Chapter 11
Current Status and Future Prospective of Genome Editing Application in Maize

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Abstract Maize (*Zea mays* ssp. *mays*) is the most world-widely cultivated agricultural crop and over the past century, its yield per unit land area has increased consistently due to both breeding efforts and improvement in management. Cross breeding, mutation breeding, and transgenic breeding are the main methodologies adopted for maize improvement. The first maize transgenic hybrids were commercialized more than 20 years ago, and till now more than 150 different events of transgenic maize have been approved for commercial cultivation. The sequencing of the maize genome and the development of advanced genomic tools provided the biologists with the theoretical information necessary to attempt the genome modification at the pre-intended genomic loci. The tremendous advances brought about by CRISPR/Cas systems from first applications to nowadays has made genome editing a powerful tool for precise maize improvement. Although many CRISPR-Cas-edited genes have been documented to improve maize traits of agronomic interest, only a few lines have been tested in field trials; additional work for determining potential breeding values of edited maize lines must be done in terms of field tests. The integration of CRISPR-Cas technology in the breeding of new maize varieties also depends on existing and future regulatory policies that will be adopted worldwide.

1 Introduction

Maize (*Zea mays* ssp. *mays*) is the most world-widely cultivated agricultural crop and a renowned experimental model plant for molecular and genetic studies. Maize domestication started about 9000 years ago from the wild grass Z. mays subsp. Parviglumis in the Balsas region of southwest Mexico [1, 2]. Morphological observations, genetic and genomic studies have elucidated how from *Z. mays* subsp. *Parviglumis* (also called "teosinte") *Z. mays* subsp. *mays* was

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A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_11

domesticated. Although a few major morphological differences distinguish these two subspecies, and genes responsible for these traits have been identified, it has emerged that several genomic regions have contributed to maize domestication [3]. Among the grasses, maize has a medium-sized genome of approximately 2.4 Gb [4], which is characterized by an exceptional genomic structural diversity. Large insertions and deletions that includes tandem repeats cluster and transposable elements are common between maize inbred lines. This structural diversity, characterized by copy number variants (CNVs) and presence/absence variants (PAVs) is important for maize adaptation and has offered a rich pool of genetic diversity to breeders for creating improved germplasm [5]. At the gene sequence level, single nucleotide polymorphisms (SNPs) are frequent in introns and untranslated regions of maize genome. SNPs surveys and subsequent quantitative traits genome wide association studies (QTL/GWAS) were adopted for linking genetic and phenotypic variations. In parallel, the sequencing of the B73 reference genome, which quality has been greatly improved during the last few years by the development of long-read sequencing (mainly by Pacific Biosciences' PacBio) single molecule real-time (SMRT) sequencing [6] have shed light on both the complexity of the maize genome and the contributions of different variations to phenotypic differences. In addition to the characterization of genetic variations, recent research in maize was aimed at understanding the relationships among gene expression, epigenetic modifications, chromatin interactions, and metabolic, proteome, and phenotype variations. The development of high-quality genome assembly tools together with the precise characterization of genomic diversity and the association of genetic variants with yield-related traits has greatly improved maize genomic research. Till now, thousands of diverse and representative maize lines have been genotyped [7] and the integration of multiple annotated reference genomes has been facilitated by comprehensive databases that store, maintain, analyze, and visualize the multi-omics data, such as MaizeGDB [8] and ZEAMAP [9]. The development of genomic technologies has allowed a deeply exploration of diversity at all levels in different environments and the identification of genes that determine maize yield: the mechanistic understanding of gene function and the precise modification of genes in different genomic background can accelerate breeding for yield in the coming years.

2 A Glance Over Conventional Maize Breeding

Over the past century, maize yield per unit land area increased consistently (over sevenfold) due to both breeding efforts and improvement in management [7]. Breeding of hybrid crops was pioneered in maize since the observation, in early 1900s, that hybrid cultivars provided higher yield compared to pure lines and open pollinated varieties [10]. The constitution of modern maize hybrids relays on

development of elite parental inbred lines and their subsequent evaluation in single cross combination. The original methods for hybrid production introduced by Shull in 1908 (referred as "pure line method of corn breeding") [11] underwent several modifications over years. This allowed both a more efficient production of inbred lines and identification of superior hybrid combinations between them [12]. In modern maize breeding, the activity of evaluation of inbred lines for hybrid performance is the most critical and expensive phase. Inbred lines are collected in heterotic groups with the aim of facilitating the identification of superior hybrid combinations [10]. Additionally, to increase the number of lines having good potential for hybrid performance, population improvement methods are adopted, and double haploid (DH) technology is used to generate homozygous lines [13]. To introduce desirable alleles into a desired elite inbred line background by genetic crosses, 8–10 selfing generations are required. Moreover, extensive background screening and evaluation of large-sized progenies are necessary to increase the chance of genetic recombination and reduce possible linkage drag effects, when the desirable trait is closely linked to an undesirable trait [7]. The advantage of DH technology relays on the much quicker development of homozygous lines compared to 8–10 generations of inbreeding by selfing or sib-crossing necessary to develop inbreds [14]. Nowadays in maize breeding programs, DH are routinely obtained by pollinating the plants with haploid inducer (HI) lines. Subsequently, chromosomes can be doubled spontaneously or artificially, by treatment with mitotic inhibitors such as colchicine, for generating DH lines.

Cross breeding, mutation breeding, and transgenic breeding are the main methodologies adopted for maize improvement. As illustrated above, in maize to introduce desirable alleles by cross breeding, and use genetic recombination to produce genetic variability are long and costly procedure. Additionally, for some agronomic traits genetic variability has been greatly reduced by domestication and directed selection [15]. To overcome these limits, mutation breeding has created genetic variation by introducing random mutations in the maize genome [16]. However, the stochastic nature of the mutations produced and the need to screen large numbers of mutant genotypes makes mutation breeding a time-consuming and laborious procedure that cannot enhance selection efficiency, even if marker-assisted breeding approach are adopted. Transgenic breeding through the transfer of exogenous genes into commercial elite varieties can accelerate the improvement of important agronomic traits. However, along with some limitations of the methodology, such as the random insertion of the transgene and the low number of sequences that can be introduce in the genome by genetic transformation, the long and costly deregulation processes, and public concerns about transgenic crops, limit the commercialization of genetically modified maize plants [17]. In the following chapter we will highlight how the introduction of targeted mutagenesis and the combination of these novel technologies with conventional breeding procedures can solve some of the main limiting factors for a more sustainable maize breeding.

3 Maize Genetic Transformation

Technological innovation and scientific discoveries have always had a big impact on maize research and breeding, and genetic transformation has been an indispensable biotechnology in both applied and basic maize research. In basic research transformation technologies were widely adopted to study gene regulation and function, mainly through the obtention of mutant plants in which the target sequences are over-expressed, expressed ectopically, downregulated or silenced. Additionally, transgenesis allows the study of gene promoters and other regulatory sequences regulating gene expression in the coding portion of the genome. As already mentioned above, in applied maize research, the introgression of beneficial target genes from one line (donor) to another (recipient) by conventional breeding requires many years of backcrossing after hybridization and may lead to linkage drug effect on the recipient line genome [7]. Conversely, genetic transformation introduces well characterized DNA regulatory and coding sequences into the plant genome. The goal to transform maize with a high efficiency technology, providing high-quality transgenic events has been essential to improve specific maize traits [18]. Initially, the production of genetically modified maize varieties has encountered enormous difficulties, mainly for the genotype-associated recalcitrance to transformation. In late 80', progresses in genetic engineering and biotechnology resulted in stable transformation of maize [19]. Fromm and colleagues stably transformed maize cells for resistance to kanamycin by electroporation-mediated DNA transfer of a chimeric gene encoding neomycin phosphotransferase. In 1987 Grimsley and colleagues reported that maize plants developed symptoms of viral infection when inoculated with strains of Agrobacterium carrying copies of maize streak virus (MSV) genomes in their T-DNA, thus demonstrating that Agrobacterium could transfer DNA to maize [20]. The first genetically transformed infertile maize plants were obtained from embryogenic cell derived protoplasts treated with plasmid DNA containing a gene coding for neomycin phosphotransferase (NPT II) driven by the 35S promoter region of cauliflower mosaic virus [21]. Finally, fertile transgenic maize plants were produced from embryogenic cell suspension transformed with the bacterial gene bar, encoding for phosphinothricin acetyltransferase (PAT), using microprojectile bombardment [22]. Although several protocols for Agrobacterium- and polyethylene glycol (PEG) protoplast-mediated transformation were developed before 2000s [23, 24] transformation efficiency and successful *in vitro* plantlet regeneration through tissue culture was highly dependent on genotypes. Therefore, hybrid lines showing the ability to produce highly transformable calluses were selected: High type II callus (Hi II), containing both A188 and B73 inbred genetic background, became one of the most widely used hybrids for maize transformation in both academic and plant industrial labs [25, 26]. Commonly for maize transformation, embryogenic callus started from immature embryos and cell suspension cultures of embryogenic callus were used [27–29]. Nowadays, although maize transformation is routinely performed, a few genotypes (A188, B104) have been reported to have acceptable transformation efficiency comparable to Hi-II, which have remained the

most popular lines for commercial transformation. For instance, the maize inbred line B73 that is the first inbred to be sequenced and an important genetic resource is strongly recalcitrant to transformation, as well as most of the commercial elite maize inbred lines. The discovery that transgenic maize genotypes overexpressing BABY Boom (ZmBbm), WUSHEL (ZmWus2) and OVULE DEVELOPMENT PROTEIN 2 (ODP2) genes can enable high transformation frequencies in numerous recalcitrant genotypes was an important milestone for maize transformation [30]. Morphogenic Regulator-Mediated Transformation (MRMT) vectors containing these morphogenetic genes can be introduced into Agrobacterium strains and used for immature embryo transformation. Through MRMT increased plant regeneration rates, recovery of transformed plants from recalcitrant genotypes, and a shortening in time needed for transformation by avoiding the callus culture step have been obtained [31]. However, since the constitutive expression of MRs can have a negative pleiotropic effect on important developmental traits, its expression has to be restricted to the embryogenesis induction step, either by excision of the MR expression cassette through a recombination system or driving the expression of MRs using specific promoters [31].

The first maize transgenic hybrids were commercialized more than 20 years ago, and till now more than 150 different events of transgenic maize have been approved for commercial cultivation or food/feed use. Most of the released events concerns simple traits, such as herbicide tolerance, insect resistance, modified product quality, pollination control system and abiotic stress tolerance [32]. It is a matter of fact that applying transgenic approaches for the improvement of complex traits is difficult, because these traits are controlled by numerous genomic loci with a small effect and are strongly influenced by the environment. Evidently this represents a limitation for maize improvement because the limitation concerns the integration of biotechnology and traditional breeding in the improvement of complex traits [33]. One more limitation of transgenic approaches is that foreign DNA integrates into random sites of the host genome. Random integration of transgene might affect the transgene expression, although some recent observations did not prove the assumption of this risk [34]. Moreover, from the first transgenic hybrid commercialization new techniques were developed, new regulations were adopted, and despite their significant beneficial impact on modern agriculture, public perception is still controversial about transgenic crops. The high costs necessary for the deregulation of genetically modified commercial maize plants can be afford only by the largest agricultural biotechnology companies, with a consequent increasing concentration of maize seed providers [35].

4 Mutagenesis in Maize

Traditionally in maize, mutagenesis has been an impressive useful tool for both broadening genetic variation and understanding gene function. Numerous strategies were developed for creating mutations and identifying genes based on phenotypes

(forward genetics). The same strategies have also been used to assignee a phenotype to target coding sequences (reverse genetics). While in early mutagenesis experiments UV and X rays [36, 37], ethyl methanesulfonate (EMS) and other chemical mutagens were used, the main strategies for comprehensive mutagenesis of maize genes comprise TILLING (Targeting Induced Local Lesions IN Genomes) [38], RNAi [39] and transposons mutagenesis. The discovery and characterization of transposon families as well as their massive presence into maize genome facilitated the development of transposable element systems, particularly useful for two maize inbreds, namely B73 and W22. In recent years these systems based on Class II "cut and paste" maize transposons were implemented for creating based genetic resources, such as Ac/Ds families and UniformMu available through MaizeGDB (https://www.maizegdb.org/) and Maize Genetics Cooperation Stock Center (http://maizecoop.crops ci.uiuc.edu/) respectively. In the genomic era maize transposons have been the premier method for gene discovery and phenotypic-related characterization in a whole -organism context [40].

The sequencing of the maize genome and the development of advanced genomic tools provided the biologists with the theoretical information necessary to attempt the genome modification at the pre-intended genomic loci in a more precise way than random mutation breeding, which can be time consuming and expensive for the large screens needed. Targeted mutagenesis initial attempts were rarely successful in maize, due to the very low frequency of homologous recombination (HR) events involving either endogenous target or exogenous donor DNA [41]. However, the adaptation to maize of Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) two novel technologies successfully applied in mammalian cells and in a few model plants, such as Arabidopsis and tobacco, allowed the first genome editing events on maize endogenous target genes. Targeted genome editing tools use nucleases to induce DNA double-strand breaks (DSBs). In plant cells, DSBs can be repaired by two main pathways, nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). The NHEJ pathway usually generates in/dels at the repair sites. Differently, when a template DNA is provided HDR can be adopted for precise sequence replacement or insertion [42]. The ZFNs are chimeric proteins with two domains: the N-terminal domain is a synthetic zinc finger-based domain that recognizes a 3-base-pair (bp) target sequence and binds to DNA; the C-terminal domain is a non-specific DNA cleavage domain using Fok1 a type IIS class of restriction endonucleases [43]. Because FokI functions as a dimer, ZFNs are designed as two ZFN monomers bound to an 18- or 24-bp sequence with a 5–7-nucleotide spacer. This spacing is a critical part of ZFN design as it allows Fok1 monomer to dimerise and create a DSB in the target sequence. A pair of Zinc finger arrays (ZFAs) binds to respective sequences targeted and get aligned in reverse fashion with each other. In 2009, Schukla et al. [44] reported the use of designed (ZFNs) that induced a double-stranded break to modify a target endogenous locus in maize. The simultaneous expression of ZFNs and delivery of a simple heterologous donor molecule allowed the targeted addition of an herbicide-tolerance gene, one of the phytic acid biosynthesis genes, namely inositol-1,3,4,5,6-pentakisphosphate kinase 1 (IPK1). ZFN-modified maize plants

could pass these genetic changes to their progeny: in developing maize seeds the targeted cleavage of IPK1 gives the characteristics of both herbicide tolerance and desired alteration of the inositol phosphate [44]. About five years later, the TALEN technology was successful applied for targeted gene mutagenesis in a proof-of-concept study in maize [45]. Similarly to ZFNs, TALENs are fusion proteins of native or artificial TAL effector DNA-binding domains and the DNA-cleavage domain of FokI. The modular TAL effector repeats can be custom-tailored into DNA recognizing domains for virtually any sequence in a genome [46]. When expressed in plant cells, the paired TALENs recognize and bind to two adjacent, opposite subsites, enabling the FokI domains (homo- or heterodimeric) to dimerize to an approximately 50–60-bp target sequence within a 14–18-bp spacer, which is necessary for its function. In maize, TALENs were employed to generate heritable mutations at the glossy2 (gl2) locus. Hi-II transgenic lines containing mono- or di-allelic mutations were produced at a frequency of about 10%. Three modified alleles were functionally tested in progeny seedlings, demonstrating that they conferred the glossy phenotype. The authors reported that the integrated TALEN T-DNA segregated independently from the loss of function gl2 alleles in most of the events, generating mutated null-segregant progeny in T1 generation [45].

These results confirmed ZFNs and TALENs as new strategies for maize genome editing in basic science, with potential in breeding applications. However, owing to construction complexity, high off target rate of ZFNs and high costs of result screenings they had limited applications in maize till now.

5 The CRISPR Technology Application in Maize

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system, comprising CRISPR repeat-spacer arrays and Cas proteins, is an RNA- mediated adaptive immune system in bacteria and archaea. It provides defense against invasive genetic elements by cleaving their nucleic acid genome [47]. The detailed description of the system and its classification in classes and types based on Cas genes are provided in other chapters of this book. Here we will focus on the CRISPR system that has been developed and improved for genome editing in maize, which is generally based on RNA-guided interference with DNA. Briefly, the CRISPR/Cas9 approach uses an RNA-guided endonuclease to generate DNA DSBs at the target sites of the plant genome. The type II CRISPR/Cas9 system adopted in plants requires the hetero-duplex RNA of CRISPR RNA (crRNA) and auxiliary trans-activating crRNA (tracrRNA) to guide the non-specific nuclease, Cas9, for DNA cleavage. In Cas9 the nuclease domains RuvC and HNH cleave the complementary DNA strands [48]. The crRNA and tracrRNA can be further replaced by a single chimeric guide RNA (sgRNA) [47] that can recognize any genomic locus that is followed by a 5′-NGG protospacer-adjacent motif (PAM), and a 20-nt sequence preceding the PAM directs the Cas9 and cleave the target sequence by complementary base pairing. Differently from ZFNs and TALENs that

require sophisticated protein engineering to define precise target site recognition, Cas9-sgRNA components are simple to design and clone. Additionally, the fewer limitations of potential target sites in plant genomes make the CRISPR/Cas system widely applicable. When using this technology, the NHEJ repair pathway is the preferred way to disrupt genes by producing small indels at specific sites in target genes. Moreover, NHEJ can also be exploited to produce insertions of donor DNA sequences in a homology-independent manner [49]. An important choice to make for the application of this technology in maize is the system of delivery to cells of editing reagents, which include DNA, RNA, and ribonucleoproteins (RNPs). Protoplast transfection is commonly uses for transient expression, while Agrobacterium-mediated transfer DNA (T-DNA) transformation, or particle bombardment are the delivery methods of choice to produce edited plants.

In 2014 Xing et al. reported the development of a toolkit to facilitate transient or stable expression of the CRISPR/Cas9 system and generate mutants bearing multiple gene mutations in a variety of plant species, which comprised maize [50]. This system was composed by a CRISPR/Cas9-based binary vector set and a sgRNA module vector set and was validated using maize protoplasts and maize transgenic lines for the targeting of the same maize genomic DNA site (ZmHKT1). In the same year, a comparison between two systems of targeted mutagenesis TALENs and the CRISPR/Cas was conducted by Liang and colleagues [51]. Five TALENs targeting the genes ZmPDS, ZmIPK1A, ZmIPK, ZmMRP4 and two sgRNAs targeting the ZmIPK gene were constructed. The efficiency in inducing targeted mutations was similar for the two systems: 13.1% CRISPR/Cas and 9.1% TALENs, respectively, in maize protoplasts.

The first detailed report in a scientific journal of Cas9-gRNA genome editing technology application in maize, with direct delivery of sgRNA in the form of RNA molecules, was published by Svitashev et al. in 2015 [52]. For targeting five different maize genomic regions, the biolistic transformation of maize immature embryos was conducted with DNA vectors expressing a maize codon optimized Cas9 endonuclease and sgRNAs, with or without DNA repair templates. The genomic regions were located upstream of the liguleless1 (LIG1) gene, at male fertility genes (Ms26 and Ms45) and acetolactate synthase (ALS) genes (ALS1 and ALS2). Following transformations, the authors could identify mutations at all sites targeted, as well as plants containing biallelic multiplex mutations at LIG1, Ms26, and Ms45. When immature embryo cells containing pre-integrated Cas9 were directly used for biolistic delivery of sgRNAs (as RNA molecules) targeted mutations could also be detected. The same authors also reported the editing of ALS2 gene and the recovery of chlorsulfuron-resistant plants, using either single-stranded oligonucleotides or double-stranded DNA vectors as repair templates yielded. Moreover, RNA-guided Cas9 endonuclease- generated double-strand breaks at a site near LIG1 stimulated insertion of a trait gene by homology-directed repair. In all cases of genes knockouts, edits, and insertions, T1 plants genetic analysis showed that the mutations followed a Mendelian segregation in subsequent generations.

CRISPR/Cas9 has been also applied for the targeted knockout of the endogenous ZmPSY1 gene in maize T0 transgenic plants [53]. Interestingly in this work, the

authors configured and optimized the CRISPR-Cas9 system for targeted genome editing in maize and accurately tested the activity of their customized system. Appropriate vectors to express Cas9 and sgRNAs for maize were generated. Cas9 open reading frame was maize codon-optimized and driven by maize Ubiquitin2 promoter. Additionally, to the N terminus of Cas9 a monopartite SV40 nuclear localization signal (NLS) was added and, to further facilitate nuclear localization, a bipartite nucleoplasmin nuclear localization signal (BiNLS) was added to the Cas9 C terminus. Similarly, the authors accurately studied the maize promoter that was used to transcribe the short noncoding sgRNA. A reasonably high mutation rate was firstly established in maize protoplasts. Subsequently, the mutations occurred in germ cells and were transmitted to the next generation with high efficiency. No off-target effect could be detected at the computationally predicted putative off-target loci and no significant difference between the transcriptomes of the Cas9 expressed and non-expressed lines was reported [53].

In maize, multiplex gene editing was achieved by expressing Cas9 together with multiple gRNAs, each targeting different sites and using conventional delivery methods. In principle, single construct containing more than one guide RNA can accelerate and improve the transformation procedures. Two procedures have been developed: the first was based on a multi-guide RNA activated by a single promoter and processed by tRNA motif-mediated self-cleavage into several sgRNAs [54] the second based on tandem repeats of different U3 and U6 promoters each controlling one guide RNA [55]. In both cases, the mutations resulting from targeted mutagenesis were mainly deletions or insertions of a few nucleotides probably due to NHEJ.

In the works described above, to produce edited maize plants, transformation mediated by Agrobacterium or particle bombardment was used for delivering CRISPR/Cas DNA and a selectable marker gene into recipient maize cells and these DNA constructs were stably integrated into the plant genome. However, this strategy of stable integration might increase off-target changes, gene disruption and plant mosaicism, as well as limit commercial applications. Transgene-free edited plants can be selected through genetic segregation by selfing and crossing, which is time consuming in maize hybrid breeding. To avoid these negative effects, transgenic maize plants with pre-integrated Cas9 nuclease have been generated and used for delivery of sgRNAs in the form of RNA molecules. However, this strategy requires the specific development and characterization of Cas9 pre-integrated lines. Transient gene expression of DNA constructs in protoplasts could represent an alternative approach for achieving transgene-free editing in plant. Till now, maize protoplast transient transformation experiments serve mainly for the evaluation of the efficiency of different Cas9 and sgRNA designs, due to the lack of an efficient protocol for the regeneration of maize plants from protoplasts. In 2016 Svitashev [56] and colleagues reported for the first time the biolistic delivery of in vitro assembled Cas9–sgRNA ribonucleoproteins (RNPs) into maize embryo cells and regeneration of plants with both mutated and edited alleles. Purified Cas9 protein pre-assembled with in vitro transcribed gRNAs were delivered into maize immature embryo cells. The previously studied four genomic regions, liguleless1 (LIG), acetolactate synthase (ALS2) and two male fertility genes (MS26 and MS45) [52] were

targeted. Using this method of delivery, they produced DNA- and selectable marker-free maize plants with mutated alleles at high frequencies [56]. As observed in other systems, delivery of RNP complexes led to a significantly decreased undesired mutation frequencies in comparison to DNA vectors. While the frequency of off-site mutations was about 50% for MS45 target site for both immature embryos and mature plants when Cas9 and sgRNA were delivered on DNA vectors, off-site mutations were not detected in regenerated plants when RNPs were used. These results demonstrated that Cas9–gRNA delivered as RNP complex has a significant advantage over DNA vector delivery by promoting high mutation frequencies in a more precise manner also in maize, as already observed for other organisms and plant species [57, 58].

Although involving genes and loci of agronomic interest, many of the initial works using CRISPR/Cas9 were proof-of-principle studies to test genome editing different strategies and efficiency for applications in precision breeding. For an efficient targeted genome editing in maize, the promoters for driving both Cas9 and sgRNA expression were proven to be an essential factor. In early works, the maize ubiquitin gene promoter was used in construct containing a maize codon optimized Cas9. In the same experiments the rice U3 or wheat U3 promoters were used for driving sgRNAs [50, 51]. Different promoter combinations, such as maize ubiquitin1 and U6 promoters, CaMV35S and maize U3, and maize ubiquitin1 gene promoter and two rice U6 promoters, were utilized for Cas9 and sgRNAs in other studies [53, 55, 59] providing additional evidence that the optimization of the promoters used for the CRISPR/Cas9 system is an essential step for efficient targeted genome editing procedures. Furthermore, the mutation efficiency of CRISPR/Cas9 system appeared largely depending upon both the expressions of Cas9 and sgRNAs. Feng and colleagues observed that when the 35S promoter was used for driving the expression of a human codon optimized Cas9 a low mutation rate was obtained and most of the regenerated T0 plants were mosaic [59]. For increasing the mutation efficiency and concomitantly avoiding mosaicism, in a subsequent work they used the promoter of dmc1 gene. DMC1 was thought to be expressed specifically in meiocytes and was used for expressing the Cas9, combined with the U3 promoter for driving the sgRNA expression. The authors reasoned that utilizing these constructs gametes could be mutated, and T1 homozygous or bi-allelic mutants could have been recovered at high frequency at the three loci selected for targeting in the maize genome. However, during their transformation experiments, they realized that the dmc1 promoter could drive the Cas9 to be highly expressed also in maize callus. This observation was confirmed by expression analysis of the endogenous dmc1 gene in different maize tissues including callus. The dmc1 gene was found to have the highest expression level in tassel but was also highly expressed in callus. Using this transformation approach, the T0 plants regenerated were highly efficiently edited at the target sites with homozygous or bi-allelic mutants accounting for about 66%; mutations could be stably transmitted to the T1 generation, while no off-target mutations could be detected in the predicted loci with sequence similarity to the targeted site [60].

After having established the CRISPR/Cas9 technology platform and confirmed its efficiency for single and multiple GT in maize, subsequent studies focused on exploitation of targeted editing for hybrid-breeding technique improvement. In maize, male-sterile maternal lines are an essential prerequisite for generating high-quality commercial hybrid varieties. ZmTMS5 gene controls a thermosensitive male-sterility trait in maize and is the orthologue of the previously characterized and edited TMS5 rice gene [61]. The targeted knockout of the ZmTMS5 gene using immature Hi-II embryos transformation by particle bombardment for delivering CRISPR/Cas9 elements, produced T1 tms5 mutant plants, male-sterile at 32 °C, but male-fertile at 24 °C. T1 plants were Cas9-free through segregation and carried only the desired tms5 mutation. They provide a useful germplasm that can potentially be used to simplify hybrid maize seed production [62].

A potential application of the genome-editing technology concerns the reduction of so-called linkage drag during back-cross breeding. Direct genome-editing technology provides the opportunity of stacking favorable genes without introgression breeding. An experimental proof-of-concept to validate this strategy was provided by the work of Li and coworkers [63]. They established an RNA-guided endonuclease (RGEN) system as an in vivo desired-target mutator (DTM) in maize, to reduce the linkage drag during breeding procedure, using the LIGULELESS1 (LG1) locus as target. The RNA-guided Cas9 system showed 51.5–91.2% mutation frequency in T0 transgenic plants. The T1 plants stably expressing DTM were crossed with six diverse recipient maize lines producing 11.79–28.71% of mutants. Furthermore, the analysis of F2 plants showed that the mutations induced by the DTM effect were heritable. The results were confirmed by the phenotypical characterization of the mutant plants in the field [63].

A further major technical limitation of utilizing gene targeting technologies resides on the recalcitrant nature of most elite maize inbred lines for genetic transformation. To facilitate GT techniques, initials functional tests and transformations are usually done in maize lines with relatively high transformation efficiencies, such as Hi-II and B104. Subsequently, the selected and desired transformation events must be introgressed into elite commercial inbred lines, through at least six back-crossing for achieving more than 99% background purity, requiring additional work and time.

Doubled haploid (DH) technology based on in vivo haploid induction (HI) is an important modern approach for maize breeding. Therefore, there is the need to identify candidate genes underlying HI in maize genome for their detailed functional characterization. Several genes involved in HI were recently validated through CRISPR–Cas9 system [64–66].

In 2018 Wang and colleagues reported the validation of a new strategy for the development of a haploid-inducer mediated genome editing system (IMGE) for accelerating maize breeding. This system uses a maize haploid inducer line carrying a CRISPR/Cas9 cassette targeting for a desired agronomic trait to pollinate an elite maize inbred line. The pollination can generate genome edited haploid maize plants in the elite background. During the process, HI genome is degraded, and no editing

tools are present in edited plants [67]. Within two generations homozygous pure DH lines improved for the desired trait could be obtained, avoiding repeated crossing and backcrossing used in traditional breeding for introgressing a desirable trait into elite commercial backgrounds [67]. Similarly, Kelliher and colleagues co-opted the aberrant reproductive process of haploid induction (HI) to induce edits in nascent seeds in maize and other monocot and dicot species. Their method, named HI-Edit, made direct genomic modification of commercial crop varieties possible, and was tested in field and sweet corn using a native haploid-inducer line. Also in this case, edited haploid plants lack both the haploid-inducer parental DNA and the editing machinery and could be used in trait testing and directly integrated into commercial variety development [68].

5.1 Novel Approaches for Maize Trait Improvement

Based on experimental proof-of-concept reported above, the application of CRISPR/Cas technology not only allows to modify the agronomic traits of interest through the insertion or deletion of single or few nucleotides, but it can also facilitate maize breeding by inserting new alleles in the genome without any linkage drag. Additionally, precise gene modifications can generate novel allelic variants by knock-ins and replacements, thus having great value for crop trait improvement Moreover, knock-in can be used to alter multiple elite traits by stacking genes in a single variety. CRISPR/Cas- systems are currently being applied for enhancing yield, product quality, resistance to diseases and abiotic stress [69].

Novel allelic variants for breeding drought-tolerant plants have been generated in maize [70]. Starting from the observation that maize transgenic plants constitutively overexpressing ARGOS8, which is a negative regulator of ethylene responses, have reduced ethylene sensitivity and improved grain yield under drought stress conditions [70], new variants of ARGOS8 were generated employing CRISPR-Cas technology. Precise genomic DNA modification at the ARGOS8 locus was produced by inserting the native maize GOS2 promoter into the 5′-untranslated region of the native ARGOS8 gene or was used to replace the promoter of ARGOS8. The modified ARGOS8 variants was highly expressed in all plant tissues and increased grain yield under flowering stress conditions when plants were grown in the field [71].

CRISPR–Cas9 can be used for editing of cis-elements, such as in promoters, alternatively to the generation of weak alleles by targeting coding regions. The fine-tuning of gene expression by editing of cis-regulatory elements can lead to quantitative trait variation. Weak promoter alleles of CLV3/EMBRYO-SURROUNDING REGION (ZmCLE) and ZmFON2-LIKE CLE PROTEIN1 (ZmFCP1) were engineered in maize with the aim to create quantitative variation for yield-related traits [72]. For CLE and FCP1 promoter mutagenesis, Cas9 with nine sgRNAs targeting promoter in accessible chromatin regions in developing ears were designed. Multiple maize grain-yield-related traits were successfully increased by using this strategy.

In the same work, Liu and colleagues demonstrated in an elegant way that, in addition to weak allele promoter editing, exploitation of compensation among paralogs can be used for improving maize traits through genome editing [72].

The construction of whole-genome-scale mutant libraries is a modern approach for both functional genomic studies and pre-breeding improvement. Commonly in plants mutant libraries are based on random mutations induced by different mutagenesis procedures like irradiation, T-DNA insertions, (EMS), and transposons. In all these cases many generations to stabilize loss-of-function mutations are required, and additionally the process for determining the relationship between phenotype and genotype in all mutants is a long and critical process.

Liu and co-workers [72] reported the development of a CRISPR/Cas9-based editing platform adapted to high-throughput gene targeting in maize. Li and colleagues established a low-cost optimized and quality-controlled pipelines that includes the design of guide RNAs (sgRNAs) up to the identification of targeted genes and edited sequences. They selected knowledge-driven candidate genes and screened a large number of mutants up to T1 or follow-up generations, showing that their platform allowed functional gene cloning and validation [63].

6 Prospects

In 2016, company researchers from Iowa–based DuPont Pioneer (currently Corteva) using the gene-editing tool CRISPR-Cas9, knocked out the endogenous maize waxy gene Wx1, which encodes the endosperm's granule-bound starch synthase responsible for producing amylose. Engineered maize contains starch composed exclusively of the branched polysaccharide amylopectin and not amylose. DuPont Pioneer expected the CRISPR-edited variety to have improved yields than conventional waxy maize and to commercialize the improved variety within five years [73]. Due to the high costs associated with the deregulation of genetically modified commercial maize hybrids only the largest agricultural biotechnology companies can afford these costs, with a consequent increasing concentration of maize seed providers [35]. It has been suggested that this scenario could be modified by both the benefits of a wider application of gene-editing technologies and reduced regulatory oversight of CRISPR-derived varieties in comparison to transgenic GM breeding technologies [74].

Using maize as a model species and CRISPR/Cas9 technology, a very recent interesting European initiative has developed a pipeline called BREEDIT to generate a collection of multiplex gene-edited plants [75]. BREEDIT combines multiplex genome editing of whole gene families with crossing schemes to improve maize quantitative traits. The researchers were able to knock out 48 growth-related maize genes and produced a collection of over 1000 gene-edited plants which displayed 5–10% increases in leaf length and up to 20% increases in leaf width compared with the controls. BREEDIT has the potential to generate diverse collections of mutants for the identification of allelic variants for use in breeding programs.

The tremendous advances in both basic plant research and crop breeding brought about by CRISPR/Cas systems from first applications to nowadays has made genome editing a powerful tool for precise maize improvement through multiple approaches, comprising point mutation, gene knock-out -in, allele replacement, fine-tuning of gene expression, and other modifications at any potential genome locus. It is expected that in the next years novel strategies will be designed to improve the specificity of Cas9-linked base editors, such as extending sgRNA guide sequences, and delivering base editors via RNP in many crops and in maize as well [58, 69].

Although many CRISPR-Cas-edited genes have been documented to improve maize traits of agronomic interest, only a few lines have been tested in field trials, with only CRISPR-waxy maize hybrids having had a limited diffusion in United States [76]. Thus, much additional work for determining potential breeding values of edited maize lines must be done in terms of field tests. For an efficient transfer of technologies from the bench to the field there is still the need to elucidate the genetic and regulatory architecture of important traits as well as to increase the efficiency of all steps of gene targeting and subsequent plant regeneration procedures. Finally, the integration of CRISPR-Cas technology in the breeding of new maize varieties also depends on existing and future regulatory policies that will be adopted worldwide.

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