The CRISPR Era: Unlocking Precision Breeding for Global Food Security

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ABSTRACT: The availability of genome sequences for many crops, along with advances in genome editing technologies, has created new opportunities to develop plants with almost any desirable trait. Tools such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) allow precise targeting of specific genes, but their application is costly and time-intensive since they rely on complex protein engineering steps. In contrast, CRISPR/Cas9 offers a simpler and more versatile approach, requiring only the design and cloning of guide RNAs, while the same Cas9 protein can be directed to multiple genomic sites. Following the initial proof-of-concept studies in crop plants, several Cas9 variants (e.g., Nmcas9, Sacas9, Stcas9) have been employed to enhance target specificity and minimize off-target effects. Additionally, Cas9 enzymes derived from different bacterial species have further improved the efficiency and precision of editing strategies. This review highlights the diverse CRISPR/Cas9-based approaches available for crop improvement and showcases applications aimed at enhancing tolerance to both biotic and abiotic stresses. Ultimately, these techniques pave the way for the development of non-GMO crops with desirable traits, contributing to higher yield potential under challenging environmental conditions.

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Introduction

One of the most pressing challenges facing humanity today is ensuring food security for an ever-expanding global population. By 2050, the population is projected to reach 10 billion, which will require an estimated 60–100% increase in global food production (FAOSTAT, 2016). Alongside this growing demand, agriculture is constrained by factors such as extreme climatic events, shrinking arable land, and rising biotic and abiotic stresses. To address these challenges, the development of innovative technologies for crop improvement has become essential.

Traditional genetic manipulation methods—including physical, chemical, and biological approaches (e.g., T-DNA insertion and transposons)—have played a key role in advancing our understanding of gene function and biological pathways, thereby contributing significantly to crop improvement (Ma *et al*, 2016). Over the past three decades, transgenic technologies have also been widely applied to study plant biology and enhance crop traits. However, these approaches often involve random integration of transgenes into host genomes, which can lead to instability, unintended phenotypes, and public concern, particularly when applied to food crops (Stephens & Barakate 2017).

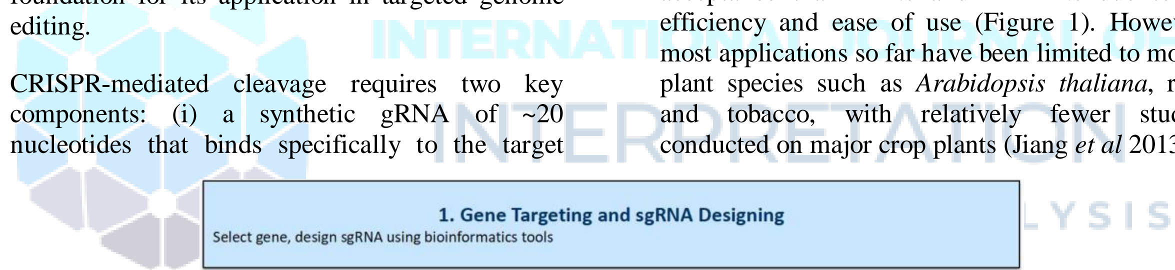
In recent years, genome editing technologies employing site-specific nucleases (SSNs) have enabled precise gene modifications in both plant and animal systems. These nucleases introduce double-strand breaks (DSBs) at targeted DNA sites, which are subsequently repaired through non-homologous end joining (NHEJ) or homology-directed recombination (HDR). These repair pathways lead to specific insertions, deletions (INDELs), or substitutions at the targeted loci (Jinek *et al* 2012). Unlike transgenic methods that cause random insertions and unpredictable traits, genome editing generates well-defined mutations, making it a powerful tool for functional genomics and crop breeding.

Moreover, genome-edited crops offer distinct advantages over traditional genetically modified (GM) plants. Since they retain targeted edits rather than foreign transgenes, these crops can be more readily incorporated into breeding programs and are often subject to fewer regulatory and consumer acceptance issues (Malzahn *et al* 2017; Waltz, 2018). This review highlights the applications and advantages of second-generation genome editing technologies, particularly CRISPR/Cas9 and its derivatives, in comparison to first-generation tools such as meganucleases, zinc finger nucleases

(ZFNs), and transcription activator-like effector nucleases (TALENs).

Clustered Regularly Interspaced Palindromic Repeats (CRISPR/Cas9)

The advent of the CRISPR/Cas9 system has transformed research in both plant and animal biology, with its genome-editing potential first demonstrated in mammalian cells in 2012 (Jinek *et al* 2012). Compared to earlier tools such as ZFNs and TALENs, CRISPR/Cas9 is simpler, requiring only the design of a guide RNA (gRNA) of approximately 20 nucleotides complementary to the target DNA sequence. The term CRISPR—originally introduced in 2002 (Jansen et al., 2002)—describes tandem repeats separated by unique non-repetitive DNA sequences, first identified downstream of the *iap* gene in *Escherichia coli* (Ishino *et al*1987). By 2005, these unique sequences were found to match foreign DNA derived from plasmids and phages, leading to the recognition of CRISPR as part of an adaptive immune system in prokaryotes (Mojica *et al* 2005; Liu *et al* 2017). This discovery ultimately laid the foundation for its application in targeted genome editing.



CRISPR-mediated cleavage requires two key components: (i) a synthetic gRNA of ~20 nucleotides that binds specifically to the target DNA and (ii) the Cas9 nuclease, which cuts 3–4 bases downstream of a protospacer adjacent motif (PAM; generally 5′-NGG) (Jinek *et al* 2012). The Cas9 enzyme contains two distinct nuclease domains: the RuvC-like domain and the HNH domain, each responsible for cleaving one DNA strand.

Since its development, CRISPR/Cas9 has been widely adopted in plant and animal genome editing. Between 2010 and 2018, nearly 5,000 publications reported its applications across biological systems. A typical CRISPR project involves a straightforward workflow: (i) identifying the PAM sequence in the target gene, (ii) designing a single guide RNA (sgRNA), (iii) cloning the sgRNA into a binary vector, (iv) introducing the construct into the host cells via transformation, followed by (v) screening and (vi) validation of edited lines (Figure 1). This simplicity enables even laboratories with basic plant transformation facilities to conduct genome-editing experiments.

In plants, CRISPR/Cas9 has gained broader acceptance than ZFNs and TALENs due to its efficiency and ease of use (Figure 1). However, most applications so far have been limited to model plant species such as *Arabidopsis thaliana*, rice, and tobacco, with relatively fewer studies conducted on major crop plants (Jiang *et al* 2013;).

Flow Chart: Basic steps for the gene mediated editing in crops

Improvements in CRISPR/Cas9 Gene Editing Technology

A key limitation of the CRISPR/Cas9 system, originally derived from *Streptococcus pyogenes*, is the occurrence of off-target cleavage. This happens when the guide RNA (gRNA) binds to genomic sequences that are partially complementary. To overcome this, various Cas9 modifications have been developed to enhance target specificity and reduce unintended cleavage, as summarized in **Table 1**.

Increasing the length of the protospacer adjacent motif (PAM) is another strategy to minimize off-target effects. Cas9 proteins from different bacterial species possess unique and expanded PAM sequences, which can further improve on-target precision. For example, Nmecas9 from *Neisseria meningitidis* recognizes an 8-mer PAM sequence (5′-NNNNGATT), which enhances specificity and lowers off-target activity (Lee et al., 2016). Similarly, SaCas9 from *Staphylococcus aureus* recognizes a 6-mer PAM (5′-NNGRRT; Ran *et al* 2015).Cas9 variants from *Streptococcus thermophilus* (St1Cas9 and St3Cas9) used to edit human genes PRKDC and CARD11 showed reduced off-target cleavage compared to SpCas9 (Muller et al., 2016). Furthermore, the engineered Cas9-VQR variant efficiently edits target sequences containing the 5′-NGA PAM (Hu *et al* 2016; Hu *et al* 2018). In addition, several CRISPR/Cas9 orthologs have been identified to further improve specificity. CRISPR-Cpf1, a class II type V endonuclease derived from *Prevotella* and *Francisella*, requires only a single crRNA for DNA cleavage and generates 4–5 nucleotide 5′ overhangs. This system has been successfully applied in both plant and animal models, showing minimal or no off-target effects (Zetsche *et al* 2017). Beyond Cpf1, around 53 other CRISPR/Cas variants have been characterized. Notably, the C2c2 nuclease from *Leptotrichia shahii* exhibits dual nuclease activity and can specifically target single-stranded RNA (Zaidi *et al* 2017).

Modification	Engineering	Application	Reference
SpCas9n (Cas9n)	Substitution of aspartate to alanine (D10A) in the RuvC domain	Allows knock-in via HDR	Cong *et al* 2013
Dead cas9 (dCas9)	Cas9 nuclease inactivation and double nicking using nickase	Nicking enhances specificity	Mali *et al* 2013
FokI Cas9 (fCas9)	Inactivated Cas9 nuclease fused with FokI nuclease	Increased on-target activity	Guilinger *et al* 2014

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Vectors for CRISPR/Cas9 Gene Editing in Plants

To alter plant genomes, Cas9 and sgRNA production within the targeted cell is necessary, much like in other animal model systems. Cas9 and gRNA are expressed in plant systems via plant-specific RNA polymerase III promoters [AtU6 (Arabidopsis); TaU6 (wheat); OsU6 or OsU3 (rice)]. To express Cas9 or Cas9 variants and gRNAs in plant systems, a number of commercially accessible vectors are available. More than 30 empty gRNA backbones in binary vectors are already available through the international, nonprofit Addgene plasmid repository2. The gRNA of interest can be inserted into the gRNA scaffolds and plant RNA polymerase III promoter found in the empty gRNA backbones.

Using CRISPR to Improve Crops

Almost 20 crop species have so far embraced the CRISPR/Cas9 gene editing technique for a variety of features, such as increased yield and the ability to manage biotic and abiotic stress (Ricroch *et al* 2017). Since they detail the use of the CRISPR/Cas9 system by knocking out particular reported genes that are crucial to abiotic or biotic stress-tolerant systems, many of the published articles are regarded as proof-of-concept research. The development of disease-resistant crops is severely hampered by biotic stress caused by pathogenic microorganisms, which also contribute to 15% of worldwide food production losses and over 42% of potential yield loss (Oerke 2005). Genome editing based on CRISPR/Cas9 has been used to improve tolerance to major abiotic stresses such as drought and salinity, as well as to raise crop disease resistance. Below is a summary of how CRISPR has been used to modify the genomes of different crop species.

Monocots

More over half of the world's population depends on rice (*Oryza sativa*) as a key staple food crop. Because of its tiny genome size, it is extensively

researched and used as a model crop for monocots. Few studies have documented the use of genome editing to improve biotic and abiotic stressors for rice crop development, however numerous studies have recently shown the application of CRISPR-based genome editing in rice.

Studies of Proof of Concept

Potential PAM sites are abundant in the rice genome (1 in 10 kb) (Xie and Yang 2013). Therefore, in the near future, CRISPR technology may be utilised to target any desired feature in the rice genome. For the first time in any crop plant, Shan et al. (2013) used both protoplast and particle bombarded rice calli systems to show sequence-specific CRISPR/Cas9 mediated genomic modification of three rice genes: phytoene desaturase (OsPDS), betaine aldehyde dehydrogenase (OsBADH2), and mitogen-activated protein kinase (OsMPK2). These genes are involved in controlling responses to various abiotic stress stimuli. OsPDS and OsBADH2 showed editing rates of almost 9 and 7 percent, respectively. By creating two vectors, pRGE3 and pRGE6, that are appropriate for rice genome editing, Xie and Yang (2013) have illustrated an RNA-guided genome editing technique. Three gRNAs were used to select OsMPK5, a negative regulator of biotic and abiotic stressors in rice, for targeted mutagenesis, and the results were evaluated in rice protoplasts. A more accurate gRNA design method was used, and a low level of off-targets was reported. For a number of genes, including OsDERF1, OsPMS3, OsEPSPS, OsMSH1, and OsMYB5, the effectiveness of the CRISPR/Cas9 system in causing targeted mutation and the heritability in mutant rice lines were assessed (Zhang *et al* 2014). For many genes with no or one bp off-target mutation, the T0 generation showed a wide range of mutation rates (21–66%), while the T2 generation showed up to 11% of homozygous mutants. The activation-induced cytidine deaminase (Target-AID) method (Shimatani *et al* 2017) allowed for targeted base editing of the herbicidal gene, C287 in rice. This method used dCas9 combined with cytidine deaminase to base edit without introducing DSBs. Similarly, Zong *et al* (2017) showed that maize, wheat, and rice could all have their genomes precisely edited. Using the BE3 base editor, Li *et al* (2017a) showed how to base edit the rice OsPDS and OsSBEIIb genes. The BE3 base editor is an enhanced genome editing tool that combines cytosine deaminase, uracil glycosylase inhibitor (UGI), which prevents base-excision repair, and nicked cas9 (ncas9-a D10 mutation in cas9). This work showed that base editing may be used successfully to rice. As recently shown in rice and Arabidopsis (Zhang *et al* 2016; Shen L. et al., 2017), CRISPR/Cas9 has made it simple to multiplex genome editing of an almost infinite number of genes (Lowder *et al* 2015). For each genetic transformation in rice, Shen L. *et al* (2017) used a single binary vector to successfully modify eight agronomic genes. The isocaudamer technique using intermediate vectors was used to ligate the genes. The analysis revealed fewer off targets and suggested that a sgRNA cascade might not have an impact on the CRISPR/Cas9 mutation rate.

Functional Analysis of Genes Associated with Abiotic and Biotic Stress

It has been effectively demonstrated that a CRISPR/Cas9 targeted mutation in rice's ethylene response factor, OsERF922, increases resistance to Magnaporthe oryzae-caused blast disease (Liu *et al* 2012). For Xanthomonas oryzae pv. Oryzae to infect rice and induce bacterial blight, the expression of the disease susceptibility gene OsSWEET13 is necessary (Zhou *et al* 2015). Two OsSWEET13 knockout mutants that target its promoter have been created using CRISPR/Cas9 technology, improving indica rice's resistance to bacterial blight disease (IR24). Plant annexins are important for both plant growth and defence against environmental stressors. OsAnn3 CRISPR knockouts were used to study the crucial role that the rice annexin gene (OsAnn3) plays under cold stress (Shen C *et al* 2017). Under cold treatment, it was discovered that T1 mutant lines' survival was lower than that of wild-type plants.

Two or more genes regulate a number of essential properties, including yield and resistance to abiotic stress. Many research have tried to map these quantitative areas (quantitative trait loci, or QTL) that affect agronomically significant features in crop improvement projects. In order to create better-performing cultivars, these discovered QTL regions were introgressed into elite lines. If the QTLs are closely related, this introgression is laborious, and adding non-target areas to the elite line may have negative consequences. A powerful tool for introducing and researching uncommon mutations in crop plants is the CRISPR/Cas9 system. A CRISPR-based QTL editing technique was used to investigate the role of grain size (GS3) and grain number QTLs (Gn1a) in rice varieties (Shen *et al* 2018). The study demonstrated that various backgrounds might have very distinct and conflicting outcomes from the same QTL.

Wheat

Grown as a staple food crop all over the world, wheat is a significant cereal grain. Shan *et al* protoplasts. Additionally, it was demonstrated that the CRISPR *TaMLO* knockdown conferred resistance to the powdery mildew disease caused by Blumeria graminis f. sp. Tritici (Btg). Four lines were discovered to be altered for the restriction enzyme site out of the 72 T0 knockout MLO wheat homoeolog (*TaMLO-A*) transgenic lines that were examined for restriction enzyme digestion utilising T7 endonuclease I (T7E1) (Wang *et al* 2014). The quantity of transgenic lines produced can be enhanced or increased by effective construct delivery techniques. SSNs and the gRNA are frequently introduced via T-DNA-based delivery systems. On the other hand, amplicons based on DNA viruses seem to boost gene targeting efficiency by several times. By using wheat geminiviral-based DNA replicons [wheat dwarf virus (WDV)] for temporary and simple CRISPR/Cas9 cassette production, Gil-Humanes *et al* (2017) were able to boost the expression of the endogenous ubiquitin gene in hexaploid wheat by a factor of 12. In the future, genome engineering of complicated genomes may employ high frequency gene targeting with WDV-based DNA replicons.

According to Kim *et al* (2018), the wheat protoplasts' CRISPR/Cas9 genome editing system targets two genes linked to abiotic stress: wheat ethylene responsive factor 3 (*TaERF3*) and wheat dehydration response element binding protein 2 (TaDREB2). The T7 endonuclease assay verified that the altered genes were expressed in over 70% of successfully transfected protoplasts. Off-target mutations and transgene integration are major issues with the use of CMGE in crops. The biolistic delivery approach of CRISPR/Cas9 ribonucleoproteins (RNPs) was used by Liang *et al* (2017) to show an effective genome editing technique in order to solve these problems. While the biolistic approach of delivering RNPs will provide temporary expression and decay quickly, significantly reducing off-targets, CRISPR/Cas9 DNA will typically be integrated into the host genome and expressed steadily. Using the CRISPR/Cas9 RNP complex, two distinct genes (TaGW2 and TaGASR7) in two varietal backgrounds were altered in bread wheat. Off-target effects are significantly reduced when this complex is broken down in vivo, and the mutant bread wheat population showed no off-targets. *Liang et al* (2018) have released an extended protocol of RNP delivery. This DNA-free editing

(2014) effectively illustrated the use of the CRISPR/Cas9 technique for the TaMLO gene (Mildew resistance locus O) in wheat

technique enables the production of transgenic-free plants at T0 and circumvents laborious processes like backcross breeding for transgene removal. However, this approach has drawbacks, including as poor effectiveness rates in comparison to CRISPR/Cas9 DNA binary delivery systems due to the temporary nature of expression and the need for time-consuming mutant screening without marker selection throughout development. The RNP method will be an effective way to accomplish CRISPR/Cas9 based genome editing in crop species, particularly perennial crops, if these restrictions can be removed. Multiplexed genome editing based on CRISPR/Cas9 has been shown to simultaneously modify numerous significant agronomic parameters in model crops. The frequency of mutations and heritability produced by multiplexed genome editing in hexaploid wheat were recently published by Wang *et al* (2018). Three gRNAs were combined in a tRNA spaced polycistronic cassette under the transcriptional control of a single TaU3 promoter to target three wheat genes: *TaGW2* (a negative regulator of grain traits), *TaLpx-1* (lipoxygenase, which confers resistance to Fusarium graminearum), and *TaMLO* (loss of function, confers resistance to powdery mildew resistance). Wheat protoplasts were used to examine the editing efficiency, and next-generation sequencing was used to check the DNA for editing or mutations, followed by Agrobacterium-mediated transformation and mutant screening. Editing efficiencies were noted for the three homologous copies after statistical and phenotypic analysis was performed in the T0, T1, T2, and T3 generations. This study demonstrated that new variation in the offspring of plants expressing CRISPR-Cas9 can be attributed to transgenerational gene editing activity. For multiplex genome editing in complex crops, such as polyploid crop species, this strategy will be effective.

Regulatory Issues with Crops Produced Through Genome Editing

According to regulatory frameworks in many nations, new breeding technologies such as ZFNs, TALENs, and CRISPR do not qualify as GMOs. CRISPR/Cas9-edited crops can be grown and sold without regulatory oversight, according to the US Department of Agriculture (USDA) (Waltz, 2018). By doing this, several million dollars can be saved on obtaining GMO crop regulations for data

collecting and field testing. Additionally, it saves time because a GMO crop's release often takes many years. Additionally, it will eliminate public scepticism regarding the consumption of GMO crops. A white button mushroom (*Agaricus bisporus*) is one of the five crops now under development that USDA has determined not to regulate using the CRISPR/Cas9 technique. Browning resistance was created by utilising CRISPR/Cas9 to knock out the polyphenol oxidase (PPO) gene (Waltz, 2016). Similarly, by deactivating the natural waxy gene Wx1, waxy maize (Z. mays) with enhanced amylopectin was created and is thus exempt from GMO laws. Regulatory evaluation will also not apply to drought-tolerant soybean (Glycine max) edited for Drb2a and Drb2b genes, Yield10 Bioscience edited camelina for increased oil content, and green bristlegrass (*Setaria viridis*) with delayed flowering time obtained by deactivating the S. viridis homolog of the Z. mays ID1 gene.

In conclusion

Compared to traditional breeding, new breeding methods allow scientists to swiftly and precisely introduce the required features. Genome editing with CRISPR/Cas9 is a fundamentally innovative method. Future research will focus heavily on the use of genome editing techniques to improve crops' production, nutritional value, disease resistance, and other characteristics. It has been widely used in various plant systems during the past five years for functional research, biotic and abiotic stress management, and the enhancement of other significant agronomic features. The majority of the work done is preliminary and requires improvement, even if number of changes to this technology must increase on-target efficiency. However, CRISPR/Cas9-based genome editing will become more and more widespread and a crucial method for obtaining "appropriately edited" plants that will contribute to the aim of zero hunger and sustainably feed the expanding human population.

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