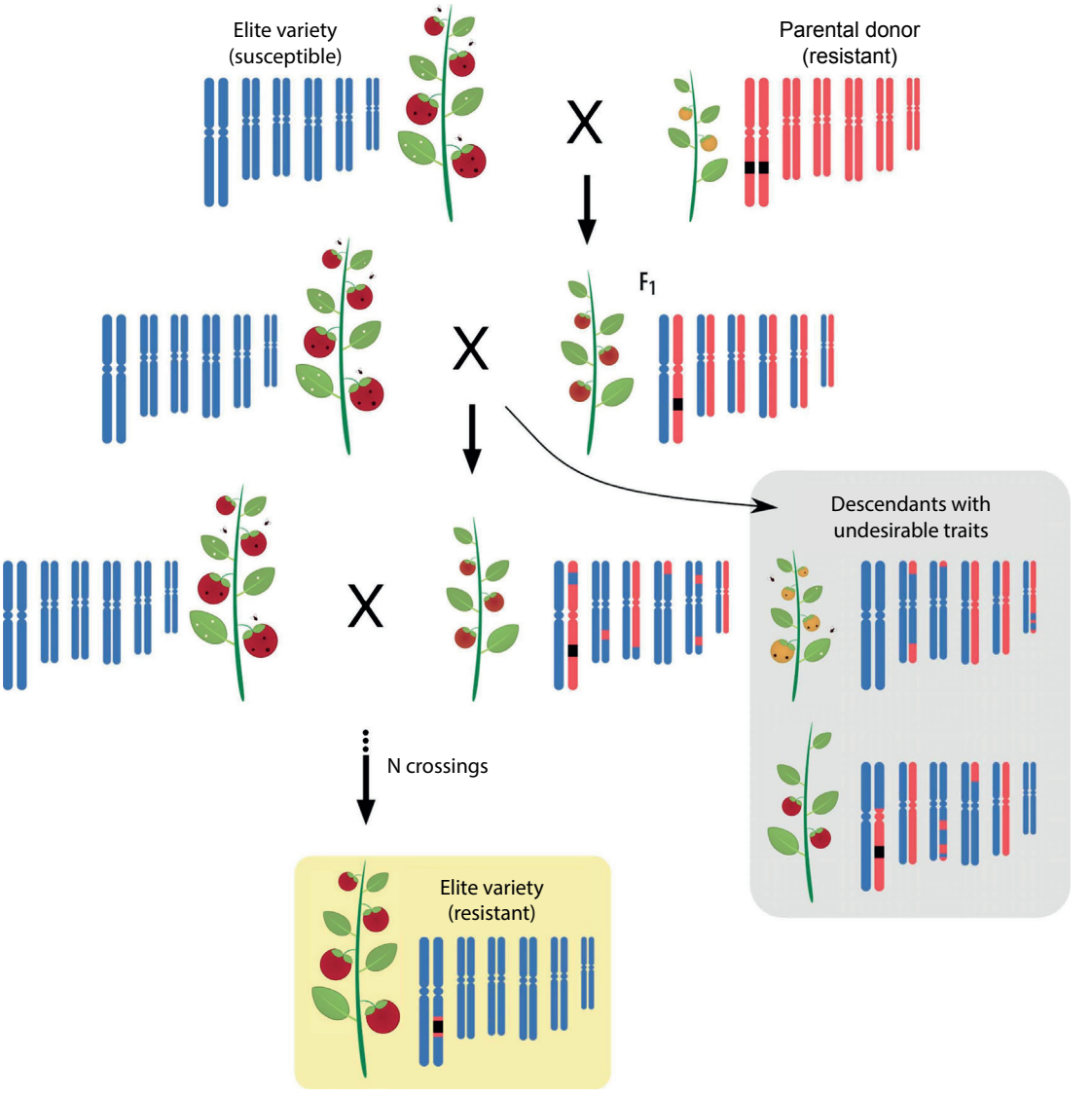

Figure 1. Representation of the introgression process in which an elite variety susceptible to a pest is crossed with a resistant donor. Blue and red chromosomes represent the genome of the elite and donor cultivars, respectively. The black region indicates the locus that confers resistance to the pest.

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

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

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

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

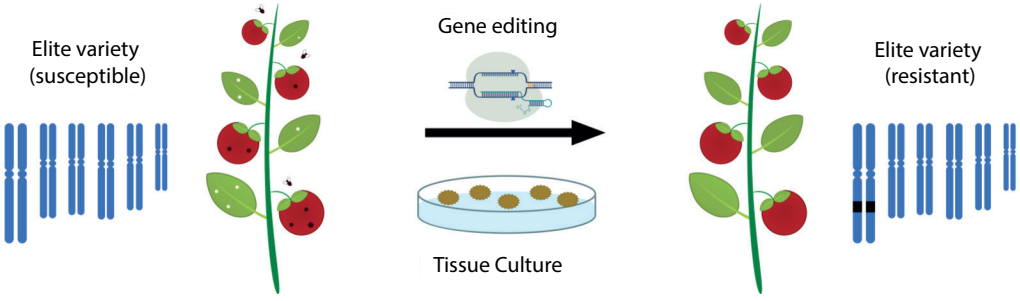

Figure 2. Improving resistance to a specific pest by genome editing without integrating exogenous DNA into the genome of an elite variety.

Genome editing methodologies

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

ZFNs (*Zinc Finger Nucleases*)

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

TALENs (*Transcription Activator-Like Effector Nucleases*)

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

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

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

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

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

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

Origin of the CRISPR/Cas technology

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

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

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

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

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

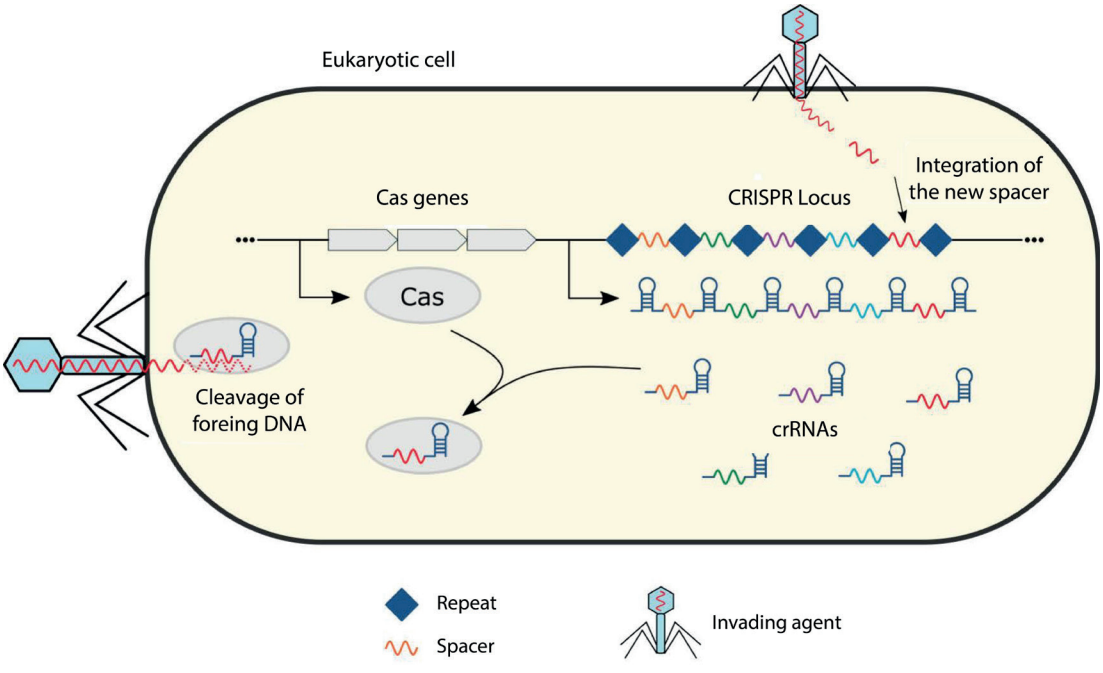

Figure 3. Schematic representation of the CRISPR/Cas immunity system in prokaryotes. A prokaryotic cell, when invaded by a virus or plasmid, can integrate part of the invading genome as a new spacer (in red) in its CRISPR locus. In a recurring infection, crRNAs derived from the CRISPR locus associate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

Source: adapted from Doudna Lab (2020).

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

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

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

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

Overview of mechanisms and enzymes

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

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

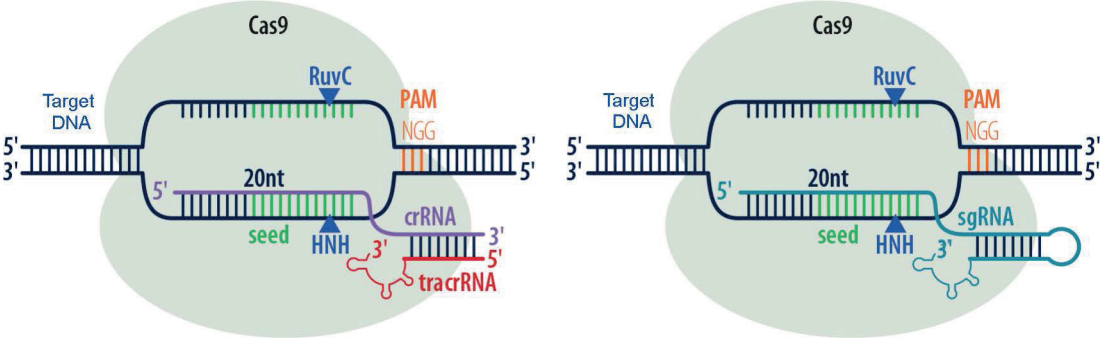

Figure 4. Comparison between the native CRISPR/Cas9 complex of *Streptococcus pyogenes* (left), containing crRNA and tracrRNA, and the optimized complex used for genome editing in eukaryotes (right) with a single guide molecule (sgRNA). Blue triangles indicate the cleavage positions in the target molecule,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

Source: adapted from Doudna and Charpentier (2014).

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

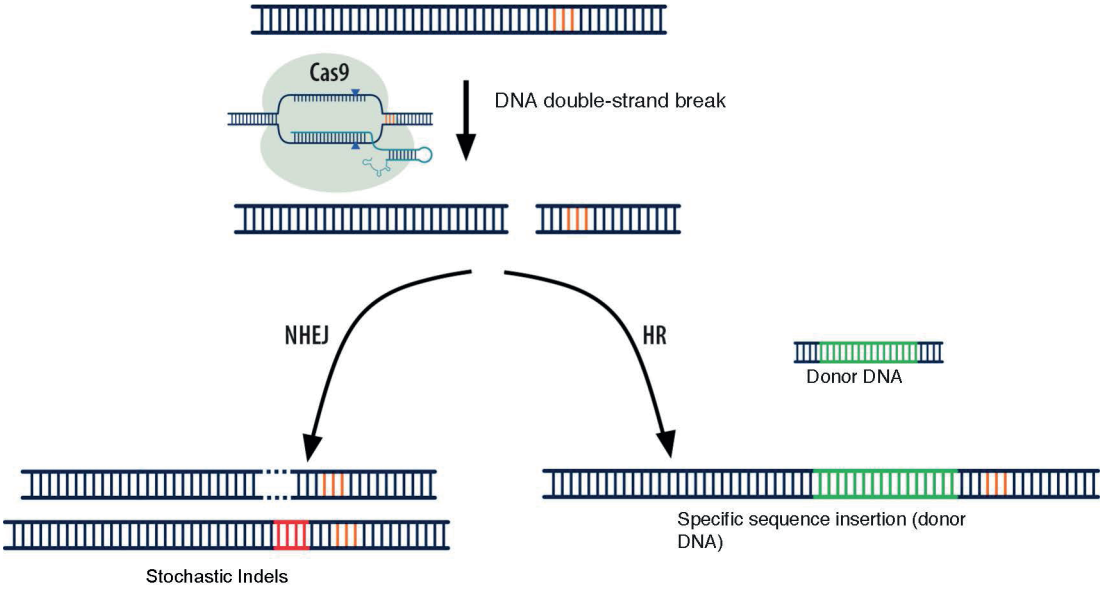

Figure 5. Schematic representation of the DNA repair mechanisms by NHEJ or HR. Dashed and red regions indicate deletions and insertions, respectively. The green region represents a sequence of interest to be integrated into the target DNA.

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

Modification and alternatives to SpCas9

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

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

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

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

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

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

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

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

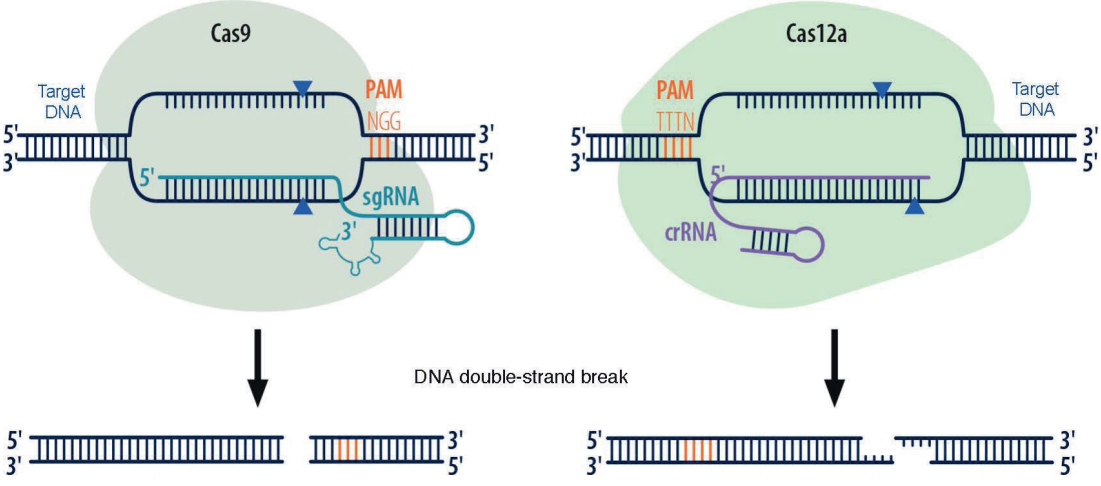

Figure 6. Representation of the main differences between the Cas9 (left) and Cas12a (right) nucleases. Blue triangles represent the cut position in the target molecule.

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

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

CRISPR derived applications

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

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

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

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

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

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

Application of CRISPR in agriculture

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

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

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

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

Productivity increase

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

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

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

Quality improvement

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

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

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

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

Elimination of undesirable traits

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

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

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

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

Resistance to biotic factors

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

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

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

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

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

Resistance to abiotic factors

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

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

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

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

Accelerated and *de novo* domestication of wild species

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

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

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

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

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

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

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

Perspectives and conclusions

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

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

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

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