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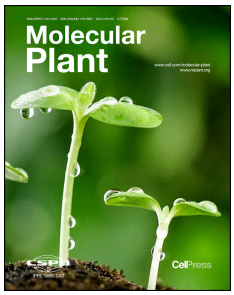

Perspectives on the application of genome editing technologies in crop breeding

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Perspectives on the application of genome editing technologies in crop breeding

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Abstract:

Most conventional and modern crop improvement methods exploit natural or artificially induced genetic variations and require laborious characterization of multiple generations of time-consuming genetic crosses. Genome editing systems, in contrast, provide the means to rapidly modify genomes in a precise and predictable way, making it possible to introduce improvements directly into elite varieties. Here, we describe the range of applications available to agricultural researchers using existing genome editing tools. In addition to providing examples of genome editing applications in crop breeding, we discuss the technical and social challenges faced by breeders using genome editing tools for crop improvement.

Keywords: Genome editing, Crop breeding, Mutations, Base editing, Plants

Introduction

Crop breeding programs mainly rely on the introgression of existing natural genetic variation into elite backgrounds, which requires substantial germplasm resources and extensive back-crossing followed by selection of the progeny lines with the best agronomic traits. The availability of beneficial alleles in nature limits the effectiveness of conventional crop breeding, although non-naturally occurring new alleles can be generated by random mutagenesis using physical, chemical, and biological means (Co^{60}, EMS, T-DNA, and transposon insertion) (Mba, 2013). Physical and chemical mutagenesis typically generates a large number of random mutations throughout the genome, along with rare chromosomal rearrangements (Oladosu et al., 2016). Mutagenesis-based breeding has produced over 3,000 commercial varieties of food crops (Oladosu et al., 2016), but the initial mutagenesis must be followed by the screening of large populations to identify mutants with desirable properties, such that the process is time consuming and labor intensive, especially for polyploid crops (Phillips et al., 2009).

As an alternative to the imprecise random mutagenesis methods, genome editing based on sequence-specific engineered endonucleases (SSNs) has recently emerged as

a powerful tool to rapidly modify plant genomes in a precise and predictable way (Gaj et al., 2013). A number of genome editing technologies have been developed, including the mega-nucleases or homing endonucleases (HEs) (Cohen-Tannoudji et al., 1998), zinc finger nucleases (ZFNs) (Bibikova et al., 2002), transcription activator-like effector nucleases (TALENs) (Christian et al., 2010), and type II clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) (Cong et al., 2013; Mali et al., 2013). These genome editing systems generate targeted DNA double-strand breaks (DSBs) in the genome (Carroll, 2014), which are primarily repaired by either the non-homologous end-joining (NHEJ) pathway or the homology-directed repair (HDR) pathway (Wyman and Kanaar, 2006). The NHEJ pathway is normally exploited to incorporate frameshift mutations in specific genomic loci but is error prone because it typically introduces small indels at the targeted site. The HDR pathway is a template-directed repair process that can be used along with an exogenous repair template to insert a custom sequence into the genome or to replace an existing genomic sequence,

Aside from the direct applications of introducing genetic mutations and performing gene replacement, genome editing technology can be used to modulate gene expression levels and modify the epigenome (Puchta, 2017). When combined with conventional breeding, genome editing technology can accelerate the introduction of desired traits and greatly reduce costs. In addition, the genetic elements required for genome editing can be removed from the genome through genetic crosses or following segregation in the progeny, which differentiates genome-edited products from genetically modified organisms (GMOs) (Mao et al., 2019). Since the first reports of successful application of genome editing technology in plants, research institutions and biotechnology companies worldwide have focused on its application for crop genetic improvement. To date, genome editing has been mostly applied to improve crop yield, quality, and stress resistance, but innovative applications are continually emerging (Zhang et al., 2018b). Here, we highlight recent progress on the genome editing of crops. We also provide insights into present and

future applications and discuss the challenges facing genome editing technologies in crop breeding.

Genome editing tools and their suitable applications

Genome engineering tools have quickly evolved during the past decade and are now routinely used by research groups. Their widespread application is having an enormous impact on basic life science research, medicine, and agriculture. Even more diverse, efficient, and easy-to-perform gene editing tools are likely to be developed in the coming years. Because genome editing technologies have been extensively reviewed elsewhere (Gaj et al., 2013; Kumor et al., 2017), we will provide a brief summary of the available tools and focus on their appropriate use in crop improvement.

a. Gene disruption

HEs, ZFNs, TALENs, and CRISPR/Cas systems generate targeted DSBs in the genome that, when repaired by the NHEJ pathway, can introduce small insertions or deletions (indels) (Carroll, 2014). If the targeted site is located in the coding region, the introduced indels frequently generate frameshifts, resulting in gene disruption. The HEs, ZFNs, and TALENs recognize genomic target sites using protein motifs, and the molecular cassettes needed for each target are technically difficult to assemble (Gaj et al., 2013). The CRISPR/Cas system, in contrast, uses base complementarity between the single-guide RNA (sgRNA) and the target DNA for recognition, which greatly simplifies the cloning process (Jinek et al., 2012). Because of its simplicity, the CRISPR/Cas system is easy to adapt to different targets and is suitable for multiplex editing by simultaneously expressing multiple sgRNAs (Cong et al., 2013); as a result, it is the preferred choice for plant genome editing (Yin et al., 2017). Following the isolation from *Streptococcus pyogenes* of the first Cas9 protein used for genome editing (SpCas9), many homologs with diverse properties have been isolated from diverse bacteria and used for genome editing. The Cas9 proteins from type II CRISPR systems can recognize G-rich PAM sequences and mainly generate DSB with blunt ends (Cong et al., 2013), whereas the Cas12a (Cpf1) and Cas12b (C2c1) proteins from type V CRISPR systems can recognize T-rich PAM sequences and

produce DSB with staggered ends (Teng et al., 2018; Zetsche et al., 2015). In addition, engineered SpCas9 or Cas12a variants have been created in order to expand target range and to improve specificity (Chen et al., 2017; Gao et al., 2017; Hu et al., 2018; Kleinstiver et al., 2016; Kleinstiver et al., 2015; Nishimasu et al., 2018; Slaymaker et al., 2016).

b. Gene targeting

Gene targeting (GT) is a strategy to replace endogenous gene fragments based on homologous recombination (HR) (Capecchi, 2005). The frequency of HR in plants is extremely low but can be enhanced by introducing DNA DSBs at the target site (Steinert et al., 2016). Simultaneous delivery of the SSN editing system and a donor DNA as repair template into the cell facilitates GT in plants, but despite the increase in efficiency provided by the DSBs, the overall HR rate is still quite low (Li et al., 2013). In theory, high concentrations of donor DNA can significantly improve GT efficiency, and thus geminiviral replicons have been used for this purpose (Baltes et al., 2014; Wang et al., 2017). Donor DNA concentration can also be increased by adjusting the ratio of CRISPR construct and donor DNA when using a biolistic approach (Li et al., 2018a; Sun et al., 2016). In order to overcome the low HR rate in somatic cells (Puchta and Fauser, 2013), driving Cas9 expression in the egg cell- and early embryo has significantly improved gene targeting efficiency in *Arabidopsis* (Miki et al., 2018; Wolter et al., 2018).

c. Base editing

Base editing is a novel tool for precise genome editing that enables irreversible base conversion at the target site without requiring DSB formation or homology-directed repair (Rees and Liu, 2018). The cytosine base editor (CBE), which converts C to T (or G to A), uses deactivated Cas9 (dCas9) or Cas9 nickase (nCas9) as a platform that directs a cytidine deaminase to the target region where it deaminates cytosines in the exposed non-target strand, creating U-G mismatches in a small base-editing window. The resulting mismatches are then repaired by the cellular DNA repair systems, leading to the formation of U-A base pairs and ultimately to T-A base pairs after replication (Komor et al., 2016). A recently developed adenine base

editor (ABE) uses a deaminase evolved from the *E. coli* tRNA adenine deaminase TadA to induce A to G (or T to C) substitutions (Gaudelli et al., 2017).

ABEs and CBEs have been successfully used for plant genome editing (Hua et al., 2018; Li et al., 2017b; Lu and Zhu, 2017; Yan et al., 2018), but the narrow base-editing window and the requirement for specific PAM sequences restrict the number of possible targets. The development of base editors with Cas9 variants that recognize different PAMs can expand the target scope (Hua et al., 2019), and the use of newly improved cytidine deaminases will increase the efficiency of the systems (Ren et al., 2018; Zong et al., 2018). Two recent reports showed that CBEs could induce genome wide off-target editing independent of sgRNA sequences, whereas ABEs are much more specific (Jin et al., 2019; Zuo et al., 2019). Moreover, the deaminases in the ABEs and CBEs also have RNA editing activities, potentially affecting tens of thousands of off-target RNAs in the transcriptome (Grünewald et al., 2019a; Grünewald et al., 2019b; Rees et al., 2019; Zhou et al., 2019). Fortunately, the problem of off-target RNA editing by ABEs and CBEs can be alleviated by engineering the deaminases without affecting the on-target DNA editing (Grünewald et al., 2019b; Rees et al., 2019; Zhou et al., 2019).

d. Gene regulation and epigenome editing

Plants have evolved sophisticated molecular mechanisms to control their transcriptomes in order to adapt to constantly changing environments. The catalytically inactive Cas9/Cas12a mutants (dCas9/dCas12a) can be used as a platform to recruit different transcriptional regulators to specific genomic loci in order to modulate gene expression in plants. dCas9/dCas12a can be fused to transcriptional activator domains for transcriptional enhancement (termed CRISPRa) or to repressor domains for transcriptional repression (termed CRISPRi) (Li et al., 2017d; Tang et al., 2017b). In these cases, trait maintenance in the offspring frequently relies on the expression of CRISPRi or CRISPRa components, which may limit their widespread application in crop breeding because the CRISPR constructs cannot be removed from future generations. In *Arabidopsis*, epigenetic modifications have been obtained using a dCas9-Suntag fusion protein; the protein recruits the catalytic domain of the human

DNA demethylase Tet1 or the *Nicotiana tabacum* DNA methylase DRM2 to demethylate or methylate, respectively, the targeted DNA (Gallego-Bartolome et al., 2018; Papikian et al., 2019)..

Recent progress in genome editing for crop improvement

The ability to introduce targeted genomic modifications makes genome editing tools very useful for engineering crop traits, and the advent of CRISPR/Cas9 has significantly boosted the application of genome editing for crop breeding (Chen et al., 2019). Much progress has been made during the past few years, and we highlight some examples here.

a. Improving single-gene traits

Single-gene traits are those that are mainly controlled by one gene. Mutations in these genes typically affect the specific trait without compromising other agronomic characteristics, making genome editing tools especially suitable in these cases. For example, rice quality traits such as amylase content (AC) and fragrance can be efficiently improved by editing the *Waxy* and *OsBADH2* genes, respectively, without affecting plant architecture or yield (Shan et al., 2015; Sun et al., 2017; Zhang et al., 2017a). Conventional breeding methods have been used to manipulate these traits because natural allelic variants exist for *Waxy* and *OsBADH2*, but the CRISPR-based approach is much faster and far less labor intensive. In contrast, Cd accumulation in rice, which can have severe health consequences for consumers, is difficult to reduce using traditional breeding approaches. CRISPR/Cas9 has been recently used to knockout the metal transporter gene *OsNramp5*, which dramatically decreases the Cd concentration in seeds without greatly affecting yield (Tang et al., 2017a). Editing a single gene, *ZmLG1*, in maize can produce upright architecture and the resulting plants can be grown at higher density in the field (Li et al., 2017a). Targeted mutagenesis of *FT2a* in soybean delayed flowering time under both short and long day conditions providing adaptation to wider geographical growing regions to the transgene-free mutant plants (Cai et al., 2018).

b. Engineering complex traits

Many important agronomic traits in crops are regulated by complex genetic networks. Rice grain yield is a relatively well-characterized complex trait, and many quantitative trait loci (QTLs) controlling yield have been identified (Xing and Zhang, 2010). Independent or multiplex editing of these QTLs can result in improved yield (Li et al., 2016b; Xu et al., 2016), although editing of the same yield-related QTL in different elite rice varieties can have inconsistent or even negative effects under field conditions (Shen et al., 2018). One important advantage of genome editing tools is their ability to incorporate some complex traits that cannot be achieved by conventional breeding technologies. The haploid induction (HI) system is a core technique of doubled haploid (DH) breeding programs. Rice lacks a natural *in vivo* haploid induction system, but genome editing of a putative orthologue of *ZmMATL* (*OsMATL*), encoding a pollen-specific phospholipase has been used to produce haploid seeds (Yao et al., 2018). Editing *OsMATL* achieved low haploid induction rate (2-6%) in different rice varieties and reduced seed-setting rate (Wang et al., 2019; Yao et al., 2018), making the haploid identification process a daunting task. Introducing additional morphologic or more robust fluorescence markers can help to identify haploid seeds, as has been widely used in the maize double haploid breeding system (Dong et al., 2018; Li et al, 2009). Two innovative rapid-breeding approaches, IMGE and Hi-Edit, which combine haploid induction with CRISPR-Cas9 mediated genome editing, can introduce desirable traits into elite inbred lines within two generations, avoiding the time-consuming crossing and backcrossing process (Killiher et al., 2019; Wang et al., 2019). These strategies will greatly accelerate the improvement of different varieties from a wide range of crops, especially for the elite commercial lines that are recalcitrant to transformation. Heterosis has long been exploited by breeders to produce high-yielding crop varieties, but the superior traits of F1 hybrids are lost in subsequent generations. Apomixis, which produces clonal progeny asexually through seeds without meiosis or fertilization, is a strategy to perpetuate the heterozygosity of F1 hybrids in crops (Hand and Koltunow, 2014). Simultaneous mutation of four genes using CRISPR/Cas9 was recently shown to be a

promising strategy for obtaining synthetic apomixis that enables clonal propagation of F1 rice hybrids through seeds (Wang et al., 2019). Heterozygosity of F1 hybrids was fixed by multiplex editing of three *MiMe* (*Mitosis instead of Meiosis*) genes (*REC8*, *PAIR1*, and *OSD1)*, while mutation of the *MTL* gene induced formation of seeds with some genotype as F1 hybrids (Wang et al., 2019). However, this approach only produced a low percentage of clonal hybrids in the progenies because of the low haploid induction and seed-setting rate caused by *OsMATL* mutation (Wang et al., 2019). Screening other Os*MATL* alleles or exploiting different haploid-inducing genes may help to improve this technology. An alternative and seemingly more efficient method to induce haploid seed formation in rice uses ectopic expression in the egg cell of the sperm cell-specific *BABY BOOM1* (*BBM1*) gene (Khanday et al., 2019). *BBM1* plays a key role in triggering embryogenesis in the zygote, and ectopic expression of *BBM1* in the egg cell can efficiently initiate parthenogenesis without zygote formation (Khanday et al., 2019). Combining CRISPR/Cas9-mediated mutagenesis of the *MiMe* genes with egg cell expression of *BBM1* enabled asexual propagation of F1 rice hybrids (Khanday et al., 2019). Interestingly, the *MiMe* phenotype in rice can be reproduced by simultaneous editing of *OsSPO11-1*, *OsREC8*, and *OsOSD*, suggesting that different sets of genes involved in meiosis can be manipulated to create the same phenotype (Xie et al., 2019).

c. Molecular domestication

The major crops feeding today's world population were domesticated from wild species thousands of years ago (Doebley et al., 2006). During the long domestication process, farmers selected for beneficial traits such as high yield and easy harvest, which are also known as domestication traits. Quantitative genetics and genomics studies have identified a number of genes controlling domestication traits in different crops (Meyer and Purugganan, 2013), making it theoretically possible to accelerate domestication of wild species or even distantly related 'orphan' crops by multiplex editing of the orthologs of main domestication genes (Zsögön et al., 2017). As a proof-of-concept, wild tomato species were *de novo* domesticated by multiplex editing of genes associated with agronomically desirable traits (Li et al., 2018d;

Zsögön et al., 2018). In addition, key domestication traits of an orphan *Solanaceae* crop, groundcherry, a distant tomato relative, can also be rapidly improved by genome editing of orthologs of tomato domestication genes (Lemmon et al., 2018). The cultivated potato is an autotetraploid tuber crop that is vegetatively propagated and difficult to improve by conventional breeding methods. Ye et al. (2018) recently used the CRISPR-Cas9 system to re-domesticate potato into self-compatible diploid lines by disrupting the self-incompatibility gene *S-RNase* (Ye et al., 2018). These re-domesticated diploid lines will be very useful for basic research and genetic improvement.

Genome editing for crop improvement: issues to be considered

Before attempting to use genome editing tools for crop breeding, researchers should consider a number of important issues, some of which are discussed below.

a. Selection of target genes

Some quality traits such as grain amylose content in rice and oil quality in soybean can be quickly improved by targeting a single gene (Haun et al., 2014; Zhang et al., 2017a). However, most key agronomic traits such as yield and biotic/abiotic stress resistance are quantitative and are controlled by many QTLs. In the case of yield, many yield-related QTLs have been identified, mapped, and subsequently cloned (Xing and Zhang, 2010; Zuo and Li, 2014), providing a rich resource of potential targets for genome editing. In contrast, very few QTLs with strong effects on abiotic stress resistance have been cloned due to the difficulty in quantitative phenotyping and to the complexity of the traits (Landi et al., 2017). QTLs identified as negative regulators of beneficial traits are the easiest targets because beneficial loss-of-function alleles can be easily generated by genome editing. In addition, molecular genetic studies have shown that some QTLs are conserved among multiple crops, such that the knowledge gained from model crops such as rice can be applied to other less-studied crops (Li et al., 2018e). Targeting of QTLs must be done with caution,

however, because many QTLs are often involved in multiple developmental processes, and modification of such QTLs can therefore have negative effects.

b. Choice of an appropriate genome editing approach

Multiple genome editing tools are now available to introduce diverse modifications in the genome, providing the opportunity to design different strategies to accomplish the desired goals.

For genes having a negative effect on the targeted trait, a complete loss-of-function allele can be easily generated by CRISPR/Cas9-mediated disruption of the coding region (Figure 1A) (Li et al., 2016b). However, it could be useful to produce multiple CRISPR-lines using different sgRNAs before evaluating the resulting phenotypes for the different mutations.

Population genetic and genomic studies have shown that a high proportion of agronomic traits are associated with DNA variations, frequently single nucleotide polymorphisms (SNPs), in the promoter regions (Li et al., 2012b). Variations in the promoter can affect expression levels, expression patterns, and/or tissue specificity of the genes. Disruption of *cis* regulatory elements in the promoter region can positively or negatively affect gene expression levels, making them good target sites for genes that regulate traits in a dose-dependent manner (Figure 2) (Birchler, 2017; Li et al., 2017c). Recent work in tomato has shown that introducing random deletions in the promoters of several yield-related genes (Figure 2A) through multiplex editing can generate quantitative variations of target traits (Rodríguez-Leal et al., 2017). Although the construction of vectors for expression of Cas and multiple sgRNAs can be cumbersome, the recently developed single-transcription-unit strategy can simplify this process in rice without compromising editing efficiency (Wang et al., 2018).

Some *cis* regulatory elements for transcription-factor binding are relatively conserved and can be predicted by online tools (Lescot et al., 2002). In this case, base editing tools may be used to substitute key nucleotides in the *cis* element to decrease or increase the binding affinity of the transcription factors and thus to modulate expression levels (Figure 2B). Compared with the relatively random indel-mutation

approach discussed above, this strategy can reduce the size of the screening population. Some pathogen virulence proteins bind to the promoter regions of host genes and subvert their expression to facilitate pathogenesis (Cox et al., 2017), providing an attractive opportunity to increase resistance by disrupting the binding sites via indel mutations or base editing (Li et al., 2012a; Peng et al., 2017).

Insertions of some transposon elements (TEs) in the promoter region, or even upstream of the promoter, can affect the epigenetic status, and thereby the expression level of agronomically important genes (Yang et al., 2013; Zhang et al., 2017b). Large fragments can be inserted either by the imprecise NHEJ or the precise HDR machineries (Figure 2C) (Li et al., 2016a; Li et al., 2018c; Li et al., 2019; Wang et al., 2017), although the efficiency of the HDR approach in plants still requires substantial improvement (Endo et al., 2016; Sun et al., 2016).

The dCas9-based epigenome editing tools, such as targeted DNA methylation and demethylation systems, can regulate gene expression in *Arabidopsis* by modulating DNA methylation levels in the promoter region (Figure 2D) (Gallego-Bartolome et al., 2018; Papikian et al., 2019), but they have yet to be applied in crop breeding. A major concern with this approach is whether the epigenetic changes induced by epigenomic modification can be accurately inherited in the following generations. Some mutations controlling agronomically important traits exert their effects at the post-transcriptional level. For example, a mismatch in the OsmiR156 binding site of *ipa1*, a beneficial allele of *OsSPL14*, disrupts OsmiR156-mediated cleavage of *OsSPL14* mRNA, resulting in ideal plant architecture (Jiao et al., 2010). MicroRNAs (miRNAs), a class of short non-coding RNAs, regulate gene expression at the post-transcriptional level by base pairing with mRNA molecules, leading to mRNA cleavage or translational inhibition (Rogers and Chen, 2013). Many agronomically and developmentally important genes in major crops are directly regulated by miRNAs (Tang and Chu, 2017). Disruption of the miRNA/mRNA base pairing can affect miRNA-mediated mRNA cleavage and thus can be used to fine-tune the expression of target genes. For this purpose, base editing tools are a good choice for introducing point mutation(s) into the miRNA binding site of the target genes without changing the amino acid

sequence of the encoded protein, an approach that takes advantage of the degeneration of the genetic code (Figure 3A) (Hua et al., 2018). Because the position and number of mismatches in the miRNA binding site greatly affect the efficiency of miRNA-mediated mRNA cleavage (Jiao et al., 2010), both ABE and CBE can edit the miRNA targets, generating allelic variants in the miRNA binding sites.

Some agricultural traits are controlled by the generation of alternative mRNA transcripts. Two well-known examples in rice are the *Waxy* gene, which controls amylase content, and *OsMADS1*, which controls grain size (Isshiki et al., 1998; Liu et al., 2018). In these cases, base editing tools can be used to alter the highly conserved intron donor (GT) or acceptor (AG) sites and to thereby interfere with mRNA splicing (Figure 3B) (Kang et al., 2018; Li et al., 2018f).

In addition to being regulated at the transcriptional and post-transcriptional levels, gene expression can be regulated at the translational level. A considerable proportion of transcripts in plant cells harbor upstream open reading frames (uORFs), which can fine-tune the translational levels of the downstream primary open reading frames (von Arnim et al., 2014). This type of translational control can be easily disrupted by using the CRISPR/Cas9 system to introduce mutations in the initiation codon for the uORFs (Figure 3C) (Zhang et al., 2018a). Although there are no available examples, it is not unreasonable to hypothesize that NHEJ- or HDR-mediated introduction of translational enhancers in the 5’ UTR can boost the translation of targeted genes (Figure 3D).

Some agronomic traits are controlled by mutations resulting in amino acid substitutions that affect the biochemical functions of encoded products. For example, some beneficial alleles of *sd1*, a rice “Green Revolution” gene involved in gibberellin biosynthesis, contain amino acid substitutions that decrease the catalytic activity of the encoded enzyme, leading to the semidwarf phenotype of most modern rice varieties (Asano et al., 2011). Base editors could be used to introduce changes in key amino acids in order to affect protein activity (Figure 1B). However, the molecular mechanisms controlling the activity of many of the proteins affecting agronomically important traits are not well understood, especially for enzymes involved in

metabolite and hormone synthesis. Key functional sites in enzymes involved in hormone synthesis (e.g., Gn1 and SD1) and quality traits (e.g., Waxy and BADH2) can be identified using CRISPR/Cas9, ABE, and/or CBE through transformation of pooled libraries of tiling sgRNA arrays (Figure 1C), which is now feasible in plants (Butt et al., 2019; Lu et al., 2017; Meng et al., 2017). Pooled screens using base editors can also generate novel resistance mutations in herbicide targets (e.g., ALS and EPSPS).

c. Selection of beneficial alleles or allele combinations

Domestication and breeding processes have significantly reduced the genetic diversity of crops such that many agronomically important genes show strong artificial selection and extremely low genetic diversity. For example, recent haplotype analysis of 120 key genes controlling yield and quality traits in rice found haplotype numbers ranging from 1 to 15 in the 3,000 rice genome panel, with 28 genes having a single haplotype (Abbai et al., 2019). It has long been recognized that the narrow genetic diversity of cultivated crops is a major cause for the yield plateau experienced in breeding programs. Genome editing tools can be used to rebuild genetic diversity in individual genes, although identification of beneficial alleles or allele combinations can be a complicated task because most agronomic traits are polygenic and regulated by complex genetic networks. As a result, the introduction of genomic changes can create imbalances in the network with unintended consequences. A well-known example is the rice *OsSPL14* gene. Strong *OsSPL14* expression increases rice panicle size and culm diameter but dramatically decreases tiller number. Optimal yield potential can only be achieved by alleles with suitable *OsSPL14* expression levels that allow the coordination of panicle size and tiller number (Zhang et al., 2017b). In addition, networks controlling a specific trait can vary among different genetic backgrounds such that the editing of the same QTL can produce different outcomes among several rice varieties (Shen et al., 2018). Genotype–environment considerations are also important when selecting ‘beneficial’ alleles. For example, weak alleles of *sd1* conferring semidwarf phenotypes have been extensively selected in rice breeding (Asano et al., 2011). However, a transcriptionally upregulated

gain-of-function allele benefits deep-water rice varieties by promoting submergence-induced internode elongation (Kuroha et al., 2018).

d. Transgenic-free genome editing

To date, *Agrobacterium*-mediated transformation and particle bombardment are the two major approaches for delivering the genome editing regents into plant cells. Once the desired mutations are introduced into the plant genome, the transgenic cassette can be eliminated from the offspring, as it is no longer required for trait maintenance and the presence of genome editing tools increases the risk of off-target editing (Mao et al., 2019). This transgene-free feature of genome-edited crops increases social acceptance and facilitates commercialization. Transgene-free edited plants can be easily obtained by traditional methods such as segregation in selfing or back-crossing populations of edited lines, although such methods can be time-consuming for some polyploid and perennial crops. To accelerate the isolation of transgene-free edited lines, researchers have developed a number of efficient and easy-to-perform methods, including the fluorescence marker-assisted selection system and the suicide gene-based programmed self-elimination system (Gao et al., 2016; He et al., 2018). For perennial, self-incompatible, or vegetatively propagated crops, however, transgene segregation in the offspring is time-consuming or even impossible. DNA-free genome editing approaches that do not require integration of exogenous nucleic acids into the plant genome can be used to avoid the need for transgene elimination. *In vitro* transcribed RNAs for CRISPR components or *in vitro* assembled Cas9 ribonucleoproteins (RNPs) have been delivered into protoplasts and immature embryos of several plant species to perform genome editing (Liang et al., 2017; Svitashev et al., 2016; Woo et al., 2015). Because no selection pressure is applied in the regeneration process using these approaches, the DNA-free editing systems have low editing efficiencies, and large populations must be screened for the targeted mutations (Liang et al., 2017; Zhang et al., 2016). Moreover, plant regeneration from protoplasts has been achieved in only a few crop species.

Genome editing for crop improvement: Challenges

Public institutions and biotechnology companies are investing considerable human and financial resources into the development of genome editing for crop breeding. However, a number of important technical challenges remain to be solved, and social acceptance and regulatory issues will play an important role in the commercialization of genome-edited crops.

a. Efficient delivery of genome editing tools into plants

Gene editing components have been delivered into plant cells as DNA, RNA, or RNP using diverse methods such as protoplast transfection, *Agrobacterium* transformation, and particle bombardment (Liang et al., 2017; Zhang et al., 2016). Irrespective of the delivery method, genome edited cells must be regenerated into full plants using time-consuming and often difficult tissue culture methods. While some crops have well-established tissue culture-based transformation methods, transformation for other crops can be very difficult, time-consuming, or impossible (Altpeter et al., 2016). Even for crops with an established transformation method, many elite varieties remain recalcitrant to transformation due to poor regeneration ability, as is the case for many cereals (Altpeter et al., 2016). A recent technological advance using ectopic expression of the plant morphogenic regulators *Baby boom* and *WUSCHEL* during *Agrobacterium*-mediated transformation greatly improved the regeneration efficiency of mature seeds and leaf segments of recalcitrant maize varieties, as well as of immature embryos in sorghum and calli in sugarcane and *indica* rice (Lowe et al., 2016).

An important challenge is the application of gene editing technologies to species or varieties without available transformation methods, including wild relatives of major crops, orphan crops, and non-crop species with high nutritional potential. Delivery of genome editing components to germline or shoot meristem cells is a promising strategy to obtain gene-edited offspring in non-transformable species. Zhao et al. (2017) recently used magnetic nanoparticles as DNA carriers to deliver foreign DNA into plant pollen; the researchers reported that, following pollination, the exogenous DNA in the transfected pollen could integrate into the genomes of the progeny with a

low frequency. The validity and utility of this delivery strategy, however, have yet to be confirmed by any follow-up reports. Carbon nanotubes have shown potential for delivering biomolecules into tissues and organs of intact plants of several species (Demirer et al., 2019). Interestingly, DNA carried by the carbon nanotubes induced strong protein expression without transgene integration, highlighting the potential of using this nanomaterial for performing transgene-free editing in a wide range of plant species. A crucial question is whether this delivery method can be successfully applied to regenerative cells, shoot meristematic cells, or other types of plant germline cells.

b. Improved understanding of genetic networks controlling key agronomic traits in crops

Next-generation DNA sequencing technologies have generated an immense amount of genomic data including full genome sequences for many species (Bolger et al., 2014; Ling et al., 2018). Given that the availability of genomic sequence is no longer the limiting factor, the challenge is to understand the extensive and complicated genetic networks controlling agronomic traits and their interaction with environmental factors. For some model crops such as rice and maize, much progress has been made in understanding the genetic basis of yield- and quality-related traits (Ikeda et al., 2013; Li et al., 2018b; Miura et al., 2011), but knowledge about stress resistance lags behind (Landi et al., 2017). In many other crops, key genes controlling major agronomic traits remain unknown, making genetic improvement by molecular approaches extremely difficult. In some instances, knowledge gained from model plant species may be transferred to crops, assisting researchers in the selection of target genes. Future understanding of agronomic traits will be aided by population-level genomic approaches combined with different “omics” databases and the application of gene editing tools (Kujur et al., 2013).

c. Simultaneous manipulation of multiple traits

Conventional and genome-based breeding methods evaluate multiple agronomic traits during the selection process. As a result, elite commercial lines bred by these methods pyramid many superior alleles that confer improvements in yield, quality,

and resistance to biotic and abiotic stresses (Zeng et al., 2017). However, genome editing tools are limited in the number of targets that can be simultaneously manipulated. Although CRISPR/Cas9 and CRISPR/Cas12a systems show multiplex editing capabilities, in practice only a small number of sgRNAs (fewer than 10) have been expressed in plants (Miao et al., 2018; Wang et al., 2018), such that only a few traits can be simultaneously improved. This limitation can be alleviated by pyramiding beneficial alleles created by genome editing tools through genetic crosses and marker-assisted selection (Wu et al., 2018), or alternatively, by sequential editing (Demorest et al., 2016).

d. Precise editing

An exciting feature of genome editing technologies is the possibility of custom tailoring non-natural alleles to achieve improvements that are not possible with the available natural genetic variation. For example, swapping the maize *ARGOS8* promoter with the *GOS2* promoter can increase yield under drought stress without imposing a yield penalty on crops grown under well-watered conditions (Shi et al., 2017). In rice, resistance against multiple strains of *Xanthomonas* sp. can be achieved by stacking different TAL effector-binding sites in the promoter of *R* genes (Romer et al., 2009). Finally, replacement of the endogenous abscisic acid (ABA) receptor PYR1 with a variant containing several amino acid changes allows activation of the ABA response in plants treated with the agrochemical mandipropamid, such that the plant drought resistance can be induced by chemical spray (Park et al., 2015). Creating such beneficial but complicated alleles by genome editing requires high precision. Although high precision can be obtained with HDR-mediated gene targeting, the relatively low efficiency of the HDR pathway in plant cells and the lack of efficient delivery methods for DNA repair templates seriously limit its adoption (Steinert et al., 2016). A number of improvements are being developed including interference with the NHEJ repair pathway genes such as KU70/80 and LIG4 (Endo et al., 2016) and the use of geminiviral systems to increase the levels of donor DNA (Baltes et al., 2014; Wang et al., 2017). It was recently reported in rice that RNA transcripts localized in the nucleus can serve as repair templates for HDR-mediated,

precise gene replacement (Li et al., 2019). Compared to the conventional DNA donor repair templates, RNA templates can accumulate to high levels through active transcription in the nucleus, providing obvious advantages as repair templates for HDR in plants. An alternative approach that uses the egg cell- and early embryo-specific *DD45* gene promoter to drive *SpCas9* expression and sequential transformation in *Arabidopsis* has shown potential for increasing the efficiency of HDR-mediated gene editing (Miki et al., 2018), but has yet to be applied to crops.

e. Government policy towards genome edited crops

Genome editing is a biological mutagenesis method, and like chemical and physical mutagenesis methods, its application in crop breeding is not troubled with ethical and off-targeting issues. Although there are many technical challenges to overcome, the biggest potential obstacles for the adoption of genome editing tools in agriculture are public acceptance of the technology and government regulatory policies. In April 2016, the US Department of Agriculture ruled that gene edited mushrooms and corn did not need to be regulated by traditional genetic-modification policies; the ruling increased the rate at which gene-edited crops are marketed and gave US companies a first-mover advantage (Waltz, 2016). In contrast, gene-edited crops have been classified as equivalent to genetically modified (GM) organisms by the Court of Justice of the European Union (ECJ), and this decision effectively blocks the development of gene-edited crops in the EU (Callaway, 2018). Most countries still lack a clear and consistent regulatory policy for gene-edited plants. Even though policies can be put in place for strict regulation of gene-edited crops, the enforcement of those policies will be extremely difficult or simply impossible because most gene editing events cannot be differentiated from ‘natural’ mutations.

Concluding Remarks

The new developments in CRISPR/Cas technologies have widened the scope of genome-editing possibilities to include base substitutions and gene targeting and the regulation of gene expression. These developments have expanded the array of crop-improvement tools available to agricultural scientists, but the use of any genetic technology for crop improvement requires functional information on the genetic

networks controlling important agricultural traits. In many cases, that information is not yet available. The development of high-throughput DNA sequencing technology and the establishment of a large number of "omics" databases will facilitate the identification of useful targets for genome editing in plants.

Although impressive progress has been made (Kumlehn et al., 2018), genome editing still must overcome important challenges to its widespread application in crop breeding, such as the establishment of efficient and genotype-independent delivery methods and the improvement in gene targeting efficiency. At present, genome editing has been mostly used in species with available transformation methods, which represent a very small fraction of the plant kingdom. For those plant varieties and genotypes that cannot be transformed, the development of efficient delivery methods is a priority.

Genome editing provides an invaluable tool for high-precision molecular breeding of crops, with the potential to support a quantum leap in agriculture for a world in desperate need to produce more food with less environmental impact. Aside from its precision, genome editing can lower the cost of crop breeding and accelerate the production of new high-yielding, stress-tolerant, nutrient-use efficient and more nutritious varieties.

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Author Contributions

K.H. and J.Z. drafted the manuscript. J.B., C.M., F.K. and B.L. edited and gave suggestions for the manuscript. J.-K.Z. conceived this article and revised the manuscript.

Conflict of interest statement

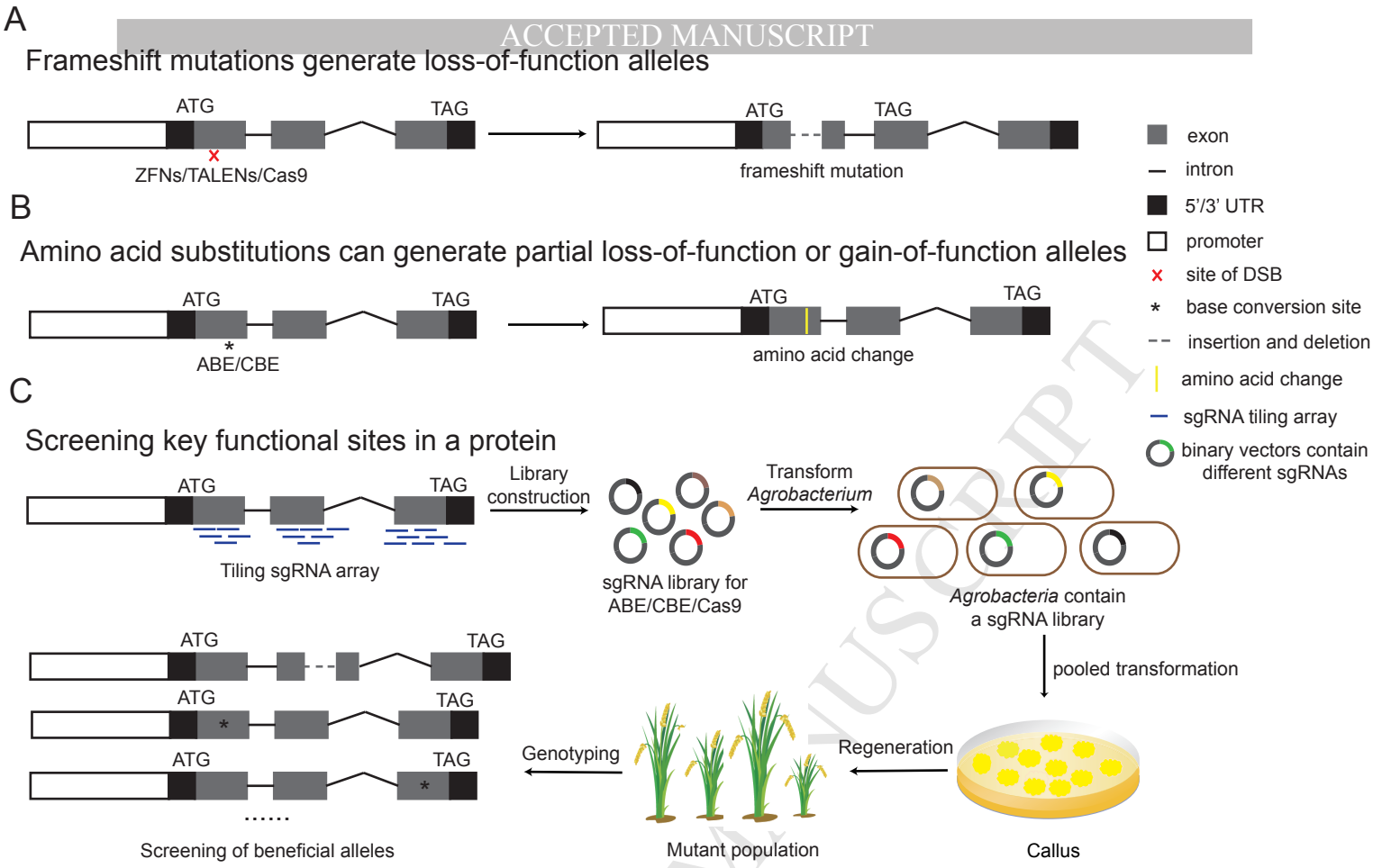
The authors declare no conflict of interests.

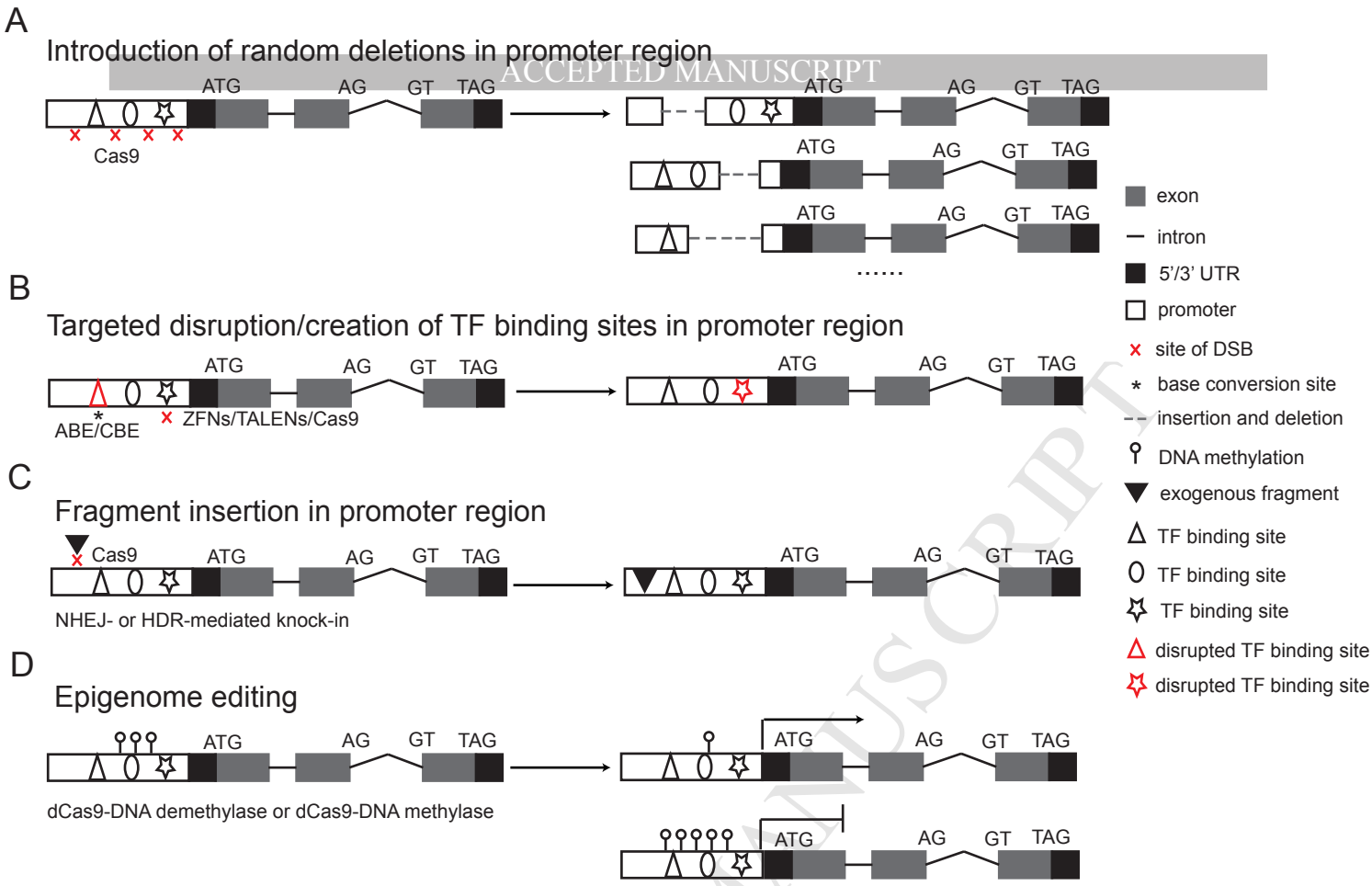
Figure legends

Figure 1. Using genome editing tools to generate mutations that affect protein function. **A.** ZFNs/TALENs/CRISPR/Cas9 can generate loss-of-function alleles by introducing DSBs in the coding region of targeted genes. **B.** Proteins with known key functional sites can be targeted with base editors to produce specific amino acid changes, generating partial loss-of-function or gain-of-function alleles. **C**. For uncharacterized proteins, key functional residues can be identified by functional screening through transformation of pooled libraries of tiling array of sgRNAs (using either ABE, CBE or Cas9). The sgRNA tiling array is designed to contain hundreds of sgRNAs covering the entire coding region of the targeted gene. The sgRNA tiling array can be pooled for vector construction and plant transformation.

Figure 2. Using genome editing tools to regulate gene transcription. **A.** Creating random deletions in the promoter region can generate allelic series with different expression levels. **B.** Targeted disruption/creation of transcription factor-binding sites can generate predictable changes in gene expression. **C.** NHEJ- or HDR-mediated fragment insertion in the promoter region can affect gene expression levels/patterns. **D.** Alteration of DNA methylation levels in the promoter region by epigenome editing tools can activate or repress gene transcription.

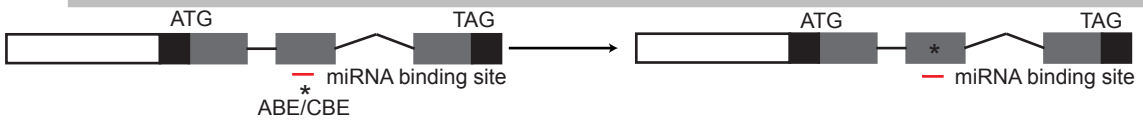
Figure 3. Using genome editing tools to modulate gene expression at the post-transcriptional (**A** and **B**) or translational level (**C** and **D**). **A.** ABE/CBE can introduce point mutations in the miRNA-binding sites of targeted genes to perturb miRNA-mediated mRNA cleavage or translation regulation. **B.** Base editors can mutate conserved intron donor AG and acceptor GT sites, interfering with mRNA splicing. **C.** Effect of upstream ORFs can be eliminated by disrupting the start ATG codon. **D.** Insertion of translational enhancer *cis* elements by NHEJ- or HDR-mediated knock-in to enhance translational levels.





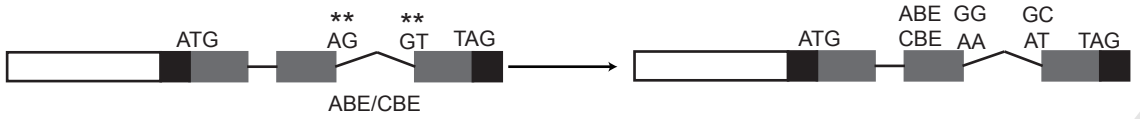
A

Base editing of miRNA binding sites interfere with miRNA-mediated regulation



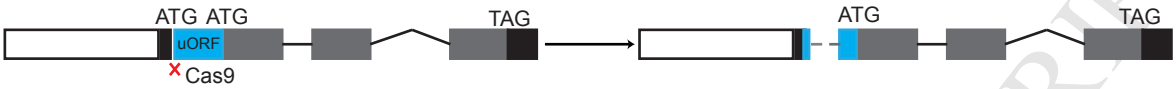
B

Base editing of intron donor or acceptor sites interfere with mRNA splicing



C

Elimination of uORF provides translational deregulation



D

Insertion of translational enhancer increases translation