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ADVANCEMENTS IN GENOME MANIPULATION OF MILLETS: APPROACHES, APPLICATIONS AND CHALLENGES

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ABSTRACT

Several initiatives have been made recently with both conventional and modern breeding methods to enhance millet crops. As a result of these efforts, we now know more about how to target particular genes to make improved crops. Crop improvement is necessary to provide enough food for the world's rising population. New genome modification technologies, that include CRISPR/Cas9, have enormous potential. Rapidly developing CRISPR/Cas9 is an effective genome modification system which is currently used in a broad range of species, particularly agricultural and model plants. Research on plant breeding has been transformed by it. Many millet crops, including finger millet, foxtail millet, pearl millet, and sorghum, have been successfully grown using this method. There are now numerous ways to acquire desired features due to the development of genome-editing techniques and the availability of complete genome sequencing for many crops. With an emphasis on millet crops, this paper outlines the ways in which CRISPR/Cas9 can be utilized to enhance crops. It also emphasizes developments in precise base editing and geneome expression modulation, and talks about recent developments that make it possible to edit many target genes at once. The paper also discusses obstacles such as transformation efficiency, the requirement for certain promoters, and moral and legal concerns with the commercial release of crops modified by genome editing.

***Key words*:** Genome editing, CRISPR/Cas9, transformation efficiency, specific promoters, millets.

Introduction

Conventional breeding methods, including hybridization and markerassisted selection (MAS), has employed to improve crops by utilizing genomes and quantitative trait loci (QTLs). However, these techniques depend on the existing natural genetic variation. New advances in genomic manipulation, particularly with the advancement of nucleases that create double-stranded breaks, (DSBs) at particular genomic locations, have revolutionized the field. These nucleases include homing endonuclease (HEs), zinc finger nucleases (ZFNs), transcription activatorlike effector nucleases (TALENs), and most notably, the CRISPR/Cas9 system. The CRISPR/Cas9 system is notable for its exceptional precision, affordability, and efficiency (Krishna *et al*., 2022). This technology has enabled effective manipulation of genes difficult to alter through conventional methods, revealing their roles in various processes. CRISPR/Cas9's attributes make it particularly useful for millet crops. Latest improvements in genome editing technologies is anticipated to address the shortcomings of transgenic approaches, potentially leading to their replacement in commercial applications, as noted in recent literature (Mishra and Zhao 2018). Millets, known for their nutritional benefits, have been a dietary staple since ancient times. Nevertheless, despite their importance for global food security, progress in genetic and genomic research on millets has fallen behind that of other cereals, mainly due to limited resources and inefficient transformation methods. While genome editing has made significant strides in principal crops, its application in millets has been minimal, with foxtail millet being a notable

exception. Nevertheless, recent developments in genome editing hold promise for improving millet crops, with the potential to increase yield, stress resilience, and biofortification. This review underscores the potential of genomicmanipulation in creating enhanced millet varieties, despite the ongoing challenges.

Fundamental Tools for Genome Editing

Genome editing has become a cornerstone of modern biology, enables exact alteration of DNA in different organisms. Over the last two decades, major advancements have been made in genome editing techniques that utilize enzymes specifically engineered to locate and break DNA fragments. These DNA cuts trigger natural repair mechanisms that allow for precise changes. This method is especially potent for making targeted alterations in plants. Tools such as homing endonucleases, zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), oligonucleotide-directed mutagenesis, and CRISPR nucleases have revolutionized plant genome editing (Tyagi *et al*., 2020).

Meganucleases (MgNs)

Meganucleases, additionally called as the homing endonucleases (HEs), are naturally found enzymes that detect lengthy DNA sequences (typically more than 14 base pairs). They were among the first tools applied to genome editing (Curtin *et al*., 2011). Regardless of their uniqueness, their widespread use has been limited by the small number of homing sites in most organisms, as well as the difficulty in engineering the enzymes to target new DNA sequences. The limitations of MgNs were addressed by ZFNs.

Zinc Finger Nucleases (ZFN's)

In 2005, ZFNs proved among the earliest genome-editing tools successfully used on plant DNA. These nucleases are specifically designed to cut targeted DNA sequences, making them highly useful for gene modification (Carroll, 2011). ZFNs consist of two primary components: zinc finger protein regions which attaches to specific DNA sequences and a nuclease region that breaks the DNA. Such nucleases are created by combining an exclusive zinc-finger protein (Cys2-His2 zinc-finger) and the cleavage region of the FokI endonuclease restriction enzyme (Kim *et al*., 1996).

The FokI catalytic domain.

The catalytic domain of FokI requires dimerization for DNA cleavage. This means two ZFN binding events must happen at the correct spacing and orientation, allowing the formation of the FokI dimer, which ensures the accurate cutting of DNA. Typically, these binding events enable the targeting of DNA sequences between 18 to 36 base pairs. However, ZFNs have significant drawbacks, such as the complexity of their manufacture, decreased affinity for AT-rich areas, and the potential for off-target impacts, which might decrease their accuracy in genome editing (Miller *et al*., 2007).

Targeting any DNA sequence in the genome is now possible because to advancements in ZFN-based genome editing. ZFNs do, however, have certain drawbacks, such as being difficult to produce, having a poor affinity for AT rich regions, and having the ability to attach any segment of DNA other than the target location, which can have an off-target effect.

Oligonucleotide-directed mutagenesis (ODM)

An innovative gene-editing method designed to achieve precise, site-directed mutations. This technique uses synthetic oligonucleotides, typically 10 to 100 bp in length, that are homologous with the targeted DNA sequence but contain a single base-pair difference. As such mismatched oligonucleotides switch to the target region of the genome, the cell's natural DNA repair systems detect the mismatch and initiate the desired nucleotide repair. This causes targeted single-nucleotide alterations, as seen in plants including *Zea mays*, *Brassica napus*, *Nicotiana tabacum*, *Oryza sativa*, *Linum usitatissimum* and Arabidopsis thaliana, where ODM has been used to create herbicide resistance (Tyagi *et al*., 2020).

Transcription Activator-Like Effectors Nucleases (TALENs)

TALENs are another advanced tool used for precise genome editing. First applied to plant genome editing in 2011, it represents a second generation of genome editing technologies. The TALEN system evolved from transcription activator-like effectors (TALEs), that are protein molecules produced by bacteria belonging to the *Xanthomonas species*. TALE proteins, like eukaryotic transcription factors, can attach to specific DNA sequences and be designed to regulate gene expression (Boch and Bonas, 2010).

TALENs, like ZFNs, the building of unique proteins for each target DNA sequence, resulting in a modular system which can be adapted to new DNA sequences. TALENs are created by combining a region that binds to DNA composed of conserved TALE repeats with the FokI nuclease region. Once the DNA-binding region of TALENs recognizes the target sequence, the FokI domain dimerizes at the spacer region, inducing a double-stranded break (DSB). DSBs are subsequently fixed by the cell's

Table 1: CRISPR/Cas9 and Gene Transformation Technologies in Millets.

Technology	Target Gene/Genome	Trait/Focus	Outcome	Reference
CRISPR/Cas9	SvLes1	Seed shattering	Verified role of SvLes1 in seed shattering	Mamidi *et al*., 2020
Agrobacterium-mediated transformation	Phosphate transporters (SiPHT1;2, SiPHT1;3, SiPHT1;4)	Root characteristics	Downregulated phosphate transporters, improved root traits	Ceasar *et al*., 2017
Genome sequencing	Foxtail millet, pearl millet	Genome assembly	Facilitated crop development	Bennetzen *et al*., 2012
Agrobacterium-mediated transformation	Transgenic finger millet	Genetic transformation	Produced transgenic finger millet	Antony *et al*., 2011
Genome sequencing	Proso millet, small millet, kodo millet	SNP identification	Identified thousands of SNPs contributing to stress resilience	Johnson *et al*., 2019
EMS-induced mutagenesis and CRISPR/Cas9	PDS, PHYC	Yield and stress tolerance	Modified PDS and PHYC genes in foxtail millet	Peng and Zhang 2021

basic repair systems, allowing targeted genomic changes. Although TALENs enables a high level of precision, the complexity involved in designing custom proteins for each target sequence, as well as their relatively large size, can limit their broad application.

CRISPR/Cas9 Based Genome Editing

The CRISPR/Cas9 protocol, a advanced genome-editing mechanism, was adapted for gene editing in 2013 and is known for its precision in creating DSBs at specific genomic location (Abdallah *et al*., 2015). Despite limitations like dependence on the PAM sequence, CRISPR/Cas9 is easy to utilize, less expensive, and allows for multiplex editing of multiple genes simultaneously. Variants like high-fidelity Cas9 and nickase Cas9 was created to prevent off-target effects.

CRISPR/Cas9 and gene transformation technologies are being explored to improve millets, which serve a significant role in security of food. The SiPDS gene was selected using the CRISPR-Cas9 technology, which was administered via protoplast transformation, which regulates the carotenoid biosynthesis pathway, show casing the utility of protoplast-based genome editing. Agrobacterium-mediated transformation has also been utilized in foxtail millet, targeting the SiMTL gene to develop haploid inducer lines (Cheng *et al*., 2021), and the SiPHYC gene, a major controller of photoperiodic flowering, leading to early flowering with a heading date of 39 days. These research illustrate the promise of genetic modification to improve important agronomic attributes in millet.

Precise Genomic Modification in Millets

Genomic modification in millets has been challenging due to their complex genetics and traditional transformation limitations. However, CRISPR/Cas9 technology allows for precise gene alterations to increase features including stress tolerance, nutritional quality, and yield. For example, CRISPR/Cas9 has been used to edit pearl millet genes related to pathogen response, like β-aminobutyric acid (BABA) and PgMPK4 (Melvin P. *et al*., 2015). A key challenge in genome editing is the reduced efficiency of Homology-Directed Repair (HDR), which has been addressed using geminivirus replication to enhance repair events. This approach has been successfully used to revive several millet species, such as finger, pearl, and foxtail millet.

In finger millet, transgenic varieties have been developed using Agrobacterium-mediated transformation, introducing genes like prawn pin for fungicide resistance, chi11 for leaf blast resistance, and others that provide salt and drought tolerance. Additionally, finger millet plants expressing OsZIP1 have shown increased zinc and magnesium accumulation (Choudhary *et al*., 2023). For delivering larger HDR templates, biolistic transformation has been effective, allowing precise gene insertions across several millet species.

CRISPR/Cas9-based genomeic manipulation in millets

Key attributes:

Genome editing or genomic maipulation in millet crops, particularly using CRISPR/Cas9, has focused on improving production, bifortification, and resistance to biotic and abiotic stresses. Key objectives include increasing food output and providing biofortified food for the growing population. Genome editing has enhanced resistance to pathogens and improved traits like herbicide tolerance and nutritional quality.

Resistance against bacterial disease:

Bacterial species cause significant agricultural damage by triggering plant diseases, and managing these pathogens is challenging due to the absence of effective

agrochemicals and asymptomatic infections. Current approaches include genomic resistance, agronomic methods, and biocontrol compounds. Bacteria produce harmful metabolites that damage plants, but CRISPR/Cas technology has proved promise in combating these infections.

In millet crops, Infections caused by bacteria such as leaf spot, stripe, streak, and stalk rot pose serious threats. Though research on CRISPR/Cas for bacterial resistance in millet is still in its early stages, the success of the technology in other crops like rice and citrus shows promise. Integrating CRISPR/Cas9 into millet breeding programs could help combat pathogens like *Pseudomonas* and *Xanthomonas*, improving immunity to both biotic and abiotic stresses (Krishna *et al*., 2022).

Resistance against fungal disease:

Millets are particularly susceptible to a range of fungal infections, causing significant yield losses. Common diseases like smut and rust affect all millet varieties, while others such as blast, blight, sheath rot, and brown spot target specific small millets. Blast disease caused by *Pyricularia grisea* can result in up to 90% yield loss in foxtail, finger, and other millets. Downy mildew, rust diseases, and grain smut also contribute to widespread losses (Choudhary *et al*., 2023).

To combat these fungal infections, transgenic approaches have been explored. In *Eleusine coracana*, the antifungal protein (PIN) gene and the rice Chitinase11 gene (Chi11) were introduced using biolistic and Agrobacterium-mediated transformations, respectively, conferring resistance to leaf blast disease (Ignacimuthu and Ceasar 2012). However, no transgenic varieties resistant to both finger and neck blast have been reported. Future efforts, including screening more antifungal genes and gene pyramiding, could help develop broader fungal resistance in *Eleusine coracana* (Antony *et al*., 2018).

Enhanced Quality and Yield:

Next-generation sequencing technologies has improved stress tolerance, high-quality, high-yield, or trait-improvement cultivars are being developed through gene editing utilizing the CRISPR/Cas9 system. A genome-wide association analysis has revealed many marker-trait relationships for a variety of agronomic characteristics, including grain yield.

The below table outlines key genetic findings and genome-editing applications in millet crops. It showcases various traits, such as nutrient content, yield, and stress tolerance, which have been improved using advanced techniques like transcriptome analysis, genome-wide association studies (GWAS) and CRISPR/Cas9. Each row presents the specific crop, the traits or genes identified, the technology used, and references, offering insights into how these genetic advancements are contributing to millet improvement. The number of papers illustrating the application of CRISPR/Cas9 to target many genes at once in millet plants to achieve ideotype is anticipated to rise significantly in the upcoming years.

Advances in CRISPR/Cas9 Based Approaches:

Recent advancements in CRISPR/Cas9 technology have expanded the possibilities for precise genome editing across various plant species. Innovative methods now allow for multiplex genome editing, enabling tandem targeting of multiple genes. This is specifically valuable for achieving complex traits in crop improvement. The following table outlines several advanced CRISPR/Cas9-based approaches, highlighting key methodologies, their applications in different crops, and relevant references that validate their effectiveness.

In the lack of Integration Gene Expression and DNA Transfer

Transfer DNA / T-DNA View:

Plant cells typically integrate transgenes into their genomes; nevertheless, understanding Agrobacterium-mediated T-DNA delivery is vital for achieving transitory Activation of genome tools for editing and regeneration genes without integration. Agrobacterium and plant genes both play essential roles in T-DNA integration, but little is known about how to avoid it. A mutant VirD2 protein in Agrobacterium can transport T-DNA transiently and with low integration. Synthetic non-integrating VirD2 proteins could improve transient T-DNA delivery.

In particle bombardment, carefully engineered gold nanocomposites or chemically coated particles can enables transient nuclear translation without genetic fusion. To prevent integration during HDR-mediated gene

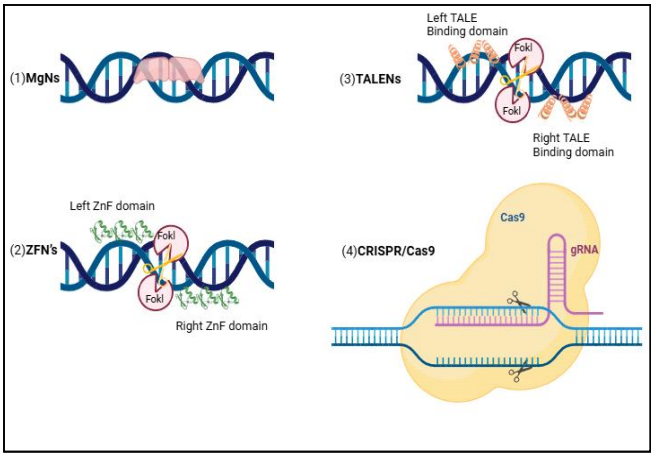

Fig. 1: Tools for genome editing.

editing, single-stranded DNA has been employed, but further advancements in template design and delivery are needed to ensure consistent results across different species. Selectable markers and reporter genes are useful in counter-selecting integrated events, while self-excising DNA templates can further prevent integration. DNA-free genome editing is evolving as a valuable approach for crop improvement, enabling precise modifications without leaving behind transgenic markers (Woo *et al*., 2015).

Cas9 Alternatives for More Precision:

Presently, more precise genome editing is achieved through Cas9 variants. To minimize off-target effects and enhance homologous recombination (HR), approaches like Cas9 nickase (nCas9), dead Cas9 (dCas9), and Cas9 fused with the FokI cleavage domain are used. The conserved RuvC and HNH nuclease domains of the Cas9 enzyme cleave the complementary and non-complementary DNA strands, respectively, guided by the gRNA. Cas9 nickase, created by mutating the catalytic residues (D10A in RuvC and H840A in HNH), cleaves only a single strand of the target DNA, resulting in a nick. When paired with two sgRNAs targeting nearby sites on opposite strands, nCas9 produces a double-strand break (DSB), which is repaired by non-homologous end joining (NHEJ), leading to mutations at the target site and reducing off-target effects. In the case of dCas9 fused with the FokI monomer (fCas9), DNA cleavage occurs only when two gRNAs bind to adjacent regions with proper orientation and spacing, further reducing off-target cleavage. found that the off-target/on-target modification ratio with fCas9 was 140 times lower than that of wild-type Cas9 and 1.3 to 8.8 times lower than Cas9 nickase.

Advances in Promoter Selection and Transformation Strategies for Millet Genome Editing

Multiple kinds of promoters are utilized to control the expression of CRISPR modules. These promoters are classified into four categories: constitutive, tissue- or developmental stage-specific, inducible, and synthetic.

Techniques for Genetic Transformation in Genome Editing:

Particle bombardment, protoplast transformation, and agrobacterium-mediated transformation are the three primary transformation techniques. Many people believe that agrobacterium-mediated transformation is the simplest and primarily practical. Alternatives such as protoplast transformation and particle bombardment can overcome the host-dependent specificity problems that arise with Agrobacterium-mediated transformation. However, these techniques have limitations; protoplast transformation require skilled handling, and particle bombardment calls for specialized facilities.

Methods of validate the construct

Protoplast:

Protoplast transfection is a fast, cost-effective method for screening genome-edited plants and assessing mutation frequencies, but it is not yet optimized for all crops. It allows the introduction of DNA constructs via PEG-mediated transfection or electroporation. This technique has been successfully used in millet species to test genome editing tools like CRISPR/Cas9, TALENs, and ZFNs. It functions as a valuable tool for optimizing Cas9 and sgRNA designs. Methods for millet protoplast isolation and transfection have been developed and applied in research like BiFC and genome modification (Xiang *et al*., 2004).

Agroinfiltration Methods:

Agroinfiltration is an effective way for delivering gene sequences into cells of plantsby infiltrating recombinant agrobacteria into leaf spaces. While a small portion of T-DNA integrates into host chromosomes, leading to stable transformation, the non-integrated DNA produces a large amount of transient recombinant protein. Techniques like syringe and vacuum infiltration, among others, can be used Sharma *et al*., (2020) used agro in filtration in sorghum to create a simpler temporary transformation test and confirmed gene editing in sorghum leaves using GFP as a marker. Monocot sorghum presents challenges as a host for Agrobacterium due to its waxy cuticle, high silica content, and limited intercellular space, but these issues were addressed through agroinfiltration-based genome editing.

Hairy Roots Validation:

Agrobacterium rhizogenes, like *A. tumefaciens*, induces hairy root growth and transfers T-DNA into plant genomes through the root-inducing plasmid. It facilitates the rapid generation of genetically modified hairy roots, which grow quickly and are easy to maintain (Cho *et al*., 2000). This method has been applied to crops like antirrhinum, *Saussurea involucrata* (Fu *et al*., 2005), tomato, soybean (Kereszt *et al*., 2007), and Medicago (Boisson-Dernier *et al*., 2001). However, a transformation system for millets using A. rhizogenes has not yet been developed, making it crucial to create an effective system for genetic advancements in millets.

High Precision Base Editing:

The CRISPR/Cas system makes knockouts simple to create, yet accurate base editing is still difficult because

Table 2: Key Genetic Loci and Genome Editing Applications in Millets for Enhancing Nutritional Content, Yield, and Stress Tolerance.

Millet Crop	Category	Genes/Traits Identified	Technology/Study Method	Reference
Finger millet	Nitrogen Usage Efficiency (NUE)	EcHNRT2, EcLNRT1, EcNADH-NR, EcGS, EcFd-GOGAT, EcDof1	Genome editing for NUE	Gupta *et al*., 2013
Finger millet	Nutritional Elements	10 loci linked to calcium, zinc, phosphorus, sulfur, magnesium, etc.	GWAS using GBS-ddRAD	Jaiswal *et al*., 2019
Finger millet	Yield and Nutritional Traits	Grain yield (GY), flag leaf width (FLW), thousand -grain weight (TGW)	GWAS	Jaiswal *et al*., 2019
Finger millet	Days to Maturity, Grain Yield, and Seed Protein Content	Genetic variables linked to these traits	Integrated breeding combined with genomics	Tiwari *et al*, 2020
Finger millet	Micronutrient Content	Genetic regulation of micronutrient content	GBS and GWAS	Puranik *et al*., 2020
Finger millet	Days to Maturity, Grain Yield, and Seed Protein Content	Genetic variables linked to these traits	Integrated breeding combined with genomics	Tiwari *et al*., 2020
Finger millet	Ca transporter and accumulation	330 genes related to Ca transporter (28 CaM ATPase, 145 CaMK1, 125 CaMK2, 29 CAX1, 3 TPC1)	Transcriptome analysis	Maharajan *et al*., 2021
Pearl millet	Iron and Zinc Content	29 million genome-wide SNPs used to map iron, zinc, yield tolerance	Pearl Millet Inbred Germplasm Association Panel (PMiGAP)	Srivastava *et al*., 2020
Foxtail millet	Yield and Nitrogen Responsiveness	Genetic loci linked to yield and nitrogen traits	GWAS	Bandyopadhyay *et al*., 2022

the NHEJ repair pathway naturally prefers to be used over HDR. For target-specific recombination in HDR, a donor template and CRISPR/Cas reagents must be added. In order to modify DNA bases without resulting in double-strand breaks, new methods have been developed. These techniques involve chemically changing bases via a redesigned Cas protein. For instance, utilizing APOBEC deaminase, the first base editor created in 2016, dCas9 directed by sgRNA can change cytidine to uracil. U-G mismatches result from this, which are finally fixed to T-A pairs (Komor *et al*., 2016). Later, BE2 and BE3 systems were created; BE3 used Cas9 D10A nickase, which functions similarly to activation-induced cytidine deaminase (AID), to increase efficiency (Hess *et al*., 2017). Certain sites undergo targeted mutations produced by CRISPR-X and other systems (Hess *et al*., 2016; Ma Y. *et al*., 2016). Adenine deaminases have also been modified to introduce exact modifications that aren't present in living things normally. By early termination of gene expression, BE3 systems facilitate the introduction of early stop codons by CRISPR-STOP and iSTOP, hence permitting gene knockouts. These systems aim to change certain codons into stop codons, such as TGG, CAA, and CGA (Billon *et al*., 2017). To assist researchers in locating induced stop codons in eukaryotic genomes, a database has been generated.

Gene Expression Modulation:

Gene expression regulation is essential for genetic engineering, synthetic biology, and research. Early methods used synthetic transcription factors, such as zinc finger-based activators, to control gene expression. The development of CRISPR/Cas9 has revolutionized transcriptional regulation by allowing precise modulation of endogenous gene expression. Cas9 proteins connected to domains of transcriptional effectors can either activate or repress genes, and the system's ability to target multiple genes at once enables simultaneous transcriptional activation using multiple gRNAs (Pinera *et al*., 2013).

Challenges for Genome-Editing in Millets:

Within the discipline of plant science, GE

advancements and their uses are constantly growing. Notwithstanding recent advancements in genome engineering's specificity and ease of use, difficulties still persist. There are significant obstacles that need to be overcome for this technology to reach its full potential.

Polyploidy:

To enhance the usage of geneome editing in crop plants, recombination frequency needs significant improvement. Overcoming the limitations of polyploid plants requires targeting specific gene sequences and avoiding conserved regions to ensure precise editing (Anderson *et al*., 2014). Polyploidy, where organisms acquire extra chromosome sets, complicates gene editing due to complex genomes and multiple gene copies. This is especially challenging in polyploid crops like wheat, where compensatory gene copies hinder the success of gene knockouts. Editing all gene paralogs may sometimes be necessary but reduces efficiency (Li *et al*., 2017). New breeding techniques utilizing CRISPR without background mutations are made possible by recent CRISPR-Cas9 research in tetraploid oilseed, which successfully edited two ALCATRAZ (ALC) homoeologs to create a transgenic plant with four mutant alleles (Braatz *et al*., 2017).

Transformation Efficiency:

The efficiency of crop transformation is essential in advancing plant genome editing for agriculture. Traditional methods like particle bombardment and Agrobacterium-mediated transformation has widely used but face challenges, especially in monocots, due to genotype dependency, T-DNA integration, and laborious plant regeneration processes (Chowdhury *et al*., 2022). Emerging delivery methods, such as nanomaterials like carbon nanotubes (CNTs) and nanoparticles, give assurance by facilitating gene delivery without tissue culture. One such technique, pollen magnetofection, introduced in 2017, uses magnetic nanoparticles to transport DNA into pollen, producing transgenic plants without tissue culture, though its reproducibility is debated. CRISPR/Cas9 delivery via nanomaterials could potentially avoid classification as GMOs due to their non-integrating properties (Demirer *et al*., 2019). However, more efficient transformation techniques for key crops are still needed, with methods like gene gun, liposome-mediated transformation, and macro-injection requiring further validation for better results (Aglawe *et al*., 2018).

Off-site targeting:

This is relevant to the toxicity that off-target DSBs bring to cells, which hinders the general application of these nuclease targeting systems in plants. Several potential approaches to address off-site SDN targeting have already been covered. By decreasing off-site targeting and facilitating the development of novel GE tools, a deeper comprehension of the homology-directed endogenous DNA repair mechanisms that ensue from nuclease-mediated DNA cleavage may assist to improve experiment specificity and accuracy (Aglawe *et al*., 2018).

Other challenges:

High-throughput sequencing platforms have enabled the resequencing and de novo sequencing of plant genomes, generating vast amounts of SNP data, though most lack phenotypic information. SNPs with known phenotypes could be key targets for gene editing to improve crops, making HapMap projects essential for major food crops (Aglawe *et al*., 2018). Latestresearch in gennome editing involves pooled CRISPR libraries, such as those provided by Addgene, which contain thousands of plasmids with unique gRNAs targeting different genes. These libraries can generate mutant cell populations for phenotyping. However, such CRISPR libraries are not yet available for plants, and their development would benefit functional genomics and crop improvement, especially for targeting noncoding regions (Aglawe *et al*., 2018).

Cas9-Mediated Genome Editing: Reducing Off-Target Mutations with Enhanced Variants

The main concern with CRISPR/Cas9 genome editing is off-target effects, which can cause unintended DNA cleavage, leading to cellular toxicity and chromosomal rearrangements like deletions, inversions, and translocations in plants (Lee *et al*., 2012). While off-target activity is generally milder in plants compared to animals, mismatches in sgRNA sequences can still result in off-target DNA cleavage. The specificity of Cas9 is influenced by the sgRNA guide sequence and PAM sequence, with mismatches at the 5' end being tolerable for target identification. Adjusting the structure and composition of sgRNA, such as truncating it or using sgRNA nickase, can reduce off-target events effective sgRNA can be selected based on GC content for more precise editing (Ren *et al*., 2014). Techniques like Digenome-seq and GUIDE-seq have proven highly sensitive in identifying off-targets with a 0.1% sensitivity.

Research shows that CRISPR/Cas9 induces more stable mutations in rice than in Arabidopsis, with germline mutations passing through generations, while somatic mutations do not (Feng *et al*., 2014). Using germline-specific promoters, like a pollen-specific promoter, can

increase germline mutations and ensure their transmission to subsequent generations. To mitigate off-target effects, variants like fCas9, created by combining dCas9 with FokI nuclease, and modified Cas9 proteins with amino acid substitutions have shown improved specificity and reduced off-target activity (Kleinstiver *et al*., 2016).

Conclusions

With its numerous uses for genome editing, CRISPR has emerged as one of genetic engineering's most useful technologies. Compared to conventional transgenic and conventional approaches, it offers faster, more accurate, and more affordable alternatives for agricultural improvement. Still, there are a number of obstacles to be solved before its efficacy in crops may be increased. For CRISPR technology to be successfully applied in crop development, several issues must be resolved. The effective use of CRISPR technology in crop development requires enges.

Transformation efficiency is a major issue that CRISPR-based genome editing must address and adjust for various crops. For the majority of millet crops, transformation strategies have been shown, despite being primarily genotype-specific. Nonetheless, there is still a lack of research on genome editing in the genetic backgrounds of high-yield commercial cultivars. The requirement for a particular PAM sequence presents another difficulty, as editing genes lacking the required PAM sequence may be challenging.

Global rules restrict the widespread application of CRISPR in agriculture, even in the face of potent genome editing tools. A easing of these constraints may encourage the growth of new, healthier crop varieties. CRISPR/Cas9-developed crops may be categorized as non-genetically modified organisms (non-GMO) because to their precise alterations, which might speed up their adoption. In the future, the use of CRISPR/Cas9 in agriculture may transform crop yield and sc a second green revolution, supplying food and nutrition security for the world's expanding population.

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