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The Plant Genome

REVIEW

# Genome editing in maize and sorghum: A comprehensive review of CRISPR/Cas9 and emerging technologies

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## Abstract

The increasing changes in the climate patterns across the globe have deeply affected food systems where unparalleled and unmatched challenges are created. This jeopardizes food security due to an ever-increasing population. The extreme efficiency of $C_4$ crops as compared to $C_3$ crops makes them incredibly significant in securing food safety. $C_4$ crops, maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) in particular, have the ability to withstand osmotic stress induced by oxidative stress. Osmotic stress causes a series of physical changes in a plant thus facilitating reduced water uptake and photosynthesis inhibition, such as membrane tension, cell wall stiffness, and turgor changes. There has been a great advancement in plant breeding brought by introduction of clustered regularly interspaced short palindromic repeats (CRISPR) gene editing technology. This technology offers precise alterations to an organism's DNA through targeting specific genes for desired traits in a wide number of crop species. Despite its immense opportunities in plant breeding, it faces limitations such as effective delivery systems, editing efficiency, regulatory concerns, and off-target effects. Future prospects lie in optimizing next-generation techniques, such as prime editing, and developing novel genotype-independent delivery methods. Overall, the transformative role of CRISPR/Cas9 in sorghum and maize breeding underscores the need for responsible and sustainable utilization to address global food security challenges.

## Plain Language Summary

This paper gives an overview of genome editing between two $C_4$ plants sorghum and maize, in regard to meeting the escalating global food security. These two crops stand out with a high productivity as compared to other plants in an era of increased climatic

**Abbreviations:** BE, base editing; CRISPR, clustered regularly interspaced short palindromic repeats; DSBs, double-stranded breaks; GABA, $\gamma$-aminobutyric acid; GMS, genetic male-sterility; MMR, mismatch repair; PAM, protospacer adjacent motif; PE, prime editing; PegRNA, prime editing guide RNA; RNPs, ribonucleoproteins; RT, reverse transcriptase; SgRNA, single guide RNA; SSB, single-stranded breaks; TALEN, transcription activator-like effector nucleases; TFs, transcription factors; Tm, melting temperature; ZFN, zinc finger nucleases; ZFP, zinc finger protein.

change. This means improving breeding within these crops provides a promising food safety with the increasing world population. CRISPR/Cas9 (where CRISPR is clustered regularly interspaced short palindromic repeats) has stood out as the most efficient gene editing technique through revolutionizing agriculture. Despite its efficiency, CRISPR/Cas9 still faces a number of challenges, such as editing efficiency and regulatory policies. We analyzed the sophisticated capabilities of prime editing. Prime editing is a cutting-edge technique in CRISPR/Cas9 technology that enables precise alterations to the DNA. Key takeaways. Prime editing holds promise in plant breeding, although further research is needed to evaluate its efficiency for larger scale.

## 1 | INTRODUCTION

$C_4$ crops, particularly maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench), have a high potential in addressing the escalating challenges of global food security (Ranum et al., 2014) due to their extreme efficiency as compared to $C_3$ plants in photosynthesis and resource usage, especially during hot climate where their potential for productivity is high (Cui, 2021). Additionally, enhanced tolerance to high temperatures, better efficiency in water use, and improved carbon fixation are distinctive advantages of $C_4$ plants over $C_3$ plants (Sage & Zhu, 2011). These advantages stem from the $C_4$ pathway's ability to regulate photorespiration, a process that reduces photosynthetic efficiency in $C_3$ plants under stress conditions (Hatch, 1987). $C_4$ plants are capable of concentrating carbon dioxide at the site of Rubisco, thus facilitating photosynthesis even in high temperatures (von Caemmerer & furbank, 2003). Stress tolerance in plants, especially tolerance against osmotic stress such as drought and salinity, is very significant in crop production. This is because these osmotic stresses trigger the physical properties of crops, such as membrane tension, cell wall stiffness, and turgor changes, thus facilitating low crop production. Likewise, an increase of 10% in photosynthetic efficiency is estimated to improve crop yields by 50% (Langdale, 2011). With this, the introduction of the $C_4$ mechanism into the most important $C_3$ plants, such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), and soybean (*Glycine max* L.), holds potential in addressing global food security (Long et al., 2015; von Caemmerer et al., 2012). Additionally, a substantial development has been made in the production of $C_4$ rice with an estimated boost in photosynthesis and yield (Ermakova et al., 2020). Furthermore, the steady increase in population with a projected 10 billion people by 2050 (Clarke & Zhang, 2013; FAO, 2021, 2018) necessitates doubling the current crop yield capacity to ensure an adequate food supply. Agricultural scientists and researchers worldwide are tirelessly working on technological and scientific innovations in these two crops to secure future global food security.

Maize is ranked the third most consumed cereal as human food globally (FAO, 2021; Venkateswaran et al., 2014), while sorghum is ranked among the five major cereals in the world (Hatch, 1987). $C_4$ and $C_4$-like species exhibit high tolerance to osmotic stress-induced oxidative stress due to the increased levels of non-enzymatic low molecular weight antioxidants (Uzilday et al., 2014), which minimize the occurrence of stress on plants, thus maintaining high yields in these plants. These plants achieve this tolerance by enhancing the presence of small antioxidant molecules within their cells (Yu et al., 2024), making maize and sorghum promising solutions to the existing climate variations that exacerbate food insecurity. The two crops face similar hurdles, such as pests, diseases, and climate changes, since they share a number of properties, necessitating technological interventions to fortify their productivity and resilience.

Hybridization has been used over decades in traditional breeding, throughout the course of crop domestication to modern heterosis, which began in maize. This led to improved yields in number of crops such as maize, wheat, rice, and sorghum (Goulet et al., 2017; Gupta et al., 2019; Kim & Zhang, 2018). However, the full potential of heterosis remains a challenge brought about by biological and procedural complications (G. Chen, Zhou, et al., 2021). Similarly, the production in some crops, particularly maize, tends to slow down in some countries where it is a staple food (Vieira, 2024), and sorghum yield still lags behind crops like rice and wheat. This is likely attributed to increased climate variations, limited genetic diversity of sorghum, and biotic stresses such as pests, pathogens, and weeds (Springmann et al., 2018), thereby necessitating further research and innovation to enhance productivity.

Scientists have made a significant milestone through the discovery of gene editing techniques, which have been utilized in maize (Gao et al., 2020), sorghum (A. Li, Jia, et al., 2018), soybean (Juwattanasomran et al., 2011), cucumber (*Cucumis sativus* L.) (Yundaeng et al., 2015), apples (*Malus domestica*) (Schröpfer & Flachowsky, 2021), rice (F. Wang, Wang, et al., 2016), banana (Musa spp.) (Tripathi et al., 2019), and

wheat (Sanchez-leon et al., 2018), production by introducing and manipulating the genetic material of organisms to improve traits. Gene editing enables the creation of foods with desirable traits, such as increased nutrition (A. Li, Jia, et al., 2018), tolerance to climate change (Blankenagel et al., 2022), improved food production efficiency (Brant et al., 2021), palatability (L. Wang, Kaya, et al., 2021), and resistance to biotic stresses predominately fungi, viruses, bacteria, and weeds (R. Xu et al., 2021; Pathi et al., 2020). For a long time, traditional breeding has been used to enhance stress-tolerant plants but due to some limitations, such as time-consuming, gene editing tends to provide promising solutions to these limitations, being precise and quick. Moreover, several countries have approved these genetically engineered crops for consumption, including Arctic apples in Canada and the United States (Okanagan Specialty Fruits, 2024), as well as tomatoes (*Solanum lycopersicum* L.) containing high amounts of $\gamma$-aminobutyric acid (GABA) in Japan (Waltz et al., 2022).

CRISPR/Cas9 gene editing (where CRISPR is clustered regularly interspaced short palindromic repeats) has revolutionized agriculture by being more efficient than previous methods (Hua et al., 2019). Identified in the 1990s as recurring DNA sequences in bacteria and archaea (Mojica et al., 1993), CRISPRs were later found to be part of bacterial immune systems protecting against viruses (Barrangou et al., 2007). The discovery of *Cas9* genes coding for proteins involved in this immune response was a crucial development. In 2012, researchers successfully elucidated the functional processes of CRISPR/Cas9 (Jinek et al., 2012), highlighting the Cas9 protein's precision in cleaving DNA. This groundbreaking research, published in the Science journal, verified that the CRISPR/Cas9 system could be programmed to target and cut specific DNA sequences (Jinek et al., 2012). This laid a foundation for the revolutionary genome editing capabilities of CRISPR/Cas9, advancing genetic engineering and biotechnology. Since then, the CRISPR/Cas9 system has been extensively applied in gene editing across various organisms, including plants such as sorghum, maize, wheat, *Arabidopsis thaliana* (L.) Heynh, tobacco (*Nicotiana tabacum* L.), and rice (Krappmann et al., 2017). Remarkably, its application in plant species like sorghum, maize, wheat, Arabidopsis, tobacco, and rice was recorded as early as August 2013 (Shan-Wang, Li, Zhang, Chen, Liang, Zhang, Liu, Voytas, et al., 2013), and numerous successful publications documenting plant gene editing using this technique have since emerged.

Despite maize and sorghum being $C_4$ crops, they are closely related species in the Poaceae (grass) family (Paterson et al., 2009), sharing a significant number of orthologous genes, which reveals their common evolutionary history. These genetic similarities and efficient $C_4$ photosynthetic pathway contribute to their similar end uses and adaptability to similar environmental conditions (warm and dry). This review article will explore the transformative potential of utilizing CRISPR/Cas9 technology to overcome persistent challenges. We will examine innovative approaches, substantial progress, and ongoing efforts to enhance sorghum and maize production while emphasizing the decisive influence of the CRISPR system in shaping the future of sorghum and maize breeding. Therefore, in the following section, we put forward a brief description about genome editing.

**Core Ideas**

- The sophisticated capabilities of prime editing are inspiring, thus giving room for optimizing it in various plants.
- Revising the current regulatory laws of gene-edited crops will increase their field trials on a plot scale.
- Pollen magnetofection and nanoparticle-mediated delivery methods are promising alternatives for transformation.

## 2 | GENOME EDITING

### 2.1 | Concept and application of genome editing

Genome editing is the ability to make specific changes to an organism's DNA, thus facilitating targeted gene mutation throughout the plant genome (Lassoued et al., 2019; Puchta, 2017). This technology leads to the production of plants with desired characteristics through modification of specific genes. Currently, this technology is made up of tools such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), CRISPR/Cas9, base editing (BE), and prime editing (PE). A detailed comparison of these precision gene editing tools is provided in Table 1. TALEN and ZFN are precise but found to be time-consuming and cost ineffective. Despite the fact that both depend on engineered proteins to recognize and bind specific DNA sequences, they differ in their targeting mechanism. This makes CRISPR/Cas9 stand out as a versatile approach. It uses the guide RNA (gRNA) to guide Cas9 protein to a specific DNA sequence thus making it easier to construct in comparison with ZFN and TALEN. In maize, genome editing has been successfully applied using sgRNA:Cas through immature embryos, ribonucleoproteins (RNPs) and gene gun method (Svitashev et al., 2016), which holds promise for creating a way for crops resistant to protoplast regeneration (Hernandes-Lopes et al., 2023). PE and BE are the latest gene editing tools under the CRISPR/Cas9 system. Particularly PE, despite being in its infancy, it has been applied in a number of crops maize

**TABLE 1** Comparative analysis of gene editing techniques.

| Parameters | ZFN | TALENNs | CRISPR/Cas9 | Base editing | Prime editing |
|---|---|---|---|---|---|
| Endonuclease | Fok1 | Fok1 | Cas9 | dCas | pegRNA |
| Mutation rate | Moderate | Moderate | Low | High | Very high |
| Multiplex genome editing | Difficult | Difficult | Easy | Easy | Easy |
| Cost effectiveness | Not | Not | High | Very high | Very high |
| DNA binding determinant | Zinc finger protein | Transcription activator like effector | sgRNA | nCas | pegRNA |
| Target size length (bp) | 18–36 | 30–40 | 22 | 4–6 | 8–15 |
| Design feasibility | Difficult | Easier than ZFN | Easy | Easy | Easy |
| Target recognition efficiency | High | High | High | Very high | Very high |
| Off target | High | Low | Variable | Low | Very low |
| References | Gaj et al. (2013) | Ul Ain et al. (2015) | Shao et al. (2016) | Komor et al. (2016). | Anzalone et al. (2019) |

inclusive (Jiang et al., 2020) and shown substantial promise in genetic engineering. Having introduced the concept of genome editing, we shall dive deeply into these gene-editing technologies elaborating their potential and applications in the field of genetic engineering.

### 2.1.1 | Zinc finger nucleases

ZFN was among the first genome editing technologies that were developed in the 1990s, capable of precise mutations at specific sites (Y. G. Kim et al., 1996). It consists of a zinc finger protein (ZFP) and nuclease FokI, which was used to bind specific DNA sequences and cut DNA non-specifically respectively. The structure of a ZFN is composed of a zinc finger domain, a FokI nuclease domain, and a linker peptide. Zinc finger domains are present in many transcription factors (TFs) and able to recognize specific DNA sequences (Osakabe & Osakabe, 2015). A zinc finger domain consists of about 30 amino acids conserved $\beta\beta\alpha$ configuration (Beerli & Barbas., 2002) and therefore a typical ZFP contains several repeats. Numerous amino acids on the $\alpha$-helix surface generally interact with three base pairs in the main groove of DNA, exhibiting differing degrees of selectivity (Gaj et al., 2013). The modular architecture of zinc-finger proteins has rendered them a compelling basis for the engineering of customised DNA-binding proteins. Additionally, Peptides recognized by fingers do not support sequence-specific protein-DNA interaction. Therefore, a single ZFN cannot recognize its target DNA sequence (Carroll, 2011; Urnov et al., 2010). To recognize a target site, a ZFN with a six-finger structure is produced. The six fingers of ZFN consist of two functional interrelated domains: the recognition domain and the interaction domain (Porteus & Carroll, 2005). The recognition domain binds the target site in a sequence-specific manner, while the interaction domain recognizes the subsequent target site. ZFN has been applied to a number of plant species such as maize, apples (Peer et al., 2015) wheat, tobacco (Townsend et al., 2009), Arabidopsis (de Peter et al., 2013). Similarly, ZFNs were used to target *IPK1* gene at a specific site in the maize genome to increase gene targeting frequency (Yang & Qin, 2023). This was achieved by introducing heterologous donor DNA molecules into maize cells where 20% selected lines displayed inheritable gene targeting events inherited into the next-generation. These studies reveal that the use of ZFNs for targeted genome cleavage significantly improves HR-mediated gene targeting in plants. Even if ZFN is known for its high target binding efficiency, its use has gradually decreased in recent years. This is due to inadequate varieties of ZFPs (Gaj et al., 2013), the cost of zinc finger nuclease design is high, and the limited number of DNA sequences that can be specifically recognized (Mushtaq et al., 2018). Despite numerous successful studies, the research community has not extensively adopted the use of ZFNs in genome editing.

### 2.1.2 | Transcription activator-like effector nucleases

After the development of ZFNs, TALEN technology emerged with the ability to effectively edit live cells, thus contributing a substantial role in revolutionizing genome editing (Method of the Year, 2012; Menz et al., 2020). It was the first tool that could be designed and built with relative ease with the ability to target any specific genomic locus, guaranteeing high precision and efficiency with no protospacer adjacent motif (PAM) site restrictions (Malzahn et al., 2017). This technology is divided into two components: the TALE, which is responsible in targeting the protein to a specific DNA sequence and the nuclease for cutting the DNA. The commonly used

nuclease is called the Fok1 (Becker & Boch, 2021). TALENs are designed to target and bind to specific DNA sequences within the genome of an organism. This involves customizing Transcription Activator-Like Effectors (TALEs), originally found in plant pathogenic bacteria, to bind specifically to desired DNA sequences. Binding of TALENs induces a double-strand break (DSB) at the precise location, initiating the cellular repair machinery and enabling precise modifications to the target gene through error-prone non-homologous end joining or more precise homology-directed repair mechanisms.

Advantages a*nd application of TALEN genome editing*

TALEN genome editing offers advantages such as sequence precision, reduced cytotoxicity, ease of use, and affordability since they are structured to one nucleotide thus making them easy and less expensive to construct compared to other gene editing technologies like ZFNs (Joung & Sander, 2013). It has been successfully used in rice to produce disease-resistant varieties, such as those resistant to *Xanthomonas oryzae*-induced bacterial blight (T. Li et al., 2012), as well as in the production of aromatic rice (Shan et al., 2015; Shan, Wang, Li, Zhang, Chen, Liang, Zhang, Liu, Voytas, et al., 2013). In maize, TALENs have proven crucial for genome mutagenesis (Becker & Bouh 2021; Char et al., 2015; Liang et al., 2014; Si et al., 2015) resulting in the generation of transgenic lines with improved agronomic traits such as nutritional content, stress tolerance (Varotto, 2024). Although TALENs convey more advantages than ZFNs, they have limitations, such as, time-consuming, cost-ineffective and lower editing efficiency (Gaj et al., 2013). Due to the available alternatives such as CRISPR/Cas9, which is cheaper, easier to design, and associated with high editing efficiency. These drawbacks have restricted TALENs' wider utilization (Mushtaq et al., 2018).

### 2.1.3 | CRISPR/Cas9

The CRISPR/Cas9 technology has been used for over a decade to precisely and effectively edit plant genomes. This technology emerged just 2 years after the development of TALENs and its development has significantly transformed biotechnology research. CRISPR/Cas9 technology exploits the adaptive immunity system of the bacteria *Streptococcus pyogenes* in DNA repair to edit the genome of the targeted organism (Gan & Ling, 2022; Wiedenheft et al., 2012). This technology consists of two essential elements: a single guide RNA (sgRNA) responsible for identifying the target DNA and the Cas9 endonuclease (Ran et al., 2013), which creates DSBs at predetermined DNA locations. The Cas9 protein, initially characterized in *S. pyogenes* (Jiang & Doudna, 2017), possesses two nuclease domains, HNH and RuvC-like, which together cut both DNA strands, generating DSBs with blunt ends. This triggers the DNA repair mechanisms of the host cell, leading to targeted genetic mutations. The CRISPR/Cas9 system can be applied to any genomic site that contains a PAM (Ran et al., 2013), such as NGG (specific PAM sequence) for the widely used *S. pyogenes* Cas9 variant, near the region of interest. The Cas9 protein remains constant, while the sgRNA's guide sequence can be altered, allowing the system to target different genomic locations (Doudna & Charpentier, 2014). However, Cas nuclease has the capability to change in order to have a similar system that utilizes a different gRNA. This versatility and adaptability make CRISPR/Cas9 a widely adopted genome editing tool.

*How CRISPR/Cas9 works?*

The CRISPR/Cas9 genome editing system operates by recognition, cleavage, and repair (Shao et al., 2016). The Cas9 protein, irrespective of its role in the CRISPR/Cas9 system, only functions in the presence of the sgRNA. The sgRNA through its 5′crRNA complementary base pair component directs the Cas9 and recognizes the target sequence in the desired gene. Double-stranded breaks (DSBs) are initiated by the Cas9 nuclease at a base pair upstream to the PAM site (Ceasar et al., 2016). The Cas9 nuclease recognizes the PAM sequence at 5′-NGG-3′and once it finds the target site with the appropriate PAM, it triggers local DNA melting and the formation of RNA-DNA hybrid. Then, the Cas9 protein is triggered for a DNA cleavage (Figure 1). The complementary strand and the non- complementary strand of the target DNA are cut by HNH domain and RuvC domain respectively thus blunt-ended DSBs, which are eventually restored by the host cellular machinery (Jiang & Doudna., 2017; Mei et al., 2016).

*Advantages of CRISPR/Cas9 system*

CRISPR/Cas9 has been extensively applied to various crops, including model plants like Arabidopsis and economically important crops like maize, rice, tomatoes, sweet potatoes (*Ipomoea batatas* (L.) Lam.), and wheat (L. Dong et al., 2019; Grootboom et al., 2010; Sanchez-Leon et al., 2018; Si et al., 2015; H. Wang, Wu, et al., 2019), using different delivery explant such as roots, protoplasts, leaves, immature embryos, and mature seeds. This technology offers efficient multiplex genome editing capabilities, making it a significant advancement in crop breeding (Doll et al., 2019). Additionally, it is highly efficient, modified, and applicable in crop breeding, setting it apart from other gene editing technologies (Doll et al., 2019; H. J. Liu et al., 2020). According to Ao et al. (2018), McCormick et al. (2018), Symington and Gautier (2011), and Xie et al. (2015), the CRISPR/Cas9 system is self-contained in nature, with the ability to cut DNA strands (simplifies the gene editing process compared to older technologies that rely on separate cleaving enzymes). Furthermore, the adaptability and flexibility of the CRISPR/Cas9 system are evident in its compatibility with tailor-made gRNA

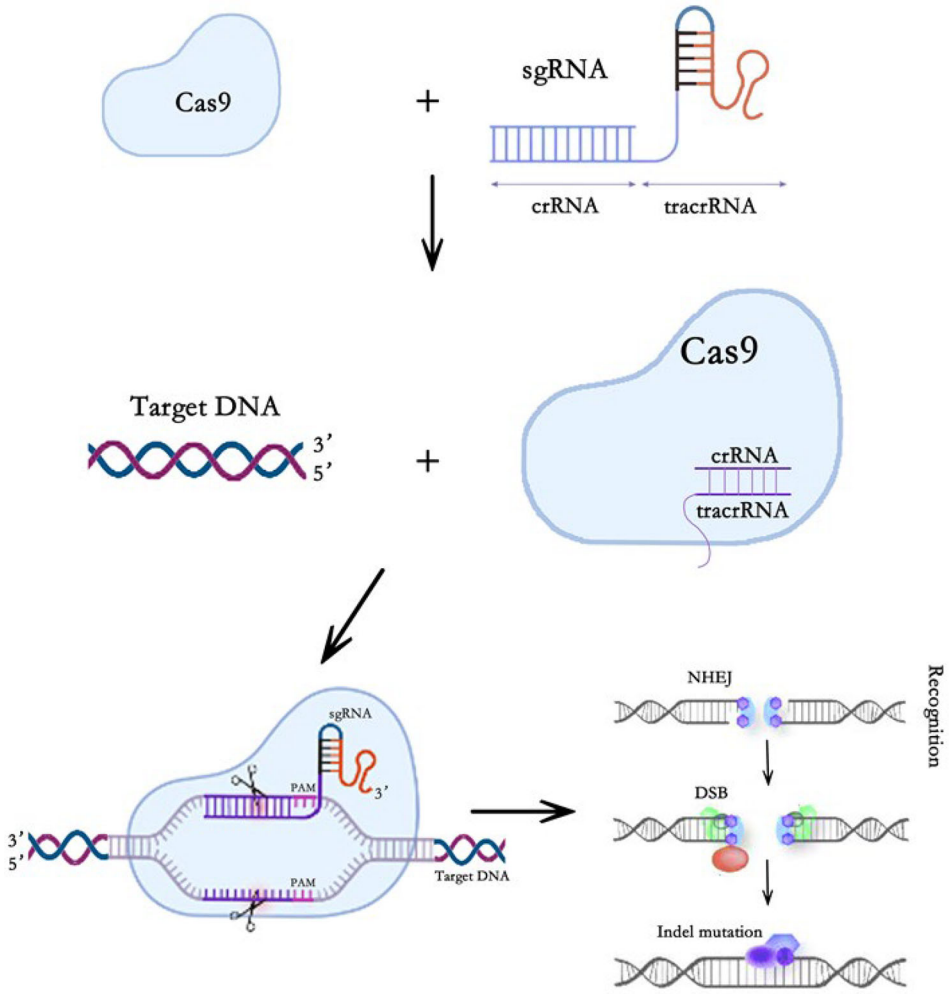


**FIGURE 1** CRISPR/Cas9 (where CRISPR is clustered regularly interspaced short palindromic repeats)mechanism. Cas9 attaches to a specific region on the target DNA, guided by the guide RNA (gRNA) and the protospacer adjacent motif (PAM). Once bound, the Cas9 induces a double strand break (DSB) allowing genetic modifications (deletion and insertion of the template DNA).

sequences designed to precisely direct the Cas9 nuclease to its DNA target. The availability of a vast repository of pre-designed gRNA sequences further strengthens its adaptability and ease of use.

The ability to obtain transgene-free mutant plants in the first generation (R. Chen et al., 2018; J. Li et al., 2016; Qi et al., 2016) is another valuable attribute of CRISPR/Cas9 system, thus enabling immediate application of edited sorghum and maize lines in breeding. It has demonstrated high efficiency in multiplex gene editing in sorghum (Ao et al., 2018; McCormick et al., 2018), enhancing the potential for sorghum functional genomics and breeding applications. For example, it has been used to target specific genes related to flowering development in sorghum (A. Li, Jia, et al., 2018), achieving a mutation efficiency of up to 100% and resulting in reliable alterations to flowering-related genes essential for controlling flowering time and crop yield.

CRISPR/Cas9 also holds promise for enhancing essential traits and new germplasm in various crops. Its applications include disease tolerance, yield enhancement, and improvement of nutritional capabilities. For instance, in wheat, the knockdown of GW2 (grain weight 2) encoding ring-type E3 results in increased grain width and length (W. Wang et al., 2018; M. Zhang, Cao, et al., 2018), while targeted mutagenesis of TasBella through CRISPR/Cas9 in wheat produces high amylose wheat with enhanced resistant starch content (J. Li, Jiao, et al., 2021). These improvements enhance both the quality and quantity of the crops. This technology has been effectively implemented in maize and sorghum, with the findings summarized in Table 2. Ultimately, the CRISPR/Cas9 system has enabled numerous technical advancements, including BE and PE, which are thoroughly examined in the subsequent sections.

### 2.1.4 | Base editing

BE technology is an innovative gene alteration under CRISPR/Cas9 framework. It directly makes targeted and irreversible base conversion without creating DSBs (Gaudelli

**TABLE 2** A number of endogenous genes targeted using CRISPR/Cas9.

| Traits | Specific trait improved | Crop | Targeted gene | Delivery method | Editing efficiency | Year of study | Reference |
|---|---|---|---|---|---|---|---|
| Quality improvement | Protein digestibility and lysine content | Sorghum | *k1C* | *Agrobacterium* | 92.4% | 2018 | (Li et al., 2018) |
| Yield improvement | Flowering | Sorghum | *sbFT* (*sb10G04510*) | *Agrobacterium* | 33.3% | 2020 | (Char et al., 2020) |
| Yield improvement | Flowering | Sorghum | *sbGA20ox5* (sb09G230800) | *Agrobacterium* | 83.3% | 2020 | (Char et al., 2020) |
| Quality and yield improvement | Lignin content | Sorghum | *CAD* | Biolistic bombardment | 25% | 2019 | (G. Liu et al., 2019) |
| Yield improvement | Leaf inclination | Sorghum | *SbLG1* | *Agrobacterium* | 33.3% | 2021 | (Brant et al., 2021) |
| Quality improvement | Fragrance | Sorghum | *SbBADH2* | *Agrobacterium* | NA | 2022 | (Suebpongsang et al., 2020 |
| Yield Improvement | Grain yield | Maize | *CLE* | *Agrobacterium* | 18.5% and 13.5% | 2021 | (L. Liu, Gallagher, et al., 2021) |
| Quality improvement | Regulate Zein protein levels | Maize | *ZmbZIP22* | *Agrobacterium* | NA | 2018 | (A. Li, Jia, et al., 2018 |
| Quality improvement | Waxy | Maize | *Wx1* | *Agrobacterium* | NA | 2020 | (Gao et al., 2020) |
| Yield improvement | Kernel weight and number | Maize | *ZmVLHP-01* | *Agrobacterium* | NA | 2019 | (Kelliher et al., 2019 |
| Yield improvement | Kernel row number | Maize | *ZmGB1* | *Agrobacterium* | NA | 2020 | (Wu et al., 2020) |
| Yield improvement | Maize kernel | Maize | *ZmNRPC2* | *Agrobacterium* | NA | 2020 | (Zhao et al., 2020) |
| Quality improvement | Phytic acid synthesis | Maize | *ZmIPK* | Protoplast transformation | 13.1% | 2014 | (Liang et al., 2014) |
| Quality improvement | Fragrance | Maize | *ZmBADH2a/2b* | *Agrobacterium* | NA | 2021 | (L. Wang, et al., 2021) |
| Quality improvement | Super sweet and waxy | Maize | *SH2, GBSS* | *Agrobacterium* | NA | 2019 | (L. Dong et al., 2019) |
| Quality improvement | Lignin content | Maize | *ZmMYB69* | *Agrobacterium* | 40% | 2022 | (Qiang et al., 2022) |
| Stress resistance | Thermo sensitive-male sterility | Maize | *ZmTMS5* | *Agrobacterium* | 48% | 2017 | (J. Li et al., 2017) |
| Stress resistance | Fungal resistance | Maize | *ZmFER1* | *Agrobacterium* | 60% | 2022 | (C. L. Liu et al., 2022) |
| Stress resistance | Moderate water use efficiency | Maize | *ZmAbh4* | *Agrobacterium* | 26.7% | 2022 | (Blankenagel et al., 2022) |
| Stress resistance | Viral resistance | Maize | *ZmGDIα* | *Agrobacterium* | 40.98% | 2022 | (Cui, 2021) |
| Stress resistance | Smut resistance | Maize | *ZmLOX3* | *Agrobacterium* | 97% | 2020 | (Pathi et al., 2020) |
| Stress resistance | Salt stress | Maize | *ZmHKT1* | *Agrobacterium* | 100% | 2014 | (M. Zhang, Cao, et al., 2018) |
| Stress resistance | Herbicide resistance | Maize | *ZmALS1 and ZmALS2* | *Agrobacterium* | 14% | 2020 | (Y. Li et al., 2020) |
| Male sterility | Male sterile | Maize | *LIG, MS26, MS45* | Biolistic bombardment | NA | 2016 | (Svitashev et al., 2016) |
| Haploid induction | Haploid maize | Maize | *ZmPLA1* | *Agrobacterium* | 2% | 2017 | (C. Liu et al., 2017) |
| Haploid induction | Haploid maize | Maize | *ZmDMP* | *Agrobacterium* | NA | 2019 | (Zhong et al., 2019) |
| Haploid induction | Haploid maize | Maize | *ZmPLD3* | *Agrobacterium* | 3%–4% | 2021 | (J. Li, et al., 2021) |

Abbreviation: NA, not applicable.

et al., 2017). This technology has recently gained quick acceptance and adaptation because of its precision, simplicity, and multiplex capabilities. Similarly, the BE technology enables precision of nucleotide substitutions in a programmable manner, without requiring a donor template (Komor et al., 2016). This has been efficiently used to induce C:G to T:A conversions at the initial stages of embryo development. The power of BE has propelled progress in genetic studies, functional genomics, and gene therapy. It can be used to introduce specific mutations, which is capable of changing the function of a gene (Komor et al., 2016). BE is an efficient technology for engineering novel traits in agriculturally important crops due to its ability to induce precise nucleotide substitutions without generating transgenes, a key to food security. The first successful use of a plant cytosine base editor converted Cas9-free rice plants to create stable and functional herbicide-resistant rice with improved yields. The plants carried a BE-generated point substitution. They added this substitution to a homozygous knockout of a target gene that encoded a D-sensitive enzyme (Zong et al., 2017). BE is a novel technology that efficiently and precisely converts one base to another in the genome of plants and animals without creating DSB. This technology has been successfully applied in both plants and animals, offering high precision and non-generation of DSB. However, further improvements are needed to enhance the scope and efficiency of editing, including overcoming off-target effects and bystander mutation generation. Artificial intelligence-based algorithms can also be used to design sgRNAs for precise modifications in crops for sustainable production in the face of global changes.

### 2.1.5 | Prime editing

PE is a CRISPR/Cas-based gene editing approach that facilitates versatile and precise alterations in the genetic code of a genome of interest (C. Lu et al., 2022). It is sometimes referred to as the search and replace genome editing tool. This latest technology operates with limited errors and off-target effects. The outstanding objective of PE is to achieve precise modifications in the genome by accurately replacing target sequences of varying lengths, thereby minimizing unintended alterations. (Vats et al., 2024)

The PE system is made up of two components: the prime editing guide RNA (pegRNA) and a prime editor (PE). The pegRNA is made up of a spacer sequence that corresponds with one strand of DNA, a primer binding site (PBS) sequence (~8–16 nt), and a reverse transcriptase (RT) template. This RT contains the desired editing sequence to be copied into the target site in the genome via reverse transcription. For PE, it operates with a modified version of the Cas9 enzyme popularly known as the Cas9 nickase (nCas9s) that can only cut one strand of DNA. This leads to the formation of an R-loop, which induces single-stranded breaks (SSBs) (Huang & Liu, 2023). RT enzyme is another component of PE that performs the required editing. The nCas9s, particularly the H840A variant, influence the binding of a pegRNA to the target site. The SSB generates a 3′ single stranded end that binds to the PBS on a 3′ extension of pegRNA. This extension contains both PBS and RT template carrying the desired genetic sequence (Anzalone et al., 2019; Chen & Liu, 2023). An RT peptide fused to the Cas9 nickase (H840A) enzyme adds deoxynucleotides to the 3′-OH end of the nicked strand using the code provided by the RT template. This synthesis creates a 3′ flap, which can compete with the original sequence at the 5′ nicked end for integration into the genome. According to G. Chen, Zhou, et al. (2021), this may lead to DNA mismatch repair (MMR), of which the cell's MMR system is capable of recognizing these mismatches and correcting them. This paves a way for the newly introduced sequence to correctly integrate into the genome (Anzalone et al., 2020), thus achieving the desired editing outcome. There is also a possibility of correcting the mismatch through returning the sequence back to original sequence, which reduces the efficiency of the PE process (G. Chen, Zhou, et al., 2021). PE differs from CRISPR/Cas9 by the mechanism of action. PE uses an SSB rather than CRISPR/Cas9 that creates a DSB in the DNA (Huang & Liu, 2023).

#### *PegRNA design and development*

PegRNA plays a crucial role in stabilizing PE efficiency. This is because the major function of PE of search and replace is based on pegRNA. Therefore, in order to create a competent pegRNA, software tools such as Plant peg-Designer have been generated. This software has been developed based on the melting temperature (Tm) of the PBS sequence. As PBS Tm approaches 30°C, it increases the efficiency of plant prime editing (PPEs), thus streamlining the pegRNA design and enhancing its precision (Vu et al., 2024). The extended expression cassettes of pegRNA and the potential formation of double-stranded complexes between PBS and spacer regions have the potential to inhibit the pegRNA expression. Therefore, augmenting the expression level of pegRNA is crucial in ensuring enhancement of PE efficiency.

Of late, efforts to augment pegRNA expression mostly in plants have been established with no pertinent studies identified in mammalian cells (Jiang et al., 2020). Jiang et al. (2020) further elaborated two ways to augment pegRNA expression in maize using the PPE3 system: utilizing a CaMV35SCmYLCV-U6 composite promoter to drive pegRNA and increase the number of pegRNA cassettes twofold. The findings indicated that employing the composite promoter substantially enhances PE efficiency from 0% to 43.8%, although doubling the quantity of pegRNA cassettes did not exhibit a notable impact on PE efficiency. Moreover, Qiao et al. (2023) subsequently discovered that increasing the

quantity of pegRNA cassettes enhances PE efficiency using the PPEmax method. H. Li et al. (2022) discovered that substituting the OsU3 promoter with the CaMV35S-CmYLCV-U6 composite promoter markedly enhances PE efficiency inside rice. The aforementioned results demonstrate that optimizing expression levels of pegRNA significantly enhanced PE efficiency.

*Advantages and application of PE*

PE offers accuracy, precision, and flexibility (Huang & Liu, 2023; Lin et al., 2020) in gene editing. It has been applied in a number of crops and the results are promising; for example, the herbicide-resistant maize lines with the P165S mutation in the *ZmALS1* and *ZmALS2* genes, achieving an editing efficiency of 53.2% and 6.5%, respectively (Jiang et al., 2020). Other crops where this technology has been applied include Arabidopsis, rice, and wheat, with rice attaining an editing efficiency of about 21.8% (Jin et al., 2021; Lin et al., 2020; L. Wang, Kaya, et al., 2021). According to W. Xu et al. (2022), PE RT functional domains reported considerable improvement, where a 5.8-fold increase in editing efficiency was attained compared to the original PE. The efficiency of PE provides promise for efficient and precision plant breeding.

*Challenges encountered in the application of PE in plants*

Despite the promise, PE provides for precision plant breeding, it is still in its infancy and needs to overcome some challenges in order to display its full potential. To be precise, low editing efficiency remains a great challenge. Generally, the editing efficiency varies depending on the target loci and cell types; for example, a number of studies have shown low PE efficiency in dicots due to their conservative factor as compared to monocots. Vu et al. (2022) revealed low PE efficiency at the plant stage in tomatoes and expounded that PE components (nCas9–RT fusion and pegRNA) might have a great contribution to its low activity just as it is being discussed by Lu, Yuming et al. (2021), Perrroud et al. (2022), and L. Wang, Kaya, et al. (2021). L. Wang, Kaya, et al. (2021) reported a PE efficiency of about 0.06% ± 0.03% for correcting a mutated allele of the avrRpt2 gene of the bacterial plant pathogen *Pseudomonas syringae* that was co-infiltrated with agrobacteria carrying PE tool in tobacco leaves, and 0.07% ± 0.12% at a genomic site in *Arabidopsis* protoplasts.

Second is ineffective delivery of PE system in the target cells. The size of full-length PE presents a big challenge to its safe in vivo delivery since it prevents its integration into a single adeno-associated virus vector system. Thus, approaches such as optimizing pegRNA, manipulating cellular DNA repair pathways, increasing the targeting scope, and developing PE delivery strategies need improvement so as to enhance the PE efficiency in different plant species.

## 3 | PRE-REQUISITES OF CRISPR/Cas9 TECHNOLOGY IN SORGHUM AND MAIZE ADVANCEMENT

Gene knockout using CRISPR/Cas9 entails the construction of a gRNA and the introduction of both the gRNA and Cas9 protein into the target cell to enable the deletion and insertion of donor DNA. This technology has been effectively implemented in numerous plant species. To guarantee its success, specific conditions must be taken into account as discussed in the following sections.

### 3.1 | Selection of an appropriate Cas9 protein

The Cas9 protein, an endonuclease responsible for facilitating modifications to the genome through DSBs, holds significant importance. In basic plant biology studies, wherein the focus lies in gene function analysis, the *Cas9* gene is typically tagged with a molecular tag. This allows for protein detection/purification and includes a nuclear localization signal to aid the Cas9 protein's nuclear localization (Mazumdar et al., 2016).

Choosing the appropriate, modified Cas9 enzyme is crucial. Various Cas9 enzymes with distinct capabilities are available, such as spCas9 (*S. pyogenes* Cas9), which is the most widely used Cas9 protein (Lim et al., 2022; Wright et al., 2023; D. Zhang et al., 2020; A. Zhang, Liu, Wang, et al., 2019), and saCas9 (*Staphylococcus aureus*), known for its intracellular trafficking (Collias & Beisel, 2021; Menz et al., 2020). Smaller Cas9 protein variants are advantageous as they facilitate improved cellular absorption and target DNA recognition specificity, thereby enhancing efficiency. SpRYCas9, a recent variant, operates with near-zero PAM sequence requirements, thanks to the activity of NG-Cas9, outperforming SpCas9 that solely recognizes the 5′-NGG-3′ PAM sequence within a 20-nt DNA target site (Ahmad, 2023). Compatibility with the delivery system is also essential when selecting a Cas9 variant.

#### 3.1.1 | Alternative Cas nuclease

Additionally, there are several other Cas nucleases, such as Cas12a, Cas12b, Cas3, Cas13a, and Cas10 (Kumar et al., 2022; Assou et al., 2022). Other Cas proteins include StCas9 (*Streptococcus thermophilus*), NmCas9 (*Neisseria meningitidis*), and FnCpf1 (*Francisella novicida*) (Akella et al., 2021; He et al., 2022), each targeting different PAM sequences. Cas enzymes from different species have diverse recognition sequences, expanding the range of target sites and resulting

in varying sizes of insertion and deletion indels (Huang & Puchta, 2021).

For example, Cas12a has demonstrated relevance in precise editing due to its ability to differentiate among PAM sequences, leading to the formation of sticky ends instead of blunt ends (Ahmad, 2023).

## 3.2 | Promoters selection

Promoters are regulatory sequences situated upstream of gene coding regions that modulate the functional activity of genes and encompass distinct cis-acting elements (Kummari et al., 2020). They serve as binding sites for proteins that facilitate the start and control of transcription. Promoters are molecular biological clocks essential for determining targeted gene expression (Potenza et al., 2004), serving as critical regulatory checkpoints for gene transcription recognized by TFs (Smale & Kadonaga, 2003). TFs attach to particular cis-acting regions located on the corresponding promoter sequences via RNA polymerase, hence regulating the expression of downstream genes (Hernandez-Garcia & Finer, 2014). The targeted efficacy of CRISPR/Cas9 is based upon the codon optimization of Cas9 and the promoters (Guo et al., 2018).

The choice of promoters significantly influences tissue specificity and the level of expression of Cas9 and gRNA in plants (Ma et al., 2016; Sapara et al., 2024). The germline-specific promoters for Cas9, such as those controlling expression in egg cells and early embryos, are capable of enhancing the frequency and heritability of mutations in Arabidopsis. Similarly, this approach illustrated great significance in soybean (Z. P. Wang et al., 2015; Zheng et al., 2020). The *maize ubiquitin promoter* (*ZmUbi1*) has proven incredibly effective in recent years for sorghum and maize transformation (Brant et al., 2021). Additionally, the Pol III promoters U3 and U6 are greatly considered due to their broad range of tissue activity and ability to produce high levels of sgRNA (Kor et al., 2023; Ng & Dean, 2017). Likewise, using the maize ubiquitin1 gene promoter and two rice U6 promoters for rice codon-optimized Cas9 and sgRNAs, mutations in the targeted genes of approximately 70% were reported. Among these mutations, biallelic mutations of a gene were found at a frequency of about 22%–58% for four different genes (Char et al., 2017). Furthermore, the maize dmc 1 promoter and U3 promoter, along with Cas9 and sgRNA, respectively, demonstrated a 100% mutation efficiency in the transgene-positive calli. This demonstrated high genome editing efficiency at the targeted site (Feng et al., 2018). Additionally, endogenous U6 promoters increased CRISPR/Cas9 editing efficiency in sorghum and showed great potential for use in other cereals (Massel et al., 2022). However, the effectiveness and quality of different promoters differ, resulting in varying gene expression levels in designated tissues of maize and sorghum. CRISPR technology has gained prominence as an alternative to traditional plant breeding approaches. Therefore, efficiency of genome editing can be enhanced by maximizing the varied spectrum of the promoters as well as selecting the appropriate promoter for a specific explant in genome editing.

## 3.3 | gRNA design strategies

The gRNA and the Cas9 protein are powerful tools in manipulating the genome (Jinek et al., 2012). A well-designed gRNA ensures that the Cas9 is directed to the right specific site to facilitate deletion and insertion of the donor DNA. A number of studies have demonstrated how the gRNA and experimental conditions are the causal agents for Cas9 off-target activities (Fu et al., 2013; Hsu et al., 2013). This implies that for the success of CRISPR/Cas9 experiment, the design of gRNA is very important (Chari et al., 2015). These studies provide qualitative data, although the understanding of specificity determinants is incomplete and requires a vast number of possible imperfect sgRNA:DNA interactions to disclose sequence features for prediction of off-target activity (Doench et al., 2016). Recently, efforts have been made to develop computational tools that can assist in designing the gRNA. Such as the CRISPR direct, which enables efficient selection of the target site with limited off-target effects (Naito et al., 2015). This is because to achieve a successful gRNA, there should be maximum on-target activities while maximizing potential off-target effects, which can be challenging to balance the two. These tools are being designed to help achieve the best target sites other than unintended sites (Wilson et al., 2018). Research further explains that future models may have the capability of predicting both the CRISPR/Cas9 editing and the outcome (Bae et al., 2014; Yao et al., 2017).

## 3.4 | Choosing an effective delivery system

An effective delivery system is critical in introducing CRISPR/Cas9 elements, such as the Cas9 gene and gRNA, into maize and sorghum cells for genome editing. This enhances the efficiency, accuracy, and accessibility of editing efforts in these essential crops (L. Liu, Gallagher, et al., 2021; Miller et al., 2023). Common delivery methods include Agrobacterium-mediated, particle bombardment, and PEG-mediated transformations, each with advantages and limitations in different crops. Particle bombardment, a widely employed delivery method since the 1990s, has been notably effective, as evidenced by the proliferation of commercially released transgenes (Christou, 1994). This technique remains pivotal in biotechnology and has even been applied in "nanobiolistics" (O'Brien et al., 2011). A 25% efficiency was achieved in knocking out the *CAD* gene

in sorghum using particle bombardment (Liu et al., 2019) while also producing superior maize by targeting the *ZmIPK* gene for knockout (Liang et al., 2014). Protoplast transformation has been used in a number of crops along with CRISPR/Cas9, such as Arabidopsis thaliana, tobacco, lettuce (*Lactuca sativa* L.), and rice (Woo et al., 2015), receiving a transformation efficiency of up to 46%. Agrobacterium-mediated delivery has been widely used in >20 species of plants, demonstrating high editing efficiency compared to other methods (Sandhya et al., 2020). For example, an editing efficiency of 92.4% in sorghum by knocking out the *k1C* gene using Agrobacterium-mediated delivery was achieved (A. Li, Jia, et al., 2018). Similarly, the CRISPR/Cas9 binary vector with two gRNA expression cassettes targeting the *ZmHKT1* gene achieved 60% of transgenic lines with 100% editing efficiency in maize (Xing et al., 2014). However, some crops lack suitable characteristics for regeneration and culture (Hamada et al., 2017), which makes the transformation process challenging and laborious (L. Liu, Gallagher, et al., 2021). Therefore, the discovery of delivery methods that simplify the genome editing process is necessary since delivery methods are key to efficiency and accuracy in this process. Pollen magnetofection-mediated delivery and nanoparticle-mediated delivery methods show promise for future utility and are capable of replacing traditional tissue culture process (Sandhya et al., 2020), which tends to be tiresome and time-consuming. Nanomaterials are capable of diffusing into the plasmid DNA without external assistance (H. Wang, Wu, et al., 2019), making them suitable for CRISPR/Cas9 applications in various plants. These delivery methods are deeply reviewed under limitations associated with the application of CRISPR/Cas9 in sorghum and maize.

## 4 | APPLICATION OF CRISPR/CAS9 IN SORGHUM AND MAIZE

### 4.1 | Quality enhancement

#### 4.1.1 | Nutritional, digestibility, and palatability enhancement

CRISPR/Cas9 technology has emerged as a powerful tool for improving sorghum, maize, rice, wheat, tomatoes, and bananas' nutritional content, digestibility, and palatability. Researchers have strategically modified genes responsible for nutrient uptake and synthesis, resulting in elevated levels of essential nutrients such as iron and zinc (Ibrahim et al., 2021) in wheat and vitamin A in golden rice (O. X. Dong et al., 2020). Furthermore, technology has been used to enhance the digestibility of sorghum kernels by disrupting the outer layer structure of the kafirin body, resulting in improved protein quality and digestibility (A. Li, Jia, et al., 2018). These advancements not only increase the nutritional value of sorghum but also improve its palatability. Importantly, mutants with altered $\gamma$-kafirin structure exhibit robust early-stage development compared to the wild type. The use of the CRISPR/Cas9 editing system to target the *k1C* genes has shown promise in reducing Kafirin levels, improving protein quality, and enhancing digestibility. These advancements not only reinforce the digestibility of this essential crop but also pave the way for the rapid development of transgene-free, improved sorghum cultivars. Development of transgene-free plant varieties greatly addresses public perception, disapproval of gene edited crops, and solves regulatory challenges regarding food safety. This is essential for the widespread adoption and success of genetically edited crops. This approach holds significant promise for enhancing the quality and nutritional value of sorghum, an important crop worldwide (Jambunathan, 1980; X. Li et al., 2023). In maize, the disruption of the *Wx* gene using CRISPR/Cas9 resulted in the creation of 12 elite inbred lines of waxy maize variants (Gao et al., 2020), with approximately 100% increase in amylopectin content in these maize grains. Waxy maize has desirable properties, including enhanced palatability because it tastes nice and it is fluffy; it is also widely utilized as a thickener in various food products due to its unique texture. Other benefits of waxy maize include its young and early harvest, which can contribute to food and nutritional instability.

#### 4.1.2 | Production of fragrant sorghum and maize

The demand and market value of fragrant food resources have contributed to exploring emerging technologies to enhance fragrance in crops such as maize, sorghum, cucumber, soybean, and rice (Juwattanasomran et al., 2011; Tang et al., 2021). One such technology is CRISPR/Cas9, a precise genome-editing tool used to modify specific genes responsible for fragrance production. For maize and sorghum, CRISPR/Cas9 can target and enhance the expression of genes involved in the biosynthesis of volatile compounds that contribute to their aroma. For instance, fragrant maize was successfully created through inactivating the betaine aldehyde dehydrogenase (*BADH2*) genes through genome editing (L. Wang, Kaya, et al., 2021). The market value of fragrant sorghum is expected to increase due to its potential appeal to consumers and its application in the brewing industry, particularly in producing Chinese traditional vinegar and different types of liquor (F. Wang, Wang, et al., 2016). Similarly, researchers achieved a breakthrough in creating fragrant cultivated sorghum using the CRISPR/Cas9 technology to increase 2-acetyl-1-pyrroline accumulation in seeds and leaves (D. Zhang et al., 2022). These advancements not only increase the sensory appeal of these crops but also have

significant market implications. Enhanced fragrance can lead to higher consumer preference and potentially increase the market value of these crops (Suebpongsang et al., 2020), opening new opportunities for farmers and food producers.

## 4.2 | Increasing yield

One of the primary goals of scientists is to increase crop yields, and yield-related traits are often described based on factors such as grain weight, grain size, panicle size, and grain number (Voss-Fels et al., 2019). Knocking out the *ZmCEP1* gene enhanced plant height, ear length, kernel size, and 100-kernel weight. The *ZmCEP1* gene is involved in nitrate and sugar transport into the kernel (Xu et al., 2021). Leaf inclination angle is another essential agronomic trait in cereals like rice, maize, and sorghum, as it can influence plant yield and planting density. Furthermore, a change in the angle of incidence by 10˚ for example, from 50˚ to 60˚, leads to a sudden difference in intercepted direct radiation by 22%, which definitely affects the level of photosynthesis and other physiological processes such as leaf temperature, energy balance (Yang & Qin, 2023; Yu et al., 2024). For photosynthesis to take place, light energy is so crucial, so a disruption in leaf angle may affect the rate of photosynthesis and definitely the plant yield. Several studies have explored the association between leaf angle and erectness, which can improve light capture and $CO_2$ diffusion efficiency, ultimately enhancing photosynthetic efficiency (Wang & Li, 2011) as well as affecting the plant growth and biomass (Hu et al., 2019). In sorghum, successful knockout of the *Liguleless1* gene using CRISPR/Cas9 resulted in an intermediate leaf inclination angle, providing potential for enhancing yield (Brant et al., 2021). The advancement of this technology renders its accessibility to underdeveloped countries, where food insecurity is caused by insufficient food supply resulting from low agricultural yields.

## 4.3 | Tolerance to abiotic and biotic stress

Abiotic and biotic stresses, along with climate variations, significantly affect the quality and yield of crops like maize and sorghum (Jha et al., 2020). The CRISPR/Cas9 system has demonstrated the potential to generate crops with high tolerance to abiotic stresses such as drought, salinity, cold, and herbicide by modifying stress-sensitive genes such as *ZmAbh4* (Blankenagel et al., 2022), *ZmGDIα* (C. L. Liu et al., 2022), *ZmLOX3* (Pathi et al., 2020), *ZmHKT1* (Xing et al., 2014), *ZmALS1*, and *ZmALS2* (Li et al., 2020). This potential is particularly significant when applied to already elite high-yielding but sensitive varieties, as these modifications can enhance stress tolerance while maintaining their high yield. This approach is highly precise and allows for the retention of the genomic background of a particular variety. Abiotic stresses are often governed by quantitative trait loci consisting of multiple genes (Zafar et al., 2020). For example, enhancement of the expression level of the *ARGOS8* gene using CRISPR/Cas9 gene editing negatively regulated ethylene response and improved grain yields of approximately 314 kg per hectare under drought-stress conditions (Shi et al., 2017). Similarly, CRISPR/Cas9-mediated gene editing led to the creation of *zmHKT1* mutants in maize, which exhibited increased root-to-shoot $Na^+$ delivery and enhanced salt tolerance (Jiang et al., 2022). *ZmHKT1* is a major salt-tolerance QTL and has been identified as a $Na^+$-selective transporter (M. Zhang, Cao, et al., 2018). The maize stiff1 gene encodes an F-box domain protein, and the knockout plant (Cstiff1) produced by the CRISPR/Cas9 method exhibited a more robust stalk than the unedited control (D. Zhang et al., 2020). Numerous maize varieties resistant to plant pathogens like *Ustilago maydis* and *Fusarium graminearum* have been generated, contributing to biotic stress tolerance and increased maize yield (Matsushita et al., 1999; Pathi et al., 2020).

## 4.4 | Male sterility

The CRISPR/Cas9 system has been successfully applied to create male sterile lines in major food crops worldwide, including soybean, tomatoes, maize, and rice (Barman et al., 2019). Understanding the molecular mechanisms underlying anther and pollen development has led to the identification of potential genes responsible for male sterility, facilitating the incorporation of gene editing and male sterility methods in breeding programs focused on developing hybrid crops (A. Zhang, Liu, Wang, et al., 2019). Generating hybrid seeds is easier and more important with a male sterile phenotype than using cytoplasmic male sterile lines (X. Qi et al., 2020).

Through the adoption of CRISPR/Cas9-mediated gene knockout technology, researchers have produced maize genetic male-sterility (GMS) lines and temperature-sensitive male-sterility (MS) lines (R. Chen et al., 2018; Jiang et al., 2021; J. Li et al., 2017; Svitashev et al., 2016). In addition, stable male sterile maize mutants with Mendelian genetic rules have been obtained using CRISPR/Cas9 vectors (R. Chen et al., 2018). Another RNA-seq analysis combined with CRISPR/Cas9 mutagenesis has proven valuable in identifying novel maize GMS genes that exhibit functional redundancy (Jiang et al., 2022).

## 4.5 | Haploid induction

Haploid induction boosts breeding efficiency by producing homozygous lines in crops like maize, sorghum, rice, and

wheat that are precise and uniform in a short breeding cycle. Haploid plants are made of a single set of chromosome with the ability to generate homozygous diploid plants, after being doubled (Dwivedi et al., 2015; Prasanna, 2012). To accelerate breeding efforts, CRISPR/Cas9 has been employed to knock out genes for gene function validation. Key genes in maize, such as *ZmPOD65*, *ZmPLD3*, *ZmDMP7*, and *ZmMTL*, play a crucial role in breeding haploid inducers.

The knockout of the *ZmPLA1* gene in maize resulted in the production of maternal haploid inducers, paving the way for effective haploid identification markers and the breeding of doubled-haploid crops (L. Dong et al., 2018). Furthermore, recent research suggested that the successful haploid induction system in maize could potentially be utilized in other crops such as rice, sorghum, and foxtail millet. This is supported by the high sequence similarity of 91% between maize and sorghum, as well as 73% between maize and millet (C. Liu et al., 2017).

The study also demonstrated that the incorporation of *ZmPLA1* and *ZmDMP* genes exhibited a high haploid induction rate. Their similarity implies shared roles, providing valuable insights for future investigations into the molecular mechanisms of haploid induction (J. Li, Jiao, et al., 2021; Zhong et al., 2019).

Several endogenous genes in maize and sorghum have been modified using genome editing techniques, particularly CRISPR/Cas9, with the aim of enhancing its value (see Table 2).

## 5 | LIMITATIONS OF CRISPR/CAS9 TECHNOLOGY IN SORGHUM AND MAIZE ADVANCEMENT AND FUTURE PROSPECTS

### 5.1 | Off-target effects

The application of CRISPR/Cas9 in crops faces limitations, particularly off-target effects, wherein unintended mutations can occur at other sites other than the intended target, potentially leading to adverse outcomes in the crop's genome. Off target sites are usually sgRNA dependent, and whereby Cas9 is recognized to facilitate a maximum of three mismatches throughout the entire 20-bp DNA target sequence, which increases the possibilities of off-target effects (Adhikari & Poudel, 2020). In silico tools (CasOT, Cas-OFFinder, FlashFry, Crisflash, MIT, CCTop, CFD, and DeepCRISPR) are used to identify potential off target sites and measure chances of off-target effects. These are open-source online software that can be conveniently accessed via internet (Bao et al., 2021). The predictions of these software tools entirely rely on sgRNA sequences thus, the results are usually biased toward sgRNA-dependent off-target effects, which may require experimental validation. Second, are experimental tools categorized in three methods; Cell-free methods, Cell culture-based methods, and in vivo methods. Cell free methods (Digenome-seq, DIG-seq, Extru-seq, SITE-seq, and CIRCLE-seq) reconstitute nuclease reaction on DNA that are extracted from the cells. Digenome-seq, DIG-seq, Extru-seq, are usually expensive since they require high sequencing coverage and difficult to detect Cas9-mediated large deletions. In such a scenario, scientists developed SITE-seq, which requires a much less sequencing coverage and adds a selective biotinylation reaction on the cleaved genomic site to enrich them before sequencing (Cameron et al., 2017). However, SITE-seq tool provides about 10% positive hits that are certified by the targeted sequencing, this makes its rate of accuracy in finding off target sites low (Kim et al., 2019). Similarly, CIRCLE-seq is another cell free method that is highly sensitive. The presence of Cas9/sgRNA complexes, the circular DNA fragments were selectively linearized upon Cas9 nuclease cleavage and eventually become available for high-throughput sequencing (Lv et al., 2022; Pan et al., 2022; Tsai et al., 2017). This has been demonstrated in maize gene editing, where 16–67 potential off-target sites were identified for two gRNAs with five mismatches (Lee et al., 2018). However, there are false positive frequency of CIRCLE-seq that require careful downstream validation (Tsai et al., 2017). Cell culture-based methods (whole genome sequencing [WGS], ChIP-seq, GUIDE-seq, and BLESS) were developed due to the behavior of the genome editors influenced by intranuclear context to ensure direct assessment of the off-target effects in cells. WGS provides an unbiased survey of full genome nuclease activities, identifying the clear location of the off target edit (J. Li et al., 2019). However, it comes along with excessive costs where CRISPR/Cas edited sites have to be enriched before sequencing. Similarly, in vitro assays and bioinformatics tools (Discover-seq, GUIDE-tag) are also utilized in predicting off-target activities due to their high level of sensitivity. Although they have false positives and the incorporation rate of biotin-dsDNA is relatively low (~6%) respectively, they are important in identifying off target sites.

According to (J. Li et al., 2019), off-target effects in plants are relatively low but this does not inhibit risks posed by unintended edits such as undesirable phenotypes in plants due to loss of genetic mutations (Guilinger et al., 2014). Besides, unanticipated genetic changes may cause biosafety regulatory concerns. All these methods that detect the occurrence of off-target effects in CRISPR/Cas9 have rapidly evolved of late; however, challenges still exist in balancing the accuracy and sensitivity of these tools.

Employing strategies such as enhancement of the delivery method, sgRNA improvement, Cas9 improvement, and DSB-independent gene editing can minimize the occurrence of off-target effects. Delivery methods are used in cell culture applications, such as RNP electroporation, viral transduction,

and plasmid transfection; RNP electroporation is suitable to deliver Cas9/sgRNA due to its lower off target mutations and higher on-target editing efficiency (Kim et al., 2014; Ramakrishna et al., 2014; Vakulskas & Behlke, 2019). Second, discovering new and improved Cas9 homologs that utilizes rare PAM sequences such as saCas9, st1Cas9, enhance specificity due to limited chances of binding to a non-target DNA. Similarly, enhancing the specificity of sgRNA design as well as length modification in the sgRNA extension approach, where two guanine nucleotides are usually added at the 5′ end of sgRNAs (termed 5′-GGX20) that minimize predicted off-target sites, hence effectively eliminating the majority of off-targeting events (Cho et al., 2014; Hahn & Nekrasov, 2019). Therefore, utilizing such strategies enhances the reliability of CRISPR/Cas9 technology (Mao et al., 2019).

## 5.2 | Editing efficiency

Delivery efficiency is one of the factors that greatly affect the editing efficiency of CRISPR/Cas9 technology (Chen & Gao, 2014). In the following section, we shall review delivery efficiency in details.

### 5.2.1 | Delivery efficiency

Delivery efficiency is usually determined by the choice of delivery method of CRISPR/Cas9 into the target cell, whereby low delivery efficiency caused by suboptimal delivery methods often leads to low editing efficiency. Therefore, to achieve success and a high editing efficiency in $C_4$ plants, the delivery method is a key consideration.

Biolistic bombardment, Agrobacterium, and PEG-mediated protoplast methods are commonly used for efficient genome editing. PEG-mediated protoplast has been widely used to deliver CRISPR/Cas9 particularly in plant protoplasts. Plasmids containing Cas9 and gRNA are incubated with protoplasts in the presence of polyethylene glycol (PEG), facilitating the entry of RNPs into the plant cells. This method has been used to achieve DNA-free genome editing using RNPs (Kim et al., 2017) as well as an editing frequency of up to 46% in lettuce (Woo et al., 2015). While it offers precision editing, it still faces some challenges that require alternative delivery methods to address, such as establishment of suspension cells and protoplasts isolation, and regeneration of protoplasts into whole plants in the case of recalcitrant plants (Sandhya et al., 2020).

Second is the Biolistic bombardment method that uses tungsten, gold and silver particles to carry Cas9/gRNA RNPs into the explant by applying high pressure. To achieve successful transformation, key factors such as helium pressure, particle size, and explant type must be optimized. This method has recorded successful transformation in maize, sorghum and potato (Andersson et al., 2018; Liu et al., 2019; Svitashev et al., 2015) the major advantage of this method is that it does not require CRISPR/Cas9 binary vector as other methods like agrobacterium (Table 2). It is also capable of delivering large and multiple DNA components to explants. However, it is still attached to relatively low editing efficiency, random integration patterns within the genome, it is expensive, and bombardment sites cannot be controlled.

Of all the delivery methods, Agrobacterium is the most widely used in CRISPR/Cas9 gene editing. It has exhibited the highest editing efficiency, proving to be cost-friendly and applicable across a broad range of species (Mao et al., 2019). This method uses a binary vector containing the Cas9 and gRNA expression cassette transforming it into an agrobacterium strain, which transfers CRISPR components into desired plant explants. Of late, Agrobacterium-mediated delivery of CRISPR/Cas9 has been successfully edited in >20 plant species. Monocots have successfully and frequently been edited despite their low regeneration and transformation capacity (Sandhya et al., 2020). With efforts to enhance editing efficiency through Agrobacterium mediated transformation, a CRISPR/Cas9 binary vector for both monocots and dicots was designed (Xing et al., 2014). Naim et al. (2018) reported an editing efficiency of 100% in Cavendish banana cultivar, highlighting its exceptional potential. This high efficiency is also attainable in other crops, such as maize and sorghum, highlighting the versatility and application of this method across other plant species. Moreover, it is also the most promising delivery method in woody plants as explained by (Jia & Wang, 2014; Zhou et al., 2015). Despite this method's drawback of using binary vector and integration of an alien gene into the plant genome, it has attained a wide applicability across different plant species as compared to other methods.

Additionally, all these delivery methods are associated with tissue culture, which is time-consuming and laborious. Therefore, there is a need to develop new delivery methods that do not require tissue culture. Promising alternatives include pollen magnetofection-mediated and nanoparticle-mediated delivery methods, which have demonstrated the capability to directly target the meristematic region. For instance, nanotechnology-based methods have been shown to be cost-effective and robust, enabling the transfer of genes with high efficiency and low toxicity in various plant tissues such as leaves, roots, and protoplasts (Chandrasekaran et al., 2020). Pollen magnetofection-mediated method is also capable of transferring the CRISPR/Cas9 RNPs directly into pollens. These methods have already been successfully applied in cotton, rice, and maize, offering the potential to overcome current limitations of transformation methods (Cuevas & Prom, 2020; El-mounadi et al., 2020; Hamblin & Jannink, 2011). Editing efficiency is highly determined by the delivery method;

therefore the need to develop these two potential delivery methods with the ability to deliver DNA components directly into the meristematic region will omit laborious time required for tissue culture, thus increasing the possibilities of high editing efficiency.

### 5.3 | Biosafety and regulatory policies toward CRISPR/Cas9 edited crops

The primary objective of advancing novel methodologies and advances in the application of CRISPR/Cas9 technology is to improve consumer quality of life via the cultivation of transgenic plants (Gan & Ling, 2022). Gene-edited organisms, modified via CRISPR/Cas9 technology, undergo genomic mutagenesis through deletions, substitutions, or insertions of base pairs, whereas GMOs entail the incorporation of foreign transgenes into the organism, which may or may not integrate into the genome (Callaway, 2018). Notwithstanding this essential distinction, gene-edited organisms are frequently regulated inside the identical framework as GMOs in several countries (El-Mounadi et al., 2020). The Court of Justice of the European Union recently determined that gene-edited crops are not exempt from the laws and regulations applicable to genetically modified (GM) crops (Callaway, 2018; Confédération paysanne & others v. Premier ministre & Ministre de l'Agriculture de l'Agroalimentaire et de la Forêt, 2018). This indicates that the stringent barriers established for the development of GM crops are similarly applicable to CRISPR/Cas9-edited crops, thereby deterring financing and investment in future research on CRISPR/Cas9 as a feasible plant-breeding tool. The EU's static definition of GMOs as "not naturally altered" has further influenced public view of CRISPR technology and genetic manipulation overall (Plan & Eede, 2010). The path to achieving public trust in the safety, efficacy, and advantages of GMOs is fraught with numerous social, economic, and legal obstacles (Zimny et al., 2019). Altering public perception of gene technology is essential to initiate necessary adjustments universally.

Unlike the EU, the US Department of Agriculture (USDA) excluded genome-edited plants from regulation, as long as their production does not involve plant pests (USDA, 2018). This new judgement not only emphasizes the safety and absence of dangers associated with genome-edited plants but also fosters advancement in the technology's development (Hoffman, 2021). The only genome-edited crop permitted to circumvent USDA restrictions is a CRISPR/Cas9-modified white button mushroom that exhibits resistance to browning (Waltz, 2016). The USDA has consistently financed research on CRISPR-edited plants, including rice (O. sativa) (Lee et al., 2019). Countries such as Argentina, Brazil, and Chile are already accepting CRISPR/Cas9-edited waxy maize for commercial purposes (Gao et al., 2020) as well as the GABA tomatoes in Japan (Waltz, 2022). Nonetheless, despite intermittent changes to GMO legislation and the establishment of new guidelines, Malaysia has not yet sanctioned the commercial cultivation of genome-edited crops (Singh et al., 2019). Like the EU, Malaysia's regulatory framework categorizes genome-edited crops as GMOs, making it challenging for any plants or crops to obtain permission from the system (El-Mounadi et al., 2020). Malaysia is cautious in permitting the open-field propagation of gene-edited crops; however, it has authorized over 30 instances of transgenic product imports, exclusively for consumption or processing, and has sanctioned confined field trials of transgenic plants like rubber and papaya (Singh et al., 2019).

Furthermore, reliable data on GE plants from field trials on a plot scale with repetition are often lacking around the world. This is due to the regulatory issues on GE plants (Metje-Sprink et al., 2020). Considerably, the most reported field trials are gene-edited rice varieties in Asia, specifically China (F. Wang, Wang, et al., 2016; Zhou et al., 2019). Other crops, such as tomatoes (Rodriguez-leal et al., 2017), sugarcane (Kannan et al., 2018), peanut (Wen et al., 2018), and maize, have undergone field trials using fewer varieties as compared to rice. In maize, the natural promotor of *ARGOS8*, a negative regulator of ethylene response, was replaced by CRISPR/Cas9-directed homologous recombination, which resulted in increased grain yield under flowering stress conditions in the field (Shi et al., 2017). In all these field trials conducted, no harmful effect has been recorded on the environment. Revising the current regulatory laws will lead to an increasing number of GE plants in the field as well as establish consistent scientific and technological standards for the global commercial cultivation of CRISPR/Cas9-edited crops (Ahmad et al., 2021; D. Zhang et al., 2020). Similarly, incorporating contemporary technological methods into rules originally formulated for outdated technology is not a viable path forward. Conversely, laws and regulations necessitate modernization to align with the revolutionary potential of innovation. Therefore, instead of regarding existing GMO regulations as a static framework incapable of encompassing emerging technologies like CRISPR, it is essential to change the legislation as required.

Furthermore, crops generated with CRISPR/Cas9 gene editing and other global gene editing techniques contest traditional viewpoints and definitions of gene manipulation and GMOs. Consequently, it is clear that regulatory authorities globally are continuing to adjust to the swift advancement of this technology. Similarly, 13 nations of the World Trade Organization recently released a declaration-endorsing gene editing for agricultural innovation, marking the initial step toward the creation of a global regulatory framework (WTO [World Trade Organization], 2018). Notwithstanding legal obstacles, researchers, investors, and customers should

maintain their engagement in the advancement and investigation of more advantageous crops to ensure that supply can meet the increasing food requirement.

## 6 | CONCLUSION

Despite some limitations in the application of CRISPR/Cas9 in crops, it has revolutionized the field of biotechnology, as thoroughly reviewed in the literature. The development of PE under the framework of CRISPR/Cas9 demonstrates the ability of an engineered complex for genome modifications, from targeting to both "cutting" and copying functions, all within the context of a single protein. This has expanded the horizon of what is possible with regard to genome editing. Despite its potential, further research is necessary to assess its efficiency for larger-scale alterations. Additionally, substantial consideration should be given to pollen magnetofection-mediated and nanoparticle-mediated delivery methods to address the limitations associated with the current methods. The future of sorghum-maize breeding is envisioned as a combination of precision, creativity, and commitment to addressing global food security challenges while addressing ethical considerations such as informed consent, accessibility, and equity for all countries regarding gene editing.

### AUTHOR CONTRIBUTIONS

**Mercy Jocyline Namata**: Writing—original draft. **Jingyi Xu**: Visualization. **Ephrem Habyarimana**: Writing—review and editing. **Sudhakar Reddy Palakolanu**: Writing—review and editing. **Lihua Wang**: Conceptualization; supervision. **Jieqin Li**: Conceptualization; supervision.

### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

### DATA AVAILABILITY STATEMENT

There are no original data associated with this article. Referenced data are available in the literature.

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