CRISPR – cas9 A Tool in plant breeding

Abstract

CRISPR-Cas9, a revolutionary tool for genome editing, has significantly impacted plant breeding by allowing precise genetic modifications with exceptional efficiency and accuracy. This review explores the applications, mechanisms, and implications of CRISPR-Cas9 technology in plant breeding. It outlines the molecular principles of the CRISPR-Cas9 system, focusing on its ability to target specific genes and introduce traits like disease resistance, enhanced yield, and better resilience to environmental stresses. The review highlights the advantages of CRISPR-Cas9 over traditional breeding methods, such as its speed, cost-effectiveness, and precision. Additionally, it addresses the challenges and regulatory issues surrounding its use in agriculture, including off-target effects, ethical concerns, and regulatory frameworks. The review concludes by considering the future potential of CRISPR-Cas9 in fostering sustainable agricultural practices and its role in ensuring global food security in the face of climate change and population growth.

Keywords: CRISPER-Cas9, Genome editing, Molecular basis.

Introduction

One of the most urgent challenges humanity faces today is how to sustainably feed a growing population. In addition to population growth, key factors limiting agricultural productivity and food production include extreme weather conditions, a shortage of available arable land, and increasing biotic and abiotic stresses. Technology advancements that can help with crop development can help to some extent increase yield **[5]**. In recent decades, genetic manipulation and transgenic techniques have been utilized to deepen our understanding of plant breeding principles and enhance crop improvement methods. One of the most advanced genome-editing tools developed is the CRISPR/Cas system, which was inspired by bacteria's adaptive response to bacteriophages. CRISPR stands for "Clustered Regularly Interspaced Short Palindromic Repeats," and Cas9 stands for "CRISPR-associated protein 9"**[52].** CRISPER Cas9 has been used by bacteria as an immune defence, which was a naturally occurring genome editing system. The CRISPR/Cas9 technology is globally used and has proven to be a versatile tool for editing the DNA of specific genes [**19**]. This method was created in 2012, and since then it has transformed biology research by making it simpler to examine diseases that affect Humans, animals, plants, and their associated treatments **[2]**. Due to the fact that CRISPER Cas9 is a gene found in bacteria, these bacteria "catch DNA fragments from viruses and add them to their own DNA to create segments known as CRISPER arrays" [**42**].

This genome editor tool has made it very easier nowadays to remove any targeted genes and to insert a new gene at certain position so that any desirable traits can be add [**24**]. The targeted DNA when cleaved and repaired, it is the point of interest in scientific researches. This is the technology which we can use for removing any kind of errors in the genome [**42**], Also it has been already proved that this technique of repairing defective DNA can cure the genetic disorders of a mice, human embryos can also be modified or treated in similar way [**4**]. Some of examples of this technology are such as CRISPER Cas9 has been used for tomato plant as new alleles have been developed for its physical appearance like fruit shape, size, colour etc & developed plant makeup generating novel variant very superior than existing one. other types of crops Currently, CRISPR/Cas9 genome editing has been shown to be effective on a number of significant crops, including apples [**48**], wheat [**20**], and maize, known for its relatively high transformation efficiency [**18,2**].

CRISPR/Cas9 technology has been effectively used to modify and improve various quality traits in crops, but the gene-editing revolution is still in its early stages. Despite its potential, CRISPR/Cas9 faces several challenges. According to multiple studies, one significant concern is the possibility of off-target effects, where unintended parts of the genome may be **[62].** Whole-genome sequencing data has indicated that although off-target mutations caused by CRISPR/Cas9 in plants are less common, they can still influence the desired phenotype and lead to inaccurate data interpretation. Positive off-target mutations may be retained in subsequent generations, while those with harmful phenotypic effects are usually removed during the breeding process [**59**]. Several strategies have been proposed to minimize off-target effects. First, designing highly specific sgRNAs with minimal potential for off-target interactions can significantly reduce off-targeting. Second, using high-fidelity Cas9 variants such as eSpCas9 and SpCas-HF can enhance the specificity of CRISPR systems and decrease the rates of off-target mutations [**13].**

Mechanism of CRISPR/Cas9

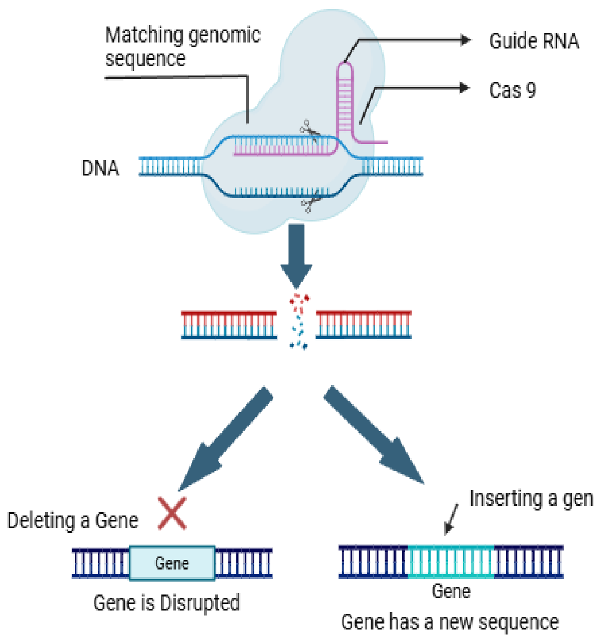
Discovered and first utilized for genome editing in 2012, the CRISPR/Cas9 system has significantly advanced research in plant and animal biology. In contrast to technologies like ZFNs and TALENs, CRISPR offers a more streamlined approach by using a guide RNA (gRNA) that is about 20 nucleotides long and complementary to the target gene sequence. This gRNA directs the Cas9 protein to the specific DNA site, where it introduces a double-strand break approximately 3–4 base pairs into the **[10].** The CRISPR-Cas9 mechanism involves three main steps: recognition, cleavage, and repair. Key components essential to this genome editing process include the guide RNA (gRNA), which directs the system to the target DNA sequence, and the Cas9 nuclease, the protein responsible for cutting the DNA **[6]**. Cas9 is directed by a specifically designed single-guide RNA (sgRNA), which contains a complementary sequence (5' crRNA) that helps it locate the desired target within the gene. Without the presence of sgRNA, the Cas9 enzyme remains inactive. Once the target is identified, Cas9 introduces a double-strand break (DSB) in the DNA, typically occurring three base pairs upstream of the PAM (protospacer adjacent motif) **[7]**. The PAM (protospacer adjacent motif) is a short, conserved DNA sequence located just downstream of the cleavage site, and its length typically ranges from 2 to 5 base pairs, varying by bacterial species. For the widely used Cas9 nuclease, the recognized PAM sequence is 5′-NGG-3′, where "N" can represent any nucleotide base. This specific sequence is essential for Cas9 to bind and initiate DNA cleavage **[3]**. The exact mechanism by which the Cas9 enzyme unwinds the target DNA sequence remains unclear. However, once Cas9 identifies the correct target site with the appropriate PAM sequence, it induces local DNA melting, which is followed by the formation of an RNA-DNA hybrid. This triggers the Cas9 protein to cleave the DNA. The target DNA is primarily cut to produce blunt-ended double-strand breaks (DSBs), with the HNH domain cleaving the complementary strand and the RuvC domain cutting the non-complementary strand. Finally, the host cell's repair machinery fixes the DSB [**25].**

Double-Strand DNA Break Repair Pathways:

The CRISPR/Cas9 system creates double-strand breaks (DSBs) in DNA, which can be repaired by two primary pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR). When no external homologous DNA template is available, the NHEJ pathway facilitates the rejoining of the DNA ends using specific enzymes. This repair mechanism operates during all phases of the cell cycle.

[46]. This is the most frequently used and efficient DNA repair pathway in cells; however, it is also error-prone, which can cause frameshift mutations or premature stop codons due to tiny random inserts or deletions (indels) at the cleavage site [**36]**. Using a homologous DNA template is necessary for HDR, which is extremely accurate. The cell cycle's late S and G2 stages are when it is most active. A significant quantity of donor (exogenous) DNA templates with a sequence of interest are needed for HDR in CRISPR/Cas9-based genome editing methods have been applied to enhance disease resistance and increase tolerance to key abiotic stresses, such as salinity and drought. Below is a list of crop species in which CRISPR has been utilized to modify the genome **[62]**.

Fig 1. Mechanism of CRISPER Cas9



CRISPER Cas9 for crop improvement

Crop improvement focuses on enhancing the quality, productivity, nutritional value, and resilience of crops against biotic and abiotic stresses. Over the years, modern agricultural technologies have greatly increased crop yields. Consumers are becoming more concerned with crop quality, as it plays a crucial role in human health by supplying important nutrients like proteins, fiber, vitamins, minerals, and bioactive compounds [**36]**. A range of techniques, including traditional crossbreeding, mutation breeding, molecular marker-assisted breeding, and genetic engineering, have been employed to improve crop quality. Among these, CRISPR/Cas systems have recently become the preferred genetic editing tool. These systems offer greater efficiency and simplicity in genome editing compared to other methods **[49]**. CRISPR/Cas9-based genome editing methods have been applied to enhance disease resistance and increase tolerance to key abiotic stresses, such as salinity and drought. Below is a list of crop species in which CRISPR has been utilized to modify the genome **[65].**

RICE

More than 3.5 billion people over the world are fed by rice (Oryza sativa L.). Different geographic areas and/or ethnic groups have various preferences for rice grain quality. Micronutrients, phytochemicals, and cooking and eating conditions are the main factors influencing quality. Grain quality evaluation is a time-consuming and difficult process that necessitates following established procedures in addition to collecting a lot of samples early in the breeding process [**12**]. The Food and Agriculture Organization (FAO) of the United Nations predicts that in order to fulfil demand, worldwide grain yields would need to rise by 70% [**31**]. It has been the focus of much research With its compact genome, rice serves as an ideal model crop for monocots. Recent advancements have showcased the practical application of CRISPR-based genome editing techniques in rice. Several studies have highlighted the potential of genome editing to enhance rice's resistance to both biotic and abiotic stresses **[21].**

Table 1. Utilization of CRISPR/Cas9 for managing abiotic stress in rice plants.

Crop	Target gene	Trait	Reference
Abiotic stress			
Rice	**OsPDs, OsMPK2**	**Different types of abiotic stress involved**	**[53]**
Rice	**OsMPK5**	**Tolerance to abiotic stress and diseases**	**[61]**
Rice	**OsPRX2**	**Resistance. Potassium deficiency tolerance.**	**[41]**
Rice	**OsHAK-1**	**Low cesium accumulation.**	**[31]**

WHEAT

Wheat (*Triticum aestivum* L.), a vital source of daily energy for millions worldwide, is one of the most essential staple crops globally. It remains the most significant source of grain for human use and is grown on more acreage than any other commercial crop to date. Population expansion and shifting dietary seems are expected to increase the demand for wheat globally, making its development essential for food security [**38**]. Wheat is the most widely cultivated and traded crop globally, covering 220 million hectares (mha), with an average yield of 350 kg per decare (at 11% moisture content), and a total global production of 773 million tons **[64]**. Due of problems with tissue culture, its large genome size, and its hexaploidy nature, wheat is not embracing CRISPR technology as widely as rice and Arabidopsis, which are the two most studied plants for gene editing [**31**]. However, the application of CRISPR -based genome engineering in wheat is expected to be greatly facilitated by the IWGSC's recent publication of a premium reference genome for wheat **[16]**. By producing CRISPR-edited mutants with improved characteristics that increase productivity under a variety of biotic and abiotic stressors, this discovery could help allay concerns about global food security in the future decades. Complex features that include several genes can be edited with new multiplex genome editing toolkits [**28**].

The TaMLO gene, which provides mildew resistance, was successfully targeted using CRISPR/Cas9 in wheat protoplasts. Knockdown of TaMLO conferred Resistance to *Blumeria graminis* f. sp. *Tritici* (Btg), the pathogen responsible for powdery mildew. Among 72 T0 knockout lines, four showed modified restriction sites confirmed via T7E1 digestion **[64]**. CRISPR/Cas9 was utilized to target stress-related genes TaERF3 and TaDREB2, achieving approximately 70% transfection efficiency, confirmed by T7E1. To reduce off-target mutations and transgene integration, CRISPR/Cas9 ribonucleoproteins (RNPs) were delivered via biolistic methods, ensuring transient expression and minimizing unintended effects **[34]**. effectively used the CRISPR/Cas9 RNP complex to modify two distinct genes, TaGW2 and TaGASR7, in two bread wheat varietal backgrounds. Off-target effects are the presence of off-target mutations was significantly minimized as the complex degrades in vivo, and the mutant bread wheat population displayed no off-target effects **[60].**

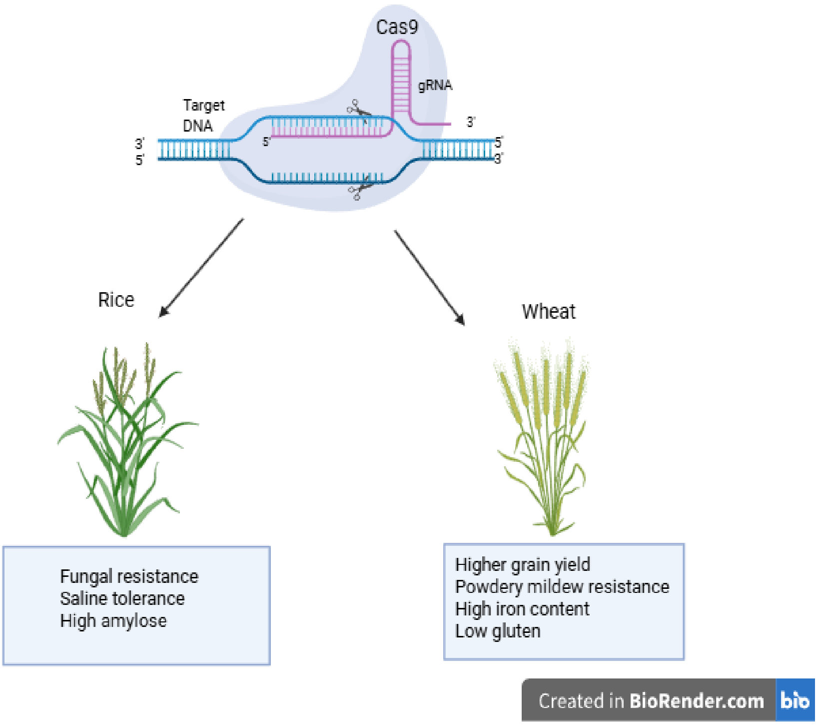


Fig.2 Utilization of the CRISPR/Cas9 system to modify agronomically significant traits in wheat and rice.

Cotton

Cotton is a primary source of natural fiber, oil, and animal feed, with its fiber derived from the seeds **[28]**. The genome of cotton is complex and contains various ploidy types; commercially grown cotton is an allotetraploid (AD) species, with a genome size of 2.5 Gb for Gossypium hirsutum (upland cotton) [**47**]. Cotton fiber consists of about 87% to 90% cellulose, a plant-derived carbohydrate, with 5% to 8% water and 4% to 6% naturally occurring contaminants. These characteristics allow cotton to endure high pressing temperatures, absorb a wide range of dyes, and remain washable [**57**]**.** The growing availability of genetic sequences has emphasized the need for fast and economical techniques to introduce targeted mutations, enabling large-scale gene functional studies in cotton. CRISPR/Cas9 gene editing has been successfully applied to a variety of major crops and model organisms, including rice, wheat, sorghum, poplar, maize, and tomato **[32].**

For phenotypic characterization, three specific locations within the GFP sequence were selected for genome editing in transgenic cotton with integrated green fluorescent protein (GFP). Two T0 plantlets exhibited homozygous modifications, while seven of the nine plants tested for gRNA2 knockdown showed bi-allelic indels **[27]**. To achieve an exact nucleotide substitution or insertion of the desired DNA sequence, the ability to create DSB at a precise target position has been further enhanced by homology-dependent repair. assessed the efficiency of genome editing in cotton by targeting the genes for Chloroplasts altera Dos 1 (GhCLA1) and vacuole H+-pyrophosphatase (GhVP) using two guide RNAs, respectively [**29**]. Mutational efficiency ranged from 47.6% to 81.8%, with deletions being the most common alteration. Due to cotton's allotetraploid nature, targeting multiple genes with CRISPR/Cas9 has proven effective. PCR and sequencing confirmed specific truncations in GhMYB25-like A & GhMYB25-like D genes, which control cotton fibre development. It was recently demonstrated that genetic engineering of the Gh14-3-3d gene confers resistance to infection by Verticillium dahliae. the transgene-free plants produced exhibited strong resistance, making them valuable germplasm for developing disease-resistant cotton varieties **[58]**.

SOYABEAN

Soybean (*Glycine max* L. Merr.) is a legume valued for its substantial protein and oil content. It is extensively utilized in both human and animal diets, biodiesel production, and a range of industrial uses. Its versatility makes it an essential crop for food, energy, and industrial sectors globally [**14**]. The growing demand for soybeans, driven by societal changes, has led to an increase in breeding soybean varieties with enhanced traits. Third-generation gene editing methods, such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated protein 9 (Cas9), are now being employed in this process **[9].**

The CRISPR/Cas9 system was successfully employed to edit the soybean genome, targeting a transgene (bar) with one sgRNA and two native soybean genes (GmFEI2 and GmSHR) using a set of six sgRNAs. The effectiveness of these sgRNAs was assessed in a hairy root system, producing positive outcomes such as small deletions and insertions at the DD20 and DD43 genomic sites on soybean chromosome 4 [**44**]. Border-specific PCR analysis at the callus stage identified targeted gene integrations via HDR. The soybean GmU6-16-1 promoter proved more efficient than the Arabidopsis AtU6-26 promoter in editing multiple homoalleles simultaneously [**20].** Through complementation and CRISPR/Cas9-mediated editing, the dominant Rj4 gene in soybean, which prevents nodulation by various strains of *Bradyrhizobium elkanii*, has been functionally validated **[30]**.

TOMATO

Tomato (*Solanum lycopersicum* L.) is a widely cultivated vegetable of considerable economic importance, consumed both fresh and in processed forms. Rich in bioactive compounds like vitamins A and C, essential antioxidants, and minerals—particularly beta-carotene and lycopene—it is recognized as a nutrient-rich food with notable health benefits [**45**]. The breeding of crops, particularly tomatoes, has been transformed by CRISPR/Cas9 technology. CRISPR/Cas9 has become the most widely favoured tool for precise, efficient, simple, and cost-effective gene editing, offering targeted modifications in specific genomic regions. Since its first application in 2013, it has been frequently used to alter tomato genotypes. The technology has been successfully employed in tomatoes for modifying various trait features related to fruit, flowers, and plant architecture [**58**].

The process of domesticating tomatoes began with small cherry-like fruits, which were later developed into larger-fruited types varieties with various traits. CRISPR/Cas9 genome editing has recently showcased these evolutionary changes [**54**]. Cas9-mediated genome editing has been applied in tomatoes to improve yield and associated traits. Modifications include inducing determinate growth through the SP (Self-pruning) gene, tripling fruit size via the FAS (fasciated) gene, altering fruit shape to oval with the Ovate (O) gene, increasing fruit number tenfold through the MULT (multiflora) gene, and boosting fruit weight using the FW2.2 gene. Moreover, gene editing has been used to induce male sterility, facilitating hybrid seed production **[67].**

ADVANTAGES OF CRISPER CAS-9

CRISPR-Cas systems have revolutionized gene editing with their natural ability to target and modify specific genes. These systems, originally discovered as part of bacterial and archaeal immune responses to viral DNA, have been adapted for programmable gene editing, enabling significant advancements in biological research and genome engineering [**52**]**.** The CRISPR-Cas9 gene-editing system, in particular, has become a go-to tool for precise genetic modifications due to its ability to cut targeted DNA sequences at precise locations, it triggers double-strand breaks that are repaired by cellular mechanisms leading to genetic changes. It has paved the way for advancements in diverse areas, including medicine, farming, and biotechnological industries **[43].** One of the major breakthroughs in plant biotechnology has been employing the CRISPR-Cas9 system to alter genomes of crops. CRISPR technology offers significant potential for enhancing agricultural productivity by allowing targeted alterations of traits such as resistance to diseases, tolerance to drought, and increased crop yields. Nevertheless, a major obstacle hindering its widespread application in plant systems is the complex and time-consuming tissue culture process needed to regenerate edited plants [**11].**

Moreover, this approach allows for gene editing to be carried out outside of aseptic conditions, which is a considerable advantage. Key elements of gene editing systems consist of Cas enzymes and guide RNAs (gRNA), can be directly introduced into somatic cells or seedlings of soil-grown plants. The CRISPR-Cas9 system then performs its function of modifying the target genes, resulting in altered gene expression. These altered shoots can then grow into full plants with the desired genetic modifications. This opens up the possibility of directly editing crops in field conditions, without the need for sterile environments, thus simplifying the process and reducing the resources required [**40].**

CHALLENGES FACED BY CRISPER-CAS 9

While CRISPR-Cas9 Has reshaped genome editing, it does come with several disadvantages when applied to plants. One of the main concerns is the potential for off-target effects, where the Cas9 protein may cut unintended regions of the genome that resemble the target sequence, leading to unwanted genetic changes [**57**]. Additionally, the efficiency of CRISPR-Cas9 can vary significantly between plant species, with some being more resistant to genome editing due to complex genomes or difficulties in regeneration through tissue culture. The dependence on tissue culture for plant regeneration is another challenge, as it is labour-intensive, species-specific, and time-consuming [**8**]. Furthermore, tissue culture can result in somaclonal variation, which complicates the selection of genetically edited plants. Incomplete or unstable gene edits are also a concern, as mutations introduced by CRISPR may not always result in the desired phenotype or be stably inherited across generations [**1].**

Another risk is insertional mutagenesis, where unintended insertions or deletions may occur during the DNA repair process, introducing additional mutations. The regulatory and Moral dilemmas surrounding the use of CRISPR-Cas9 in plants also pose significant barriers, with uncertainty regarding the classification and regulation of genetically edited crops, which may be subject to strict GMO regulations [**51**]. Moreover, unintended phenotypic effects may arise due to the complexity of plant genomes, as interactions between edited genes and Alterations in other regions of the genome might result in unexpected outcomes. Our limited understanding of plant genomes, particularly in complex or polyploid species, further complicates the accuracy of gene modification **[22]**. Nonetheless the cost of CRISPR-Cas9 itself has decreased, the infrastructure, expertise, and controlled environments required for its application remain costly, limiting its accessibility, particularly for small-scale farmers or researchers in developing regions **[68**]. Lastly, there are concerns about horizontal gene transfer, where edited genes could potentially transfer to pests, weeds, or microbes, leading to ecological issues such as the development of resistance or gene flow to wild relatives. Overall, despite its potential, CRISPR-Cas9 in plants faces challenges that must be addressed to realize its full promise in agriculture **[39].**

CONCLUSION

Genome editing represents a transformative advancement in modern plant science, offering tremendous potential for crop improvement, particularly in cases where conventional breeding methods face significant limitations. Traditional breeding approaches often struggle with long breeding cycles, limited genetic variation, and difficulty in precisely controlling specific traits. In contrast, genome editing technologies provide a precise, rapid, and efficient means of modifying DNA at targeted locations within the genome, enabling researchers and breeders to develop crops with enhanced traits in a much shorter time frame.The development and application of genome editing tools have opened new avenues for the genetic modification of plants using sophisticated and highly adaptable breeding strategies. These tools, especially those based on CRISPR/Cas systems, allow for targeted mutagenesis, enabling the alteration or silencing of specific genes linked to desirable agronomic traits. As a result, traits such as improved drought tolerance, resistance to pathogens, enhanced nutritional quality, and increased yield potential can be efficiently incorporated into crop genomes **[64].**

As genome editing technologies continue to advance, they are becoming increasingly precise, stable, and efficient, while also offering a wider range of applications in plant biology and agriculture. These tools not only allow for accurate genome modifications but also contribute to the discovery of previously unknown gene functions and complex genetic interactions. Such discoveries further enhance our understanding of plant development, physiology, and adaptation, laying the groundwork for more targeted and informed breeding programs. Among the various genome editing platforms available, CRISPR/Cas systems have emerged as the most widely adopted due to their relative simplicity, high efficiency, and ease of use. Compared to earlier techniques such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), CRISPR/Cas9 offers a more accessible and cost-effective approach to genome editing, which has led to its rapid integration into plant breeding pipelines around the world. Its ability to create precise edits without leaving transgenic footprints is particularly appealing, especially in the context of public acceptance and regulatory frameworks.

One of the most significant impacts of genome editing, particularly through CRISPR/Cas systems, is its potential to revolutionize crop breeding practices. This technology enables the development of crops that are better suited to withstand environmental stresses such as extreme temperatures, salinity, drought, and pest infestations—factors that are increasingly threatening global food production due to climate change. Moreover, it supports sustainable agriculture by facilitating the creation of high-yielding varieties that require fewer inputs, such as water, fertilizers, and pesticides **[56].**

Importantly, unlike traditional genetic modification (GMO) techniques, CRISPR/Cas-mediated genome editing often does not require the insertion of foreign DNA into the host genome. Instead, it can be used to perform precise gene knockouts, replacements, or regulatory adjustments within the native genome, resulting in plants that are genetically indistinguishable from those produced through natural mutations or conventional breeding. This characteristic not only simplifies regulatory approval in some regions but also enhances consumer acceptance. Looking ahead, genome editing holds immense promise for further innovation in agriculture. Researchers are working to improve the delivery methods of CRISPR components into plant cells, such as through nanoparticles, viral vectors, or direct ribonucleoprotein (RNP) transfer, which could further streamline the editing process. Additionally, genome editing could play a role in managing invasive plant species and improving ecological balance in agricultural systems **[26].**

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