Chapter 3

Understanding CRISPR/Cas9: A Magnificent Tool for Plant Genome Editing

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Abstract

Nowadays, it is well known that archaea organisms as well as bacteria show an important range of defense mechanisms. Among others, a unique molecular system called CRISPR/Cas (clustered regularly interspaced short palindromic repeats) helps provide protection (adaptive guided immunity) athwart foreign nucleic acids, including plasmids and viral infections. As a typical immune response, CRISPR system is based on the acquisition of genetic records provided by infectious external agents, and in this sense, a high interference upon a new infection is unchained. In relation to plant research, less than 10 years ago, efforts to understand this peculiar mechanism and the possibility of being used in biotechnological processes have been focused on obtaining atavistic changes in different transformable vegetal specimens by inducing selective mutations into a reading frame that may be translated in a given moment (i.e., ORF; open reading frame). In light of the consideration that one common use of ORFs is to assist gene prediction processes, palindromic repeats are mostly based on the directed mutations via nonhomologous end joining. Although it is true that DNA-free editing techniques are now desirable for molecular crop breeding, CRISPR/Cas as a mutational regulatory system in plant biology may offer better complex genome rearrangements.

Keywords: CRISPR/Cas9, plant genome editing, molecular crop breeding, RNA guided DNA, Cas9 nuclease

1. Introduction

Sustainable agriculture is considered to be the key for improving plant crops through genetic engineering, since random mutagenesis processes are part of conventional biotechnology techniques used for most researchers in this field [1]. CRISPR/Cas9 systems (clustered regularly interspaced short palindromic repeats-CRISPR associated) are related to a well-known CRISPR array defined by series of 20–50 bp genomic locus (i.e., unique spacers separated by direct repeats).

On the other hand, these unique spacers usually have similar length with preceded AT-rich fragments [2]. CRISPR loci were identified for the first time about two decades ago when they found a series of short genomic sequences (i.e., spacers) in *Escherichia coli* originated by viral genomes and probably due to the presence of conjugative plasmids, and in this sense, the foreign genetic material allowed the bacteria to record a kind of memory (e.g., immune system) to counteract future infections. When foreign DNA sequences match these unique spacers, they are commonly known as "photospacers" [1, 3]. Thereby, the corresponding immunization is against a foreign phage (e.g.), and when a new infection of this nature takes place in the future, the array expansion of the CRISPR is unchained and in consequence, new spacers originate from the genetic material of the phage.

According to some authors [4–6], this interesting immune system (to call it that) may be divided into three metabolic stages: adaptation, crRNA (CRISPR-RNAs) biogenesis, and interference. When a foreign DNA introduction happens, there is a selective process through the machinery that selects protospacers, which will be inserted into the CRISPR locus (insertion takes place into the leader end of the system). In the first stage of crRNA biogenesis, a transcription of CRISPR locus is observed followed by a direct processing of sequence elements (pre-crRNAs–crRNAs), all of them with the corresponding single spacer. After this stage, Cas proteins interact the crRNAs by assembling an effector complex (**Figure 1A**) [4]. This is very important since these components are intermediary elements of the interference stage where recognition of foreign DNA happens upon future infections and consequently, its degradation. It is very important to mention that a spacer acquisition creates genetic records of previous infections; as mentioned above, CRISPR immunity happens when there is an imminent detection of strange nucleic acids and consequently, the integration of foreign genetic material into the host's cells (the DNA integration occurs in the chromosome).

CRISPR systems are highly complex and diverse, and nowadays, efforts have been made to classify them into six interesting types: Type I (eight different Cas representative operons); Type II (tracrRNA; trans-activating crRNA and four Cas); Type III (eight Cas and Csm/Cmr); Type IV (four DinG/Csf); Type V (four Cas/Cpf2); and Type VI (three Cas/C2c2). In this case, operon type IV shows an extensive presence in the lack of CRISPR loci (**Figure 1B**) [4].

Due to the endless background that precede the functionality and applications of CRISPR systems in the field of genetic engineering, it has been shown that this metabolic phenomenon is extremely attractive for the molecularly directed crop improvement as well as plant genomic research. In general, the efforts that outline research for CRISPR systems within the agronomic

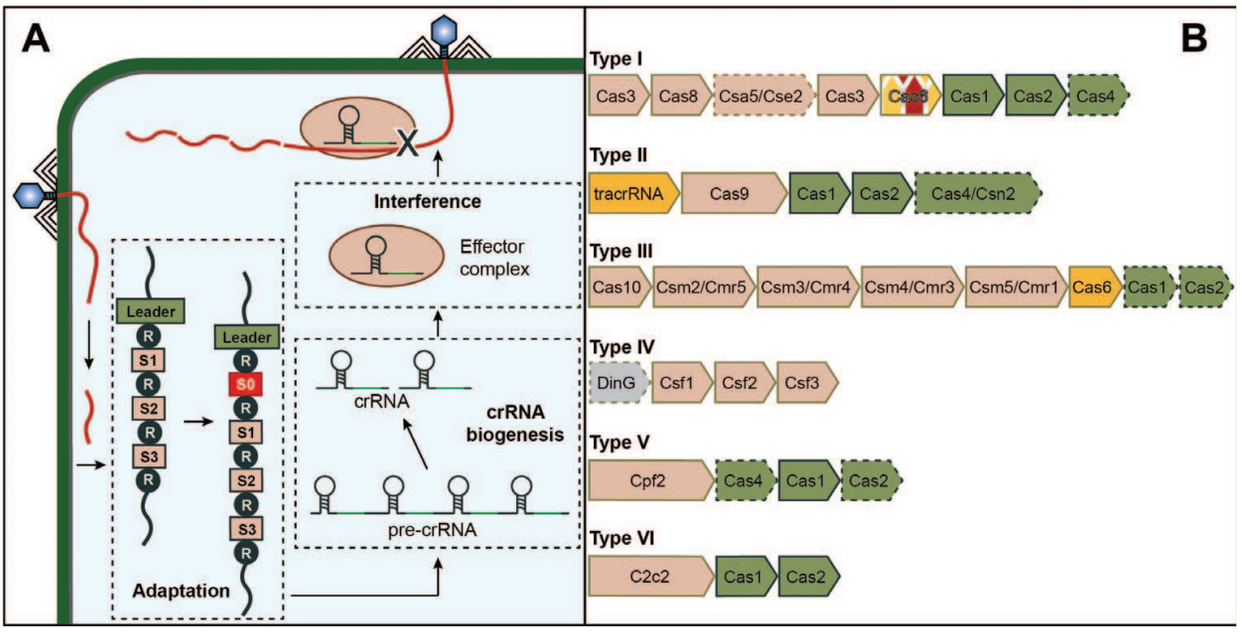

Figure 1. Functional and organizational system of the CRISPR/Cas9. (A) The process of induced immunity is carried out in three stages consisting of an adaptation, crRNA biogenesis, and interference. In the first stage, the adaptive machinery performs a selection of photospacers, and they are leader-end inserted into CRISPR locus that is subsequently transcribed during crRNA biogenesis. In a complementary way, the pre-crRNA processing into crRNAs with simple spacers is developed. Finally, the effector complex is originated through the assembly of crRNA with Cas proteins, which interacts (interference stage) in a subsequent way to the infection, and consequent degradation of the foreign genetic material. (B) Structurally, the CRISPR system is divided into six representative types (operons). Dashed outlines represent genes in some subtypes. Pink sequences represent genes related to the interference process. Yellow sequences refer to crRNA biogenesis, and the green color refers to adaptation genes. Subtypes IV are characterized by the absence of CRISPR loci. Adapted from Wright et al. [4].

sciences field have been mainly focused on plant domestication with economic and social interest. Thus, interspaced short palindromic repeats constantly open the doors to the generation and knowledge application in the area of functional genomics that jointly guide researchers toward the implementation of theoretical and applied biostrategies [7].

2. CRISPR/Cas9 mechanism: brief overview of its nature

The action mode of CRISPR/Cas9 biological system is basically based on the participation of two distinct elements: the Cas9 protein (CRISPR-associated protein 9; RNA-guided DNA endonuclease enzyme associated with CRISPR) and sgRNA (single guide RNA) [8]. Nowadays, Cas9 proteins are found mainly in different bacteria species such as *Brevibacillus laterosporus* [9], *Staphylococcus aureus* [10], and other representative species within the genus *Streptococcus* [11]. Cas9 proteins have shown two representative domains: the first one is known as HNH (nuclease-associated proteins), which is responsible for cleaving and regrouping the complementary strand of crRNA. On the other hand, the second domain known as RuvC-like (nuclease domain that cleaves complementary DNA strands) has the purpose of carrying out the cleavage of the complementary strand of dsDNA (double stranded

DNA). The nature of the sgRNA is extremely curious, since this is a kind of synthetic RNA with a length not greater than 100 bp and whose structure owns a 20 bp sequence coupled to the 5′-end that works as a guide allowing the identification of target sequences through specific adjacent motifs (i.e., PAM sequences; protospacer adjacent motifs) [8].

It is important to mention that 3′-end of the sgRNA resembles a loop structure that allows it to develop a very precise linkage with the target sequence. This is the way to structure a new complex that will be associated with Cas9 and in this sense, to perform the dsDNA cleavage that will cause double-stranded breaks (DSBs) [12]. Generally, a DSB is the result of the continuous DNA damage at chromosome level, although this is considered a completely normal phenomenon within the cell. However, the resulting by-products generated by the cellular metabolism itself such as reactive oxygen species (ROS) may interfere in the replication process due to the damage caused in the DNA. Also, environmental selective pressures as different chemical agents or UV light itself are considered other important factors involved in this process [13, 14].

When the above phenomenon has been carried out within the cell, the presence of DSBs activates the repair mechanism of damaged DNA through nonhomologous end joining (NHEJ) or homology-directed repair (HDR). In most cases, the repair of DBSs is carried out by NHEJ although the main reason why this happens is because it is the best way to make genetic insertions or deletions and consequently to give rise to a gene knockout (gene knockout is a genetic phenomenon through which an organism's gene becomes inoperative). In general, HDR is originated by the presence of an oligo template, and it activates the elimination of specific genes as well as foreign DNA (the mechanism involves the substitution of DNA sequences in a specific locus, or well, fragment sequences not found within this locus) [8, 12–15]. In addition to CRISPR, there are other methods currently used for genome editing which include the participation of peculiar endonucleases such as transcription activator-link effectors (TALEN) and zinc fingers. Through these mechanisms, a fusion between DNA-binding domains of transcription factors and the nuclease domain FokI (restriction enzyme) takes place. Beyond the application of CRISPR systems in genome editing and their regulation processes, these types of endonucleases may be used to be fused with fluorescent proteins in order to allow more specific loci location within the living cells [16, 17].

3. Cas9 enzyme: multifunctional DNA endonuclease

The Cas9 enzyme has a very peculiar structure since it consists of two distinct nodes complemented each other: an alpha-helical recognition site (REC) and an endonuclease structure (NUC) containing HNH proteins. On the other hand, Cas9 structure has RuvC-like and C-terminal domains with variable structures [18]. Both nodes are linked through anchoring-like structures mostly conformed by arginine-rich bridges and other less complex structures generally observed between residues 712–720. In this sense, there are three very important domains with alpha-helical structure that integrate the REC node (Hel-I, II, and III) and as an interesting fact; Hel domains show no structural similarity with some other known proteins to date [19].

Several studies have shown that Cas9/RuvC domains have very similar structures to retroviral integrases (virally encoded; specialized recombinases capable of catalyzing the recombination of viral DNA particles into the genome's host cell). In contrast to the above, the HNH nuclease domains show a fold-type $\beta\beta\alpha$ structure linked to a metallic cofactor that in the same way, it is linked to another HNH domain from a different endonuclease that allows the recognition through metallic ions in order to locate cleavage sites on the target DNA sequence [18]. Metallic ion-dependent restriction enzymes show highly conserved structures formed by aspartate residues and in less quantity by histidine [20].

In basal conditions, the nature of Cas9 enables the enzyme to be inactive and when a recombination between sgRNA and the corresponding REC lobe is observed, it is precisely that this natural state changes. Thus, the previous complex performs a specific search for PAMs (trinucleotide NGG) in order to identify target sequences into the double DNA strand. When a linkage has happened within the respective PAMPS, a cleavage of the hybrid DNA-RNA complex takes place thanks to HNH domain and, jointly, RuvC assists the structuring of dsSDBs (double-stranded SDBs) by cleaving the corresponding complementary sequence [21]. It is important to mention that both eukaryotic and prokaryotic cells show NHEJ and HDR mechanisms capable of repairing DSBs through the intervention of DNA ligase IV, whose nature helps regroup damaged nucleotide ends (Indels; introduction or deletion of mutations) as well as the use of complementary homologous DNA templates, respectively [21, 22].

Within the group of Cas9 proteins, there are repression effectors that are fused with a transcription activation system called dCas9 (CRISPR tool based on a modified version of the Cas9 protein). The dCas9 systems are usually combined with effector protein domains that regroup functional peptides that will target specific regions of genome loci. Thus, the resulting complex performs activation and shutdown mechanisms of gene repression, thereby; it is considered an efficient regulator of genetic information flow. This is why CRISPR/dCas9 system may be a modular platform in several cellular processes to control transcription [21, 23]. Another important fact about the dCas9 complexes is that they are able to combine with different epigenetic nature molecules, such as methylation and histone peptides [24].

On the other hand, specificity is a considerable element in genome editing tools. In the case of Cas9/gRNA (guide RNA) complexes, they have an extremely precise capacity to develop cleavages in DNA sequences, even when small mismatches may be observed into the guide template [25]. When this type of phenomenon happens, nonspecific cleavages are mostly tolerated at the 5′-ends or at a greater distance from the corresponding MAMP sequences. It has been noticed that short gRNAs (a.c. 20 bp) confer better specificity to Cas9 proteins at the target cleavage sites within the genetic editing processes [26]. Likewise, when the inactivation of Cas9 conserved domains is observed, a specific break occurs in one of the DNA strands (nickase), which leads to a splitting and loss of its double-stranded native structure [27]. In general, this type of nicks usually causes no mutations since they can be repaired in a very simple way by eliminating damaged bases through a specific repair metabolic pathway.

4. CRISPR/Cas9: a close relationship with TALENs and ZFNs

As previously mentioned, CRISPR/Cas9 system is conformed by a single monomeric protein as well as a complex RNA rearrangement. The Cas9 protein is responsible for carrying out the cleavage process, and a 20-nt fragment corresponding to gRNA is responsible for identifying target sequences [28]. Notwithstanding, the nature of TALENs and ZFNs (zinc finger nucleases) allows them to function as dimers; consequently, protein components are just required to develop the corresponding catalysis processes. A specific domain of the FokI endonuclease performs the cleavage of target sequences. On the other hand, domains corresponding to DNA binding that may be found in different polypeptides are those that confer the sequence specificity [29].

Because ZFNs show a necessary interaction with zinc fingers, it is considered that this process is experimentally complicated, and therefore, its application is usually limited within a biotechnological context, especially considering the need for nucleotide sequence specificity. However, TALENs and ZFNs are easy to design, and they are commercially available in most cases at an affordable cost [30]. TALENs can promote homologous recombination at cellular level through repetitive sequences compared to gRNA that is based on a Watson-Crick base-paring principle using target DNA sequences [28, 30]. On the other hand, TALENs and ZFNs are capable of generating DSBs through the restriction of FokI catalytic domains of different overlap sizes that may vary depending on their binding capacity. In comparison, Cas9 has two cleavage domains as previously discussed (i.e., RuvC and HNH). While it is true that ZFNs could target any sequence of interest, this process is subject to the availability of assembly platforms. Today, available molecular libraries can hold up to 100 bp sequences that serve as platforms for functional ZFNs. Thereby, TALEN's targets require thymidine residues to show an efficient functionality, which could limit their application [31].

TALEN protein arrays are exclusive for the group of plant pathogenic bacteria. The repeated sequences comprise 10–20 residue agglomerates that recognize specific DNA molecules. Each repeat has a maximum of 35 amino acids in length, and it is complemented by two adjacent amino acids (RVD, repeat-variable-di-residue) which have the function of conferring specificity to the four nucleotides that structure the DNA strands. In this way, a direct link between repeated sequences and target DNA is observed. Both TALENs and ZFNs are capable of generating DSBs in a specific region of the genome, and as previously discussed, this phenomenon is commonly used to generate gene knock out. RVD codes are used to generate TALEN repeating arrays, and therefore, an affinity of up to 96% can be observed respect to target sequences of interest [31, 32]. It is worth mentioning that TALENs show advantages over ZFNs, for example, TALENs can be extended over any sequence length that is necessary, and for the case of ZFNs, they can only be extended in a range of 9–18 bp, although it is considered that TALENs show less specificity [33].

A disadvantage of TALENs over ZFNs is their considerable size, since the extension of a cDNA sequence encoded by TALENs can be up to 3 kb, while the size per every ZFN is only 1 kb. The considerable size of TALENs could hinder the recombination process at cellular level, and therefore, these arrangements tend to be less attractive for biotechnological processes, mainly in

therapeutic applications. On the other hand, since TALENs show repetitive nature, it is usually more complicated to pack and move the molecules into the host cell using viral vectors [31].

In the case of ZFNs, it is important to highlight that in recent years, their use has increased considerably in both industrial and basic research, mainly for the generation of therapeutic adjuvants in both animal and human models. ZFNs comprise arrays of protein fusions with specific binding domains adapted to transcription factors that contain zinc finger complements. In a complementary way, its structure is also conformed by a FokI restriction domain. The zinc finger domains are able to recognize 3–4 bp sequences in the target DNA molecule. Tandem domains (tandem repeats; occurring in the DNA through the repetition of patterns of one or more nucleotides and the position of these is completely adjacent) tend to interlace with nucleotide sequences between 3, 9, and 18 bp length, and this process is repetitive throughout

Species	Cas9 codon	Trans method	Target sequence	Promoter	Mutation rate	Detection method	Ref
Arabidopsis thaliana	*Arabidopsis* (intron)	PEG protoplast	PDS3, FLS2	CaMV35SPDK, AtU6	1.1–5.6%	PCR sequencing	[36]
A. thaliana	*Arabidopsis* (intron)	PEG protoplast	RACK1b, RACK1c	CaMV35SPDK, AtU6	2.5–2.7%	PCR sequencing	[36]
Nicotiana benthamiana	*Arabidopsis* (intron)	PEG protoplast	PDS3	CaMV35SPDK, AtU6	37.7–38.5%	PCR sequencing	[36]
N. benthamiana	*Chlamydomonas reinhardtii*	Leaf agroinfiltration	Co-transfect GFP	CaMV35S, AtU6	n.a.	Pre-digested PCR	[37, 38]
N. benthamiana	Human	Leaf agroinfiltration	PDS	CaMV35S, CaMV35S	12.7–13.8%	n.a.	[39]
Oryza sativa	Rice	PEG protoplast	PDS, BADH2, MPK2, Os02g2382	2xCaMV35S, OsU3	14.5–38%	PCR	[40]
O. sativa	Human	PEG protoplast	MPK5	CaMV35S, OsU3 or OsU6	3–8%	qPCR and T7E1 assay	[14]
O. sativa	Rice	PEG protoplast	SWEET14	CaMV35S, OsU6	n.a.	Pre-digested PCR	[37, 38]
Triticum aestivum	Rice	PEG protoplast	MLO	2xCaMV35S, TaU6	28.5%	PCR + RE	[40]
T. aestivum	Human	Agro-transfect embryo immature cell	PDS, INOX	CaMV35S	18–22%	PCR sequencing	[39]
Zea mays	Rice	PEG protoplast	IPK	2xCaMV35S, ZmU3	16.4–19.1%	n.a.	[41]
Citrus sinensis	Human	Leaf agro-infiltration	PDS	CaMv35S, CaMV35S	3.2–3.9%	PCR + RE	[42]

Table 1. Examples of plant transient transfection based on CRISPR/Cas9-mediated NHEJ.

the entire genome in question. ZFNs act at two sites of the DNA sequence at the cellular level, on the forward and reverse strand, respectively. Since cleavages of specific regions within the genome can be observed, ZFNs are capable to recognize two adjacent sequences, and once the corresponding cleavage occurs, the FokI restriction enzyme domains produce a dimerization prior to the cleavage of the corresponding DNA loci. Thus, DSBs with 5′-extensions originate [31, 34, 35]. As listed in **Table 1**, some examples of experiments related to transient transfection based on CRISPR/Cas9-mediated NHEJ may be cited.

5. CRISPR/Cas9 applications in crop genetic improvement: brief overview

The relevance of agriculture for human survival is hard to underestimate. Crops provide food, fiber, and raw materials for a growing human population that faces an increasing amount of challenges, including loss and degradation of arable land as well as climate change. In this context, the rational use of all the available biotechnological tools is of paramount importance to attain a worthy life quality both in developed countries and the third world. Crop genome editing is among the most promising techniques to cope with the aforementioned agricultural challenges. However, it is worth noting that the development of those methodologies is useful not only for genetic improvement of agricultural crops but also to functional characterization of specific plant genes for basic research purposes [43].

Three short reports, published in 2013, demonstrated the feasibility of CRISPR/Cas9 system for genetic engineering of crops, based on the pioneer works of Li et al. (using *A. thaliana*), Nekrasov et al. (with *N. benthamiana*), and Shan et al. (*O. sativa* and *T. aestivum*), respectively [36, 40, 44]. After that, a plethora of research on crop genome editing has been published. The works listed below include some of the most representative studies on the matter, according to our best knowledge. However, the amazing dynamism of the field makes the presumption of exhaustiveness unattainable.

Given its undeniable worldwide relevance as a staple food, it is not surprising that rice has been one of the most studied crops in terms of CRISPR/Cas9 mediated genetic edition [14, 38, 45–48]. Among the main modifications proposed to this crop, it can be found herbicide resistance [49, 50], improved nitrogen use efficiency [51], and resistance against the rice blast disease [52].

Wheat (both durum and bread wheat) has also been subjected to extensive research in order to optimize the effectiveness of the genome editing process [53–55] as well as the acquisition of novel attributes including heritable broad-spectrum resistance to powdery mildew and other plant diseases [56].

Other crops widely used for the validation of technical improvements in CRISPR/Cas9 system are soybean [12, 57, 58] and maize [59, 60]. The latter cereal has also been modified in order to exhibit advantageous traits. For instance, this technology has generated novel variants of the ARGOS8 gene on maize. The ARGOS8 edited variants significantly increased grain yield under drought stress conditions, compared to wild-type maize, and had no yield loss under normal conditions [61].

The CRISPR/Cas9 system was also used to investigate the influence of specific genes on the phenotype development in tomato plants [51, 62, 63], as well as to achieve features of agronomic importance, such as delayed ripening of tomato fruit [64] or parthenocarpy [65]. Other members of the *Solanaceae* family reported to have undergone genetic editing via CRISPR/Cas9, include tobacco (*Nicotiana tabacum*) [38, 66], potato (*Solanum tuberosum*) [67], and petunia (*Petunia hybrida*) [68].

The CRISPR/Cas9 technology has also been used to confer molecular immunity against tomato yellow leaf curl virus (TYLCV), using *N. benthamiana* as host [69], as well as inducing complete resistance to Turnip mosaic virus (TuMV) [70], and improve the stress response in the model plant *A. thaliana* [71].

In the case of the emerging oil seed plant, *Camelina sativa*, the CRISPR/Cas9-targeted genetic edition has improved its fatty acid composition, obtaining a seed oil of superior quality on multiple levels, which besides being healthier, was more stable to oxidation and better suited for biofuel production [72, 73].

In addition to that, this technology has been used to obtain a nontransgenic cucumber strain (*Cucumis sativus* L.), resistant to cucumber vein yellowing disease, papaya ringspot mosaic virus-W, and zucchini yellow mosaic virus [74], as well as to successfully induce targeted mutagenesis in the Chardonnay grape cultivar that enhanced its endurance to powdery mildew, and to increase the golden delicious apple cultivar resistance to fire blight disease [75].

Besides the aforementioned, other crops in which the CRISPR/Cas9 technology has been optimized include barley (*Hordeum vulgare*) and *Brassica oleracea* [76], watermelon (*Citrullus lanatus*) [77], as well as the nonherbaceous sweet orange (*C. sinensis* cultivar *Valencia*) [42] and poplar (*Populus tomentosa*) [78].

Finally, we would like to stress that there is no scientific evidence whatsoever to assume that genetic modifications produced by modern biotechnological tools, such as CRISPR/Cas9, represent a higher health or environmental risk than conventional breeding techniques. However, public distrust caused by genetically modified crops has led to many countries to implement highly strict and costly regulations that make very difficult to successfully commercialize such products. Interestingly, since CRISPR/Cas9 genetic editing does not necessarily implies the incorporation of foreign DNA, according to some interpretations, the existing legislation might not be applicable to this technology. Therefore, the scientifically informed public discussion of such legal framework is imperative [43, 75, 79].

6. Conclusions and final considerations

In recent years, the progress in the development of new tools for molecular genetic research has been evident since their application by simple, versatile, and efficient experimental techniques. From all the genome edition systems based on the nucleases application, CRISPR/Cas9 is the most friendly and simple method. It is now clear that the utility of this technology for the modification of specific loci is limited only by the interest of the researcher. In coming years,

debates are expected about the best use of organic and conventional agriculture, sustainable farming, and all that coming from biotechnology. Thus, CRISPR/Cas9 technology has changed the way we see the future of agriculture. On the other hand, the implementation and easy accessibility to the CRISPR/Cas9 technology has allowed the generation of diverse molecular methodologies that constitute significant advances in the genome edition and its subsequent exploitation for agrarian and health purposes. Therefore, this technique is considered a revolutionary tool.

The major challenges for CRISPR/Cas9 technology will focus on two underlying aspects. First, the corresponding ethical or bioethical discussion, in order to demarcate what should or should not be done with this tool considering the risks that we could face by using promising technology, an even more when this is accessible and cheap. On the other hand, the legal consequences in terms of intellectual property that today literally generate wars between law firms and universities for the patents generated by thousands of investigations must be considered. Although many scientists consider CRISPR/Cas9 system as a "Holy Grail of genetic engineering," we must not lose sight of the objectivity and rationality when interpreting the consequences of its use. Additionally, demanding compliance with all the necessary safety steps before this technology becomes a trivial routine, especially if this tool is used for genome editing, and the genetic improvement of living beings must be imperative.

The features of the CRISPR/Cas9 system have allowed opening the possibility of using it to perform gene and cell therapy, in addition to its application in plant genetic improvement. In general, this technology has been used as a tool to perform point mutations, homologous recombination by HDR, and silencing and activation or repression of gene transcription. Thanks to these properties, its application has been possible for genetic monitoring, analysis of metabolic pathways, functional genomic research, generation of animal models, discovery of possible targets for disease treatments, and, even, correction of phenotypes. Another application of great importance that should continue to be developed is the generation of more precise and representative plant lines for the study of phytopathogenic diseases. Knockdown, knockout, and knocking models show the advantage of being able to be quickly and efficiently generated with this system. Also, CRISPR/Cas9 is considered a great biotechnological tool in the field of human therapies since its capacity to perform genetic level corrections/deletions, which is traduced in the possibility of regulating transcription or translation pathways.

In relation to the introduction of CRISPR/Cas9 in agricultural and environmental sciences, several studies recognize the possibilities of this technique to improve crop varieties [25, 80]. Uncertainty about safety and efficacy of genome editing requires evaluating its potential and utility by applying the precautionary principle. Research on this technology also unchains important legal and social debates among genetic engineering and genomic editing, in order to establish whether new mechanisms are needed to regulate research, confined use, voluntary release and if it is necessary to evaluate the possible impact on the environment just like in consumers' health, among other aspects. The application of the precautionary principle in any case must be done considering the available scientific evidence and raising the necessary social and economic considerations, in order to avoid a dogmatic interpretation that could undermine or stop scientific progress.

Nevertheless, despite the great potential of the CRISPR/Cas9 technique, it is also common to find evident limitations, since other alternatives have been proposed to improve genome editing with biotechnological processes [81]. Molecularly, the components of the CRISPR/Cas9 system are too large to be introduced into a viral genome (e.g.), and thus, they are most commonly used in gene therapy to transport foreign genetic material into human and plant cells. One solution for this problem is to use a smaller type of a Cas9 enzyme, obtained from *S. aureus* [82]. This enzyme is small enough to fit in the virus. The mini-Cas9 complex has been used in mice to correct the gene responsible for Duchenne muscular dystrophy [83].

The Cas9 enzyme not always cleavages where it is intended within the genome (a certain DNA sequence must be nearby for that to happen). This is easily accomplished in many genomes, but it may be a limitation in some experiments. Researchers are constantly looking for microbes to obtain enzymes with different genome characteristics to expand the number of sequences that can be modified. One of these enzymes is called Cpf1, and it can be an interesting alternative, since it is smaller than Cas9 and has different characteristics in its sequence that make it highly specific [84, 85]. Another enzyme called C2c2 is able to target RNA instead of DNA, which is why it has a great potential to study RNA and to fight plant viruses with this type of genomes [86].

Several laboratories apply CRISPR/Cas9 technology in order to eliminate specific regions in a gene sequence, thus repressing its function. Those who want to exchange a sequence for another face a more difficult task. When Cas9 cleavages the DNA, the cell makes mistakes by regrouping the loose ends, thus obtaining desired deletions. Researchers who want to rewrite a DNA sequence rely on different repair pathways that can insert new sequences (a process that occurs at a much lower frequency). Cas9 directs the sequence dictated by its guiding RNA, but does not cut it; instead, the bound enzyme changes the letters of DNA, ultimately producing a T where once there was a C [87].

In contrast to the above, recent new gene editing systems have been released using a protein called NgAgo to cleavage DNA at a predetermined site without the need for an RNA guide or a genome-specific neighbor sequence. Instead, the protein (of bacterial origin) is programmed using a short DNA sequence corresponding to the target sequence. However, laboratories have failed to reproduce the results so far, so the effectiveness of this technique cannot be affirmed [88]. There are also other genetic editing systems, some of which have existed for years. For example, scientists rely heavily on a system called lambda Red, which can be programmed to alter DNA sequences without the need for a RNA guide.

In light of the above considerations, we can finally conclude that the biotechnological tools that belong to cas9 toolbox that in synergism with new bioinformatic algorithms increase their potential in a specific and powerful way and help position this technology as a magnificent last generation method for genomic edition, which is considered a revolutionary scientific discovery for both basic and applied research including the field of plant biotechnology, even when there are inconclusive details of its application in the laboratory and very probably a gross ignorance of its nature, for now.

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Conflict of interest

The authors declare no conflict of interest regarding the conduct of this work and the results obtained from it.

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