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REVIEW ARTICLE

CRISPR-Cas mediated genome editing technologies in Plants

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ABSTRACT
Crop improvement for food and nutritional security, especially in the perspective of global population explosion and such challenges as climate change and water scarcity, have become intimidating tasks for the scientific community. The global population is predicted to reach 9.6 billion by 2050 from the current 7.3 billion. In modern agriculture, cross breeding, mutation breeding, and transgenic breeding are the primary crop improvement strategies in the present era. Conventional crop breeding, which relies on genetic variations from spontaneous mutations, physical or chemical mutagens, and recombination following hybridization, is usually cumbersome, and time-consuming, and cannot keep pace with the increasing food demand. Continuous advances will thus be required to meet these challenges and achieve sustainable agricultural production. Recent developments in CRISPR (Clustered regularly interspaced short palindromic repeats) technologies make the targeted and precise crop genome engineering a reality, and can thus speed up precision breeding for crop improvement. In the present chapter, we provide the overview of CRISPR technology, its important applications in improving abiotic/biotic stress tolerance, yield, and quality parameters in plants. We also provide an update on newly discovered CRISPR/Cas systems for use in plants. Some recent breakthrough technologies have also been highlighted providing potential for synthetic biology and crop domestication. We also discuss the implications of regulatory landscape for utilization of CRISPR technology in the developing world.
Keywords: *Genome editing, CRISPR, crop improvement, abiotic stress, biotic stress, yield, quality, precision breeding*

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INTRODUCTION

Agricultural production faces the challenge of expanding sustainably and maintaining nutritional quality under intensifying climate change. In the present scenario, the most critical challenge which the human race is facing is to provide food security for a growing population. The human population will reach to 10 billion by 2050 and to feed the world, global food production needs to increase by 60–100% [1]. The development of various technologies can contribute to crop improvement by increasing production to some extent. Genetic manipulation techniques using physical, chemical and biological (T-DNA insertion/ transposons) mutagenesis have contributed majorly in studying the role of genes and identifying the biological mechanisms for the improvement of crop species in the past few decades [2]. Recent advances in CRISPR (clustered regularly interspaced short palindromic repeats) technologies make the targeted and precise genetic manipulation of crops a reality, and can thereby accelerate the transition towards precision breeding for crop improvement. In the last decade, the use of genome editing technologies with site-specific nucleases (SSNs) has successfully demonstrated precise gene editing in both animal and plant systems. In contrast to the transgenic approach, which leads to random insertions and very often random phenotypes, genome editing methods produce defined mutants, thus becoming a potent tool in functional genomics and crop breeding. Genome edited crops have an additional advantage over transgenic plants since they 'carry' their edited DNA for the desired trait [3]. Such improved crops can be used in breeding programs and the resulting varieties can be used directly with lesser acceptability/consumption issues and relatively lesser regulatory procedures compared to conventional genetically modified (GM) crops [4]. Genome-editing tools have been used to eject precise modifications in many plant genomes. They have had a great influence on basic research as well as crop improvement. More recent modification methods, especially CRISPR/Cas, have improved the robustness of this process by allowing genetic changes to be accomplished without any integration of foreign DNA, through transient expression of a site-specific nuclease within the plant cell [5]. In practical terms, genome-editing

technologies offer a great chance for improving crops and ensuring global food security. We should grasp this opportunity to increase crop productivity and potentially save the lives of millions of people around the world, particularly in developing nations.

Genome editing is defined as a collection of advanced molecular biology techniques that facilitate precise, efficient, and targeted modifications at genomic loci (Zhang *et al.,* 2019). Genome editing using zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) has been around for two decades, but it has recently come under the spotlight through the development of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas systems [6], which provide simplicity and ease of targeted gene editing. All of these technologies use typical sequence-specific nucleases (SSNs) that can be induced to recognize specific DNA sequences and to generate double-stranded breaks (DSBs). The plant's endogenous repair systems fix the DSBs either by non-homologous end joining (NHEJ), which can lead to the insertion or deletion of nucleotides thereby causing gene knockouts, or by homologous recombination (HR), which can cause gene replacements and insertions [7].Many gene knockout mutants and some gene replacement and insertion mutants have been produced through the use of genome-editing technologies in a wide variety of plants, and many of these mutants have been shown to be useful for crop improvement. The risks involved in altering genomes through the use of genome-editing technology are significantly lower than those associated with GM [8]. Thus, the introduction of genome editing into modern breeding programs should facilitate rapid and precise crop improvement.

CRISPR technology is based on RNA-programmed DNA cleavage systems that were discovered in bacteria and archaea. CRISPR–Cas9 and CRISPR–Cas12a are the best-studied and most widely used CRISPR system [9]. Each system has two components: a DNA endonuclease (Cas9 or Cas12a) and an RNA molecule that confers targeting specificity, known as single-guide RNA (sgRNA) or CRISPR RNA (crRNA) [10]. The only prerequisite for applying CRISPR to a given target is the presence of a protospacer-adjacent motif (PAM) sequence near the site of interest. Using CRISPR for various targets thus only requires different *spacer* sequences; hence it is simple, rapid, efficient, inexpensive and versatile. Implementing a CRISPR project involves simple steps *viz.* (i) identifying the PAM sequence in the target gene, (ii) synthesizing a single gRNA (sgRNA), (iii) cloning the sgRNA into a suitable binary vector, (iv) introduction into host species/cell lines transformation followed by (v) screening and (vi) validation of edited lines.The simple steps involved in CRISPR/Cas9 mediated genome editing (CMGE) allows even a small laboratory with a fundamental plant transformation set up to carry out genome editing projects. CRISPR/Cas9 techniques have been used more extensively to edit plant genomes in the last half decade compared to ZFNs/TALENs and are reflective of its ease of use. Unlike first-generation genome editing tools, CRISPR/Cas9 genome editing involves simple designing and cloning methods, with the same Cas9 being potentially available for use with different guide RNAs targeting multiple sites in the genome. After proof-of-concept demonstrations in crop plants involving the primary CRISPR-Cas9 module, several modified Cas9 cassettes have been utilized in crop plants for improving target specificity and reducing off-target cleavage (e.g., Nmcas9, Sacas9, and Stcas9). Unlike ZFNs and TALENs, CRISPR genome editing is more straightforward and involves designing a guide RNA (gRNA) of about 20 nucleotides complementary to the DNA stretch within the target gene. To date, many crops such as rice, maize, wheat, soybean, barley, sorghum, potato, tomato, flax, rapeseed, *Camelina*, cotton, cucumber, lettuce, grapes, grapefruit, apple, oranges, and watermelon have been edited by this technique [11].

Emerging CRISPR/Cas Systems for Genome editing

The CRISPR/Cas system, comprising CRISPR repeat-spacer arrays and Cas proteins,is an RNA mediated adaptive immune system in bacteria and archaea that provides defense against phages and other invasive genetic elements by cleaving the invader's nucleic acid genome.The evolutionary arms race between prokaryotes and environmental mobile genetic elements such as phages has been going on for billions of years. This survival struggle yielded various CRISPR-type immune responses as defense mechanisms in bacteria. These CRISPR systems are classified based on the structure of CRISPR-associated (Cas) genes that are typically adjacent to the CRISPR arrays [12]. Broadly speaking, there are two classes of CRISPR systems, each containing multiple CRISPR types. Class 1 contains type I and type III CRISPR systems that are commonly found in Archaea. Class 2 contains type II, IV, V, and VI CRISPR systems [13]. Although researchers repurposed many different CRISPR/Cas systems for genome targeting, the most widely used one is the type II CRISPR-Cas9 system from Streptococcus pyogenes. In the last few years, more than 10 different CRISPR/Cas proteins have been repurposed for genome editing Among these, some of the recently discovered ones, such as Cpf1 proteins from Acidaminococcussp (AsCpf1) and Lachnospiraceae bacterium (LbCpf1), are particularly interesting [14]. In contrast to the native Cas9, which requires two separate short RNAs, Cpf1 naturally requires one sgRNA. The discoveries of 1082 aa Cas9 from Neisseria

meningitides (NmCas9) 53, li1053 aa Cas9 from *Staphylococcus aureus* (SaCas9) [15] and 984 aa Cas9 from Campylobacter jejuni (CjCas9) [16] are major forward steps toward this goal. However, the tradeoff is that these smaller Cas9 proteins require more complex PAM sequences. The SaCas9 requires a 5′-NNGRRT-3′ PAM sequence [17]. Whereas CjCas9 requires a 5′-NNNNACAC-3′ PAM sequence. Therefore, these smaller Cas9 proteins have relatively limited targeting scope and flexibility in genome targeting compared to SpCas9 despite the reduction in size. The type II CRISPR/SpCas9 system is simple and efficient, but it can only recognize DNA sequences upstream of the appropriate 5′-NGG-3′ PAMs, thus restricting potential target sites. Therefore, Cas9 variants were needed to overcome this limitation. The type V CRISPR/Cpf1 system has demonstrated great potential in this area. Cpf1 recognizes T-rich PAMs and generates cohesive ends with four or five nucleotide overhangs rather than blunt-end breaks, which complements the characteristics of Cas9 to a large extent [18]. Recently, Cpf1 from *Francisellanovicida* (FnCpf1) was used for targeted mutagenesis in tobacco and rice [19], and the Cpf1 ortholog from a Lachnospiraceae bacterium (LbCpf1) generated targeted mutations in rice [20]. A variant AsCpf1 (Cpf1 ortholog from *Acidaminococcus* sp. BV3L6) demonstrated high genome-editing efficiencies in human cells [21] but was less efficient in rice [26] and in soybean and rice protoplasts [22, 23]. When tested for their ability to induce targeted gene insertions via HR, the FnCpf1 and LbCpf1 nucleases generated precise gene insertions at a target site in rice, at a higher frequency than most other genome-editing nucleases [24]. LbCpf1 has also been used for targeted gene replacement in rice [25].

DNA free genome edited plants:

DNA-free genome editing is a groundbreaking technology, producing genetically edited crops with a reduced risk of undesirable off-target mutations, and meeting current and future agriculture demands from both a scientific and regulatory standpoint.DNA-free genome editing has been accomplished using both protoplast-mediated transformation and particle bombardment. The elimination of transgenes contributes to the achievement of precise genome editing, in which unnecessary changes do not exist in the genome. The elimination of the Cas9 gene from genome-edited plants would also prevent the induction of mutations at untargeted loci. The elimination of transgenes would also alleviate concerns about genome-edited plants. DNA-free genome editing is another promising approach for plant genome engineering without transgene integration. A ribonucleoprotein (RNP) consisting of Cas9 protein and gRNA can be used for this purpose in case of targeted mutagenesis without donor DNA templates. The RNP can be formed in vitro and transferred into plant protoplasts. Because the RNP does not contain any DNA, transgene integration can be avoided. Woo *et al.* [56] were the first to demonstrate that the preassembled Cas9-gRNA RNP complex could be directly delivered to plant protoplasts of Arabidopsis, tobacco, lettuce, and riceProtoplasts have been used as plant material for the introduction of RNP complexes in several studies [55].The successful application of RNP-mediated genome editing in protoplasts has been reported in grapevine and apple [26] wheat [27], and cabbage and Chinese cabbage [28]. As an alternative, immature embryo transformation using a biolistic method has been used in maize [29] and wheat [30]. In maize, Svitashev *et al.* [45] reported the efficiency of genome editing of 47%, when a selection marker was used, and 2.4–9.7% without selection. In wheat, Liang *et al.* [24] produced mutants with 4.4% efficiency. Importantly, both studies demonstrated a considerable reduction of off-target mutations compared to genome editing by CRISPR/Cas9 DNA. As another example, recently, Toda *et al.* [49] reported the development of a genome-editing system in which Cas9-gRNA RNP is directly delivered into rice in vitro fertilized zygotes. In addition, Kim *et al.* [12] demonstrated that purified Cas12a and gRNA could also be used to induce mutations in soybean and wild tobacco. Collectively, these observations indicate that using the RNP complex might become a prominent strategy of plant genome editing, without transgene integration and with reduced off-target effects. The results also suggest that Cas9 orthologs and variants could be used for plant genome editing, as RNP, expanding the possibility of plant genome engineering.

Applications of CRISPR for crop improvement:

The CRISPR/Cas9 system has been successfully applied in various plant species. These include not only model plants, such as Arabidopsis, but also crops, such as rice, tobacco, sorghum, wheat, maize, soybean, tomato, potato, poplar, apple and banana [31].The application of CRISPR technology in crop improvement has so far been focused on the improved crop yields, quality and stress resistance that could be obtained by simple knockout of one or several genes that confer undesirable traits [32]. For example, knocking out Gn1a, DEP1 and GS3 in rice led to enhanced grain number, dense erect panicles and larger grain size [33]disrupting the waxy gene Wx1 in maize resulted in high amylopectin content with improved digestibility that has the potential to be commercialized [34];and destroying the MLO allele generated powdery mildew-resistant wheat and tomato [35].CRISPR/Cas9 technology has been used to target FAD2 to improve oleic acid content while decreasing polyunsaturated fatty acids in the emerging oil seed plant

Camelina sativa [36]. Zhang *et al.*[65] used CRISPR/Cas9 technology to generate Taedr1 wheat plants by simultaneous modification of the three homoeologs of EDR1. CRISPR/Cas9 technology was later used to disrupt the coding region of CsLOB1 in Duncan grapefruits, resulting in crops that had no canker symptoms [37]. In the cucumber, when the eIF4E (eukaryotic translation initiation factor 4E) gene was disrupted, broad virus resistance was generated [38]; the plants were shown to be immune to an Ipomovirus (Cucumber Vein Yellowing Virus) and were resistant to the potyviruses Zucchini yellow mosaic virus and Papaya ring spot mosaic virus-W [39] by using a dead Cas9/Cas12a, CRISPR technology can also be used for gene regulation, epigenetic modification and chromosomal imaging and so on. Recently, CRISPR-mediated gene knockout has been used to maintain heterosis [40-44] which is usually lost in subsequent generations owing to genetic segregation. In rice, a genotype named MiMe (Mitosis instead of Meiosis) was produced by targeting crucial genes related to meiosis, and haploid plants were also created using CRISPR technology. In crops, many agriculturally important traits are conferred by single-nucleotide polymorphisms (SNPs) or by dominant gainof-function point mutations [45]. Such traits can now be generated by CRISPR-mediated base editing, which provides a new degree of precision in creating base substitutions and presents ample opportunities for crop improvement. CRISPR technology is best known for its ability to generate targeted gene knockouts. However, there are many essential genes that cause seedling lethality when knocked out, and many agriculturally important traits such as improved photosynthesis require gene over expression. CRISPR-mediated gene regulation provides solutions to these problems. CRISPR-mediated gene regulation has so far been focused mainly on promoters implicated in gene repression, activation and epigenetic modification. The best example of CRISPRmediated gene regulation for crop improvement comes from work in tomato, where CRISPR technology was used to mutate the promoters of genes related to quantitative traits such as fruit size, inflorescence branching and plant architecture by creating a continuum of variation for tomato breeding [46].

Base editing

Base editing is a newly developed technique for precise genome editing that enables irreversible base conversion at a specific site. A base editor is a fusion of catalytically inactive CRISPR–Cas9 domain (Cas9 variants, dCas9 or Cas9 nickase) and a cytosine or adenosine deaminase domain which converts one base to another. Several agriculturally important traits are conferred by SNPs in the genome, and base editing has played a critical role in correcting those point mutations and accelerating crop improvement. Base editing is more specific than the CRISPR/Cas9 technology [47]. BEs are mainly categorized as CBEs which can convert C to T, ABEs which convert A to G, and RBEs which convert A to I or C to U. Cytosine and adenine base editors have been successfully used in a wide range of major crops and model plants to edit specific genes conferred by single nucleotide polymorphisms. Some of the following CRISPR-based base editors are discussed below:

DNA base editors

Base editors are chimeric proteins composed of a DNA targeting module and a catalytic domain which is capable of deaminating a cytosine or adenine base in the genome [48]. There are two types of DNA base editors: cytosine base editors (CBEs) and ABEs.

Cytosine base editors

Cytosine base editors are the vectors that catalyse the conversion of cytosines to thymines. The cytidine deaminase enzyme removes an amino group from cytosine converting it to uracil, resulting in a U-G mismatch which gets resolved via DNA repair pathways to form U-A base pairs. Subsequently, a T gets incorporated in the newly synthesized strand forming T-A base pairs. This results in C.G to T.A conversion in a programmable manner. The first-generation base editor (BE1) was composed of a cytidine deaminase enzyme APOBEC1 (from rats) linked to a dCas9 by a 16 amino acid XTEN linker [49]. The second-generation base editor BE2 (APOBEC-XTEN-dcas9-UGI) was developed by adding a uracil DNA glycosylase inhibitor (UGI) to the C terminus of the DNA targeting module [50]. Subsequently, BE3 base editor was developed, the major improvement in BE3 was the replacement of dCas9 with Cas9 nickase (nCas9), which nicks the strand opposite to the deaminated cytidine. Another base-editing system, Target-AID was developed [51]. Fourth-generation base editors were efficiently used for programmable C to T conversion with reduced indel formation and increased product purity. More recently, a new plant base editor, A3A-PBE, was developed to further enhance the base-editing efficiency in plants [68].

Adenine base editors

Base-editing capabilities and study of genetic diseases were further expanded by the development of a new class of ABEs that could modify adenine bases [52]. Unlike cytidine deaminases, adenine DNA deaminases do not occur in nature. The first-generation ABEs were developed by fusing a Tad A with a catalytically impaired CRISPR/Cas9 mutant [53]. Among the series of ABEs developed, ABE7.7, ABE7.8

and ABE7.9 are considered to be the most active ABEs with a broader sequence compatibility. The seventh-generation ABEs (ABE7.10) were recommended for conversion of A.T to G.C in a wide range of targets with increased efficiency and product purity. ABEs introduce point mutations with higher efficiency and have greatly expanded the scope of base editing by enabling all four transitions (C to T, A to G, T to C and G to A) in a programmable manner. ABE-P1 (ABE plant version 1), the modified version of ABE7-10, was used for precise A.T to G.C conversion in rice plants [53]. ABE7.10 (base editors used in human cells) was adapted and optimized to an adenine base-editing system in plants to create point mutations at multiple endogenous loci in rice and wheat [54]. The plant ABE system was used to generate base-edited plants in wheat by targeting TaDEP1 and TaGW2 genes. These new base editors with different Cas9 variants have increased the scope of base editing and could be useful in rice functional genomics research in rice and other crops in the future. Most recently, a rice codon-optimized ABE-nCas9 tool was synthesized to induce targeted A-T to G-C point mutation in the rice genome [55]. The scope of base editing was expanded by development of novel ABEs using a Cas9 variant SpCas9-NGv1 that successfully induced A to G base substitutions in endogenous sites of the rice genome [56].

RNA base editors (ADAR)

Feng Zhang and his group were the first to develop RNA base editors [57]. Cas13 is a type VI CRISPR-associated RNA-guided RNase with RNA binding abilities. Among a set of Cas13 enzymes assayed for RNA knockdown activity, Cas13b ortholog from Prevotella sp. (PspCas13b) was found to be more efficient and specific in RNA binding and knockout applications. The adenosine deaminase acting on RNA (ADAR) family of enzymes mediates endogenous editing of transcripts via hydrolytic deamination of adenosine to inosine [58]. These enzymes are capable of precise base editing in RNA. This system used to edit RNA transcripts was referred to as RNA Editing for Programmable A to I Replacement (REPAIR). REPAIR presents a promising RNA editing platform with broad applicability for research, therapeutics and biotechnology.

Applications of Base Editing Tools for Plant Improvement

In plants, many genes have been edited using various BEs. As a result, precise mutations have been produced in target regions in the rice, wheat, maize, potato, watermelon, cotton, tomato, and Arabidopsis genomes [59, 60]. Different types of CBEs are also widely used in plant genome editing, particularly in rice and wheat. Several BEs, such as PBEs and hA3A-BE3, can efficiently perform base conversions in plants. A3A-PBE, generated from hA3A-BE3, can be used for mutagenesis-oriented editing [61]. Successful use of APOBEC3A (A3A-PBE) for the C-to-T base substitution was reported by Zong *et al.* [66], who showed that A3A-PBE was more efficient than was pnCas9-PBE, a standard PBE, enabling a conversion of C to T within a 7-nt editing window (from 3 to 9 bp). Additionally, pnCas9-PBE was generated for cereal plants and cloned under the maize ubiquitin-1 gene promoter. Two genes (SLR1 and NRT1.1B), known to control the plant architecture and effective utilization of nutrients in rice, respectively, were targeted and edited via base editing, which resulted in increases in the height and nitrogen use efficiency in the mutant plants [56]. Recently, an elite herbicide-resistant transgene-free wheat variety was developed by targeting the TaALS-P174 gene, with an approximately 75% mutation efficiency, and the mutants showed high tolerance to imidazolinone, sulfonylurea, and aryloxyphenoxy propionate-type herbicides [48]. To date, base-editing systems have been successfully used not only for the modification of genes of herbicide resistance in rice and wheat but also for editing ZmCENH3 and ALS genes in maize and watermelon [67, 55]. Additionally,transgene-free potato plants,with increased chlorsulfuron resistance, were produced by targeting the ALS gene using a CBE [61]. Moreover, the use of CBEs was shown to confer increased resistance to chlorsulfuron to tomatoes via the conversion of proline (CCA) to serine (TCA) in the targeted region of the ALS gene [62]. Marker-free tomato plants were also developed by editing DELLA and ETR1 genes [44]. In another study, a CBE was reported with a window from 12 to 17 in the target region of cotton [52].These findings demonstrated that base-editing tools might be effectively used for editing of target genes and might have broad applications for the improvement of plant traits.

Advantages of Base editing: Base-editing systems offer several advantages over non-DSB-mediated genome editing in plants. They are more efficient and generate far fewer undesired products than do DSB mediated systems. Multiplex or whole-gene base editing is not likely to lead to chromosomal rearrangements,such as large deletions and inversions; and they can be used to create nonsense mutations to avoid DSB-induced in-frame indels. Base-editing systems will be valuable tools for genetic research with various agricultural applications.

Limitations of base editing

Base editing is a recently developed and advanced technology. However, there is still a need for developing easier, more precise, and more convenient base-editing tools.

1. Targeting limitations: Successful base editing requires the presence of a specific PAM sequence (NGG PAM for SpCas9) and the target base must be within a narrow base-editing window [63]. This specific PAM requirement is a severe limitation which lowers the editing efficiency in plants.
2. Size of catalytic window: Cytosine deaminase base editors can potentially edit any C that is present in the wide activity window of approximately 4–5 nucleotides (or up to 9 nt). This is a severe limitation in base editors which result in low specificity and editing efficiency.
3. Off-target editing: In the base editing systems, off-targets occur when additional cytosines proximal to the target base gets edited. In a recent study,it was observed that CBEs BE3 and high-fidelity BE3 (HF1-BE3) induce unexpected and unpredictable genome wide off-target mutations in rice crop [64].The study also indicates that to minimize the off-target mutations, it is necessary to optimize the cytidine deaminase domain and/or UGI components.

Prime editing

Prime editing is a recent genome editing technology that can introduce all 12 possible types of point mutations (that is, all 6 possible base pair conversions), small insertions and small deletions in a precise and targeted manner with favorable editing to indel ratios [2]. Prime editors are fusion proteins between a Cas9 nickase domain (inactivated HNH nuclease) and an engineered reverse transcriptase domain. The prime editor protein, exemplified by PE2, is targeted to the editing site by an engineered prime editing guide RNA (pegRNA), which not only specifies the target site in its spacer sequence, but also encodes the desired edit in an extension that is typically at the 3′ end of the pegRNA. Upon target binding, the Cas9 RuvC nuclease domain nicks the PAM-containing DNA strand. The prime editor then uses the newly liberated 3′ end at the target DNA site to prime reverse transcription using the extension in the pegRNA as a template. Successful priming requires that the extension in the pegRNA contain a primer binding sequence (PBS) that can hybridize with the 3′ end of the nicked target DNA strand to form a primer•template complex. In addition, pegRNAs contain a reverse transcription template that directs the synthesis of the edited DNA strand onto the 3′ end of the target DNA strand. The reverse transcription template contains the desired DNA sequence change(s), as well as a region of homology to the target site to facilitate DNA repair.After reverse transcription, the newly synthesized edited DNA strand exists as a 3′ DNA flap that is redundant with a 5′ flap containing the original, unedited DNA sequence. Cellular DNA repair processes are thought to excise the 5′ flap, allowing the edited 3′ DNA flap to be incorporated into the target site to generate heteroduplex DNA containing one edited and one non-edited strand. Finally, permanent installation of the edit occurs through replacement of the non-edited strand by DNA repair of the non-edited strand, which can be promoted by using a simple sgRNA to direct PE2 to nick the non-edited strand. This additional nick stimulates resynthesis of the non-edited strand using the edited strand as a template, resulting in a fully edited duplex.Three versions of the prime editor system have been characterized. PE1 contains a fusion of Cas9 nickase to the wild-type Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT). PE2 substitutes for the wild-type M-MLV reverse transcriptase an engineered pentamutant M-MLV RT that increases editing efficiency by about threefold. Finally, PE3 combines the PE2 fusion protein and pegRNA with an additional sgRNA that targets the non-edited strand for nicking, further increasing editing efficiency two- to fourfold. A variant of the PE3 system called PE3b uses a nicking sgRNA that targets only the edited sequence, resulting in decreased levels of indel products by preventing nicking of the non-edited DNA strand until the other strand has been converted to the edited sequence. A major determinant of prime editing efficiency is the design of the pegRNA [2]. Prime editors are able to install point mutations at distances far (>30 bp) from the site of Cas9 nicking, which offers greater targeting flexibility than nuclease-mediated HDR with ssDNA donor templates, which typically are unable to introduce edits efficiently more than ∼10 bp from the cut site [67]. In principle, this feature also makes PAM availability less restrictive for prime editing. Prime editors have been shown to support all types of nucleotide substitutions, as well as targeted insertions and deletions, in rice and wheat protoplasts, which can generate edited plants [68]. Prime editing has demonstrated more advantages than base editing in cases where multiple cytosines or adenines were present in a base editing window, and bystander edits were unacceptable, because prime editing enabled precise single-nucleotide replacement. The target scope is also expanded in prime editing because, unlike base editing, it is not limited by the need for a PAM sequence at a suitable distance from the target nucleotides. Although prime editing offers advantages compared to other genome editing technologies, it has not yet been applied to plant cells. Prime editing would be a promising technology for plant genome engineering, especially because prime editing can achieve efficient knock-in of DNA fragments in plant cells. Generally, HDR efficiency is low in plant cells, so knock-ins of DNA fragments to target sites is difficult. However, prime editing offers a new strategy for knock-in of DNA fragments via an HDR-independent pathway.

REGULATION OF GENOME-EDITED CROPS

Genome-editing tools have been used to effect precise modifications in many plant genomes. They have had a great influence on basic research as well as crop improvement. A primary advantage of these technologies is that the transgenes initially used to induce genetic alterations can be easily removed from the genome by genetic segregation, making the resulting plants typically indistinguishable from naturally occurring genetic variants. More recent modification methods, especially CRISPR/Cas, have improved the robustness of this process by allowing genetic changes to be accomplished without any integration of foreign DNA, through transient expression of a site-specific nuclease within the plant cell [55]. The transient nature of the expression often results from the degradation of nuclease-encoding DNA constructs after they have done their job and before they can be integrated into the plant's genome. This can be achieved by using viral vectors to deliver the site-specific nuclease in the form of either mRNA, which is unstable and quickly degrades, or protein, which is not transmitted from parent to offspring [59]. The United States Department of Agriculture (USDA) has stated that CRISPR/Cas9 edited crops can be cultivated and sold free from regulatory monitoring. This can save several million dollars on getting regulations of GMO crops for the field test and data collections. In addition, it also reduces time as it usually takes several years to release a GMO crop. It also will remove the uncertainty of consuming GMO crops among the public. To date, there are five crops edited with CRISPR/Cas9 approach in the pipeline that USDA has declared not to regulate including a white button mushroom (Agaricusbisporus); resistance to browning was developed using CRISPR/Cas9 by knocking out a gene polyphenol oxidase (PPO). Similarly, waxy corn (Z. mays) with enriched amylopectin has been developed by inactivating an endogenous waxy gene Wx1 and has also been exempted from GMO regulations. Green bristle grass (*Setariaviridis*) with delayed flowering time achieved by deactivating the S. viridis homolog of the Z. mays ID1 gene, Yield10 Bioscience edited camelina for increased oil content and drought tolerant soybean (Glycine max) edited for Drb2a and Drb2b genes will also not be subject to regulatory evaluation. DuPont Pioneer is planning to release the waxy corn variety as the first commercialized genome-edited crop in 2020.

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