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Advanced Genetic Tools for Rice Breeding: CRISPR/Cas9 and Its Role in Yield Trait Improvement

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Abstract The advent of CRISPR/Cas9 has revolutionized genetic research, providing rice breeding with unprecedented precision and efficiency in genetic modification. This study synthesizes the current applications and advancements of CRISPR/Cas9 technology in rice breeding, particularly focusing on yield trait improvement. By facilitating targeted gene editing, CRISPR/Cas9 enables the modification of specific genes associated with yield, such as grain size, panicle length, and stress tolerance. Key studies demonstrate its effectiveness in enhancing grain quality and increasing overall yield by editing genes like Grain Size 3 (*GS3*) and *OsSAP*. Additionally, the technology's ability to edit multiple genes concurrently through multiplexing has expedited the development of rice varieties tailored to diverse environmental conditions and agricultural demands. Challenges remain, including regulatory hurdles, off-target effects, and the need for precise delivery systems. However, advancements in base and prime editing are addressing these issues, broadening the scope of CRISPR applications. The integration of CRISPR/Cas9 with traditional breeding methods and functional genomics is also enhancing the precision and speed of developing new rice cultivars. Continued research and interdisciplinary collaboration are essential for leveraging CRISPR/Cas9's full potential to meet global food security challenges.

Keywords CRISPR/Cas9; Rice breeding; Genetic editing; Yield improvement; Agricultural biotechnology

1 Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population, making its yield and quality critical for global food security. Traditional rice breeding methods have significantly contributed to yield improvements; however, these methods are often time-consuming and limited in their ability to introduce precise genetic changes. The advent of advanced genetic tools, particularly the CRISPR/Cas9 system, has revolutionized the field of plant breeding by enabling precise and efficient genome editing. This study aims to explore the role of CRISPR/Cas9 in rice breeding, focusing on its applications in improving yield traits.

Rice breeding has historically relied on conventional methods such as cross-breeding and selection to enhance desirable traits like yield, disease resistance, and stress tolerance. While these methods have been successful, they are often labor-intensive and time-consuming. Recent advances in molecular biology have introduced new techniques that allow for more precise genetic modifications. Among these, CRISPR/Cas9 has emerged as a powerful tool for targeted genome editing, offering unprecedented opportunities for rapid and accurate trait improvement in rice (Li et al., 2021; Rao and Wang, 2021; Liu et al., 2022).

The increasing global population and the consequent rise in food demand necessitate the development of high-yielding and resilient crop varieties. Genetic tools like CRISPR/Cas9 are crucial in this context as they enable the precise modification of genes responsible for key agronomic traits. This technology has been successfully used to enhance various traits in crops, including yield, quality, and resistance to biotic and abiotic stresses (Ahmad et al., 2020; Usman et al., 2020; Liu et al., 2021b). The ability to make specific, heritable changes in the genome makes CRISPR/Cas9 an invaluable tool for modern agriculture, facilitating the development of crops that can withstand the challenges posed by climate change and other environmental factors (Usman et al., 2021; Park et al., 2022).

The aim of this study is to provide a comprehensive overview of the applications of CRISPR/Cas9 in rice breeding, with a particular focus on yield trait improvement. This study will examine the various strategies employed to enhance yield through CRISPR/Cas9-mediated genome editing, including gene knockout, point mutations, and single base editing. Additionally, this study will discuss the recent advancements in delivery mechanisms and the challenges associated with the broader application of this technology in rice breeding. By synthesizing the current state of research, this study aims to highlight the potential of CRISPR/Cas9 to transform rice breeding and contribute to global food security.

2 Basics of CRISPR/Cas9 Technology

2.1 History and development of CRISPR/Cas9

The CRISPR/Cas9 system, originally discovered as a part of the adaptive immune system in bacteria, has revolutionized genetic engineering since its adaptation for use in eukaryotic cells. The system was first identified in the late 1980s, but its potential for genome editing was not realized until the early 2010s. The breakthrough came when researchers demonstrated that CRISPR/Cas9 could be programmed to target specific DNA sequences, allowing for precise genetic modifications. CRISPR/Cas9 can efficiently edit the genome of diverse organisms, including humans, animals and plants (Figure 1) (Fiaz et al., 2019). This technology has since been rapidly adopted in various fields, including plant science, due to its simplicity, efficiency, and versatility (Ahmad et al., 2020; Zegeye et al., 2022).

2.2 Mechanism of CRISPR/Cas9 function

The CRISPR/Cas9 system functions through a guide RNA (gRNA) that directs the Cas9 nuclease to a specific DNA sequence. The gRNA binds to the target DNA through complementary base pairing, and the Cas9 protein induces a double-strand break at the target site. The cell's natural repair mechanisms then take over, either through non-homologous end joining (NHEJ), which often results in insertions or deletions (indels), or through homology-directed repair (HDR) if a repair template is provided. This precise targeting and cutting mechanism allows for the efficient editing of specific genes, making CRISPR/Cas9 a powerful tool for genetic research and breeding (Yimam et al., 2021; Zegeye et al., 2022).

2.3 Applications of CRISPR/Cas9 in plant science

CRISPR/Cas9 has been widely applied in plant science to improve various traits, including yield, stress resistance, and quality. In rice, CRISPR/Cas9 has been used to edit genes associated with drought tolerance, grain size, and cold resistance. For instance, editing the *OsSAP* gene has shown potential in enhancing drought tolerance by modulating stress-related transcription factors (Park et al., 2022). Similarly, the *OsSPL16* gene was edited to increase grain yield by affecting pyruvate metabolism and cell cycle proteins (Usman et al., 2020). Additionally, simultaneous editing of multiple genes, such as *OsPIN5b*, Grain Size 3 (*GS3*), and *OsMYB30*, has resulted in rice varieties with improved yield and cold tolerance (Zeng et al., 2020). These examples highlight the versatility and effectiveness of CRISPR/Cas9 in addressing complex breeding challenges and accelerating the development of superior rice cultivars (Bui, 2020; Peng et al., 2020; Usman et al., 2021).

3 CRISPR/Cas9 in Rice Breeding

3.1 Early successes in CRISPR/Cas9 rice research

The application of CRISPR/Cas9 technology in rice breeding has shown significant promise in recent years. Early successes include the precise editing of genes associated with important agronomic traits, such as drought resistance and grain size. For instance, the editing of the *Oryza sativa* Senescence-associated protein (*OsSAP*) gene demonstrated the potential of CRISPR/Cas9 to enhance drought tolerance in rice. The edited plants exhibited improved survival rates and growth metrics under drought stress, as illustrated in Figure 2, highlighting the efficiency of CRISPR/Cas9 in developing stress-resistant rice varieties (Park et al., 2022). Additionally, the CRISPR/Cas9-mediated mutagenesis of the Grain Size 3 (*GS3*) gene resulted in rice mutants with significantly increased grain length and weight, showcasing the technology’s capability to improve yield-related traits (Usman et al., 2021).

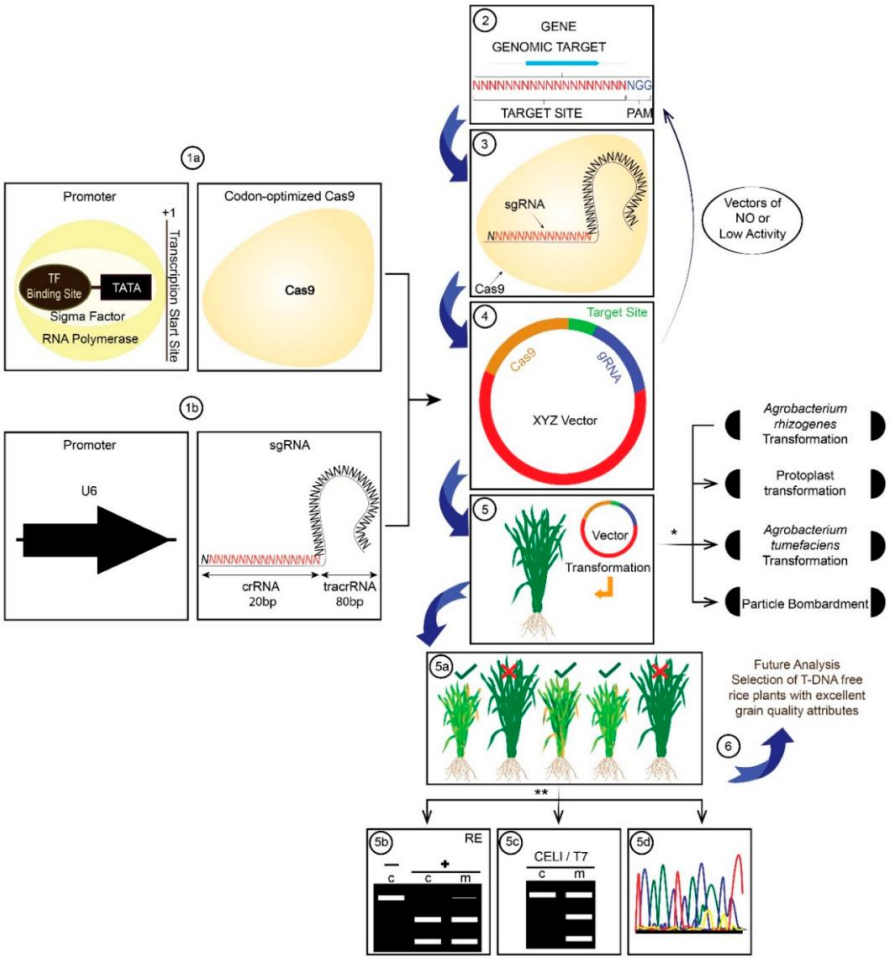

Figure 1 Basic flow chart of the CRISPR/Cas9 genome editing system (Adopted from Fiaz et al., 2019)

Image caption: the engineered CRISPR/Cas9 system consist of two components; (1a) The Cas9 endonuclease and, (1b) A single-guide RNA (sgRNA). "The sgRNA contains a spacer sequence followed by 79 nt of an artificially fused tracrRNA and crRNA sequence", (2) The spacer sequence is typically 20 nt in length, and specifically binds to the target DNA sequence containing a 5'-NGG-3' PAM motif at the 3' end, which is highly specific for the gene of interest, (3) The fused trans-activating crRNA (tracrRNA) and crRNA sequence forms a stem-loop RNA structure that binds to the Cas9 enzyme; tracrRNA hybridizes and joins Cas9. (4) Assembly of sgRNA, attached with the target sequence and the Cas9 vector construct. (5) Transformation of the vector construct into rice via different transformation techniques. (5a) Screening and selection of rice mutant plants based on phenotypic changes. (5b) Restriction enzyme site loss generating a CRISPR/Cas9 mutagenized plant line. (c, control; m, mutagenized; RE, restrictions enzyme). (5c) Surveyor Assay (CEL1 and T7 are DNA endonucleases utilized in surveyor assay). (5d) Next-generation sequencing. (6) Future analysis to obtain T-DNA-free plants, and further experiments to prove phenotypic changes cast by the knockout of the gene under investigation. * Different techniques for the vector construct transformation. ** Regeneration and screening of transgenic plants for gene editing events (Adopted from Fiaz et al., 2019)

3.2 Targeted gene editing for yield traits

CRISPR/Cas9 technology has been instrumental in the targeted editing of genes to enhance yield traits in rice. By focusing on specific genes that regulate grain size, panicle length, and stress tolerance, researchers have been able to create rice varieties with superior agronomic performance. For example, the simultaneous editing of *OsPIN5b*, *GS3*, and *OsMYB30* genes led to the development of rice mutants with increased panicle length, enlarged grain size, and enhanced cold tolerance. These modifications resulted in higher yields and better stress resistance, demonstrating the multifaceted benefits of targeted gene editing (Zeng et al., 2020). Furthermore, the editing of the *GS3* gene alone has been shown to increase grain length and weight by over 30%, further emphasizing the potential of CRISPR/Cas9 in yield trait improvement (Usman et al., 2021).

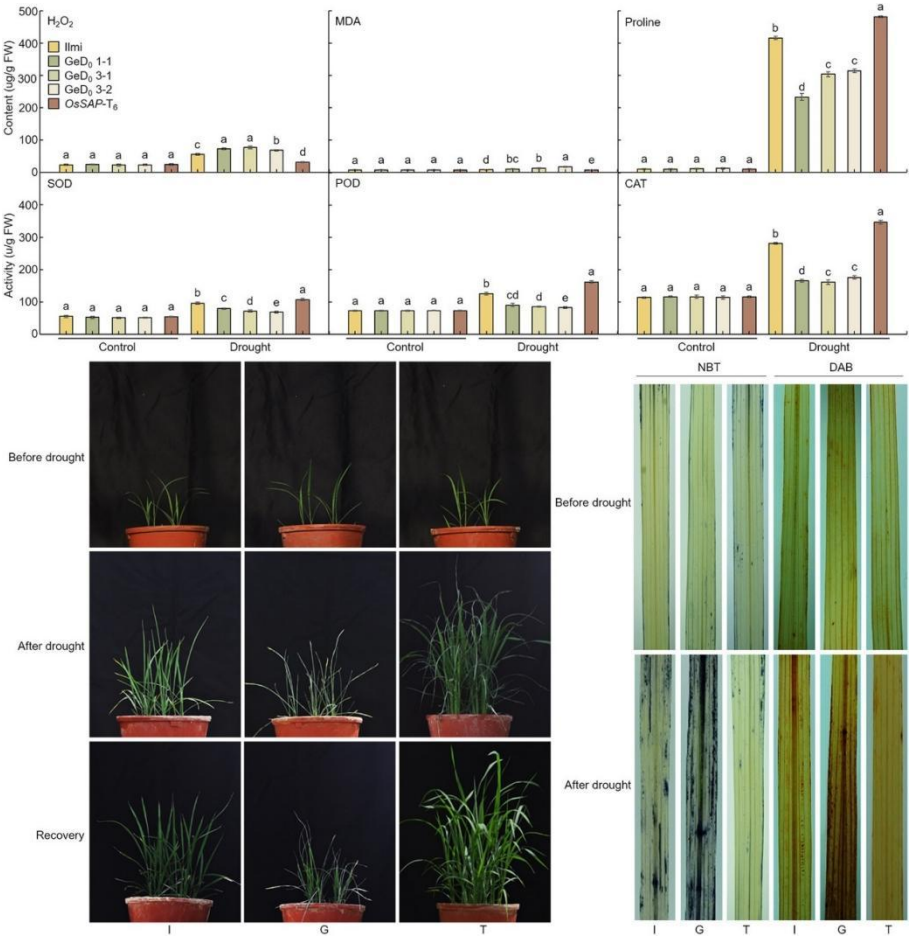

Figure 2 Effects of *OsSAP* on cellular antioxidants and reactive oxygen species (ROS) levels in Ilmi, GeD, and *OsSAP*-T_6 lines under normal and drought conditions (Adopted from Park et al., 2022)

Image caption: under drought conditions, the effects of *OsSAP* on the level of endogenous hydrogen peroxide (H_2O_2), the accumulation of malondialdehyde (MDA), the accumulation of proline (proline), the activity of superoxide dismutase (SOD), the activity of peroxidase (POD), and the activity of catalase (CAT). Data presented are expressed as mean ± SD from five independent biological experiments per line. Bars represent means ± SE. Means denoted by the same letter are not significantly different ($p < 0.05$) as evaluated by Duncan's multiple range test. The GeD lines showed reduced drought tolerance, whereas the *OsSAP*-T_6 lines showed increased drought tolerance before drought stress, and 20 days after germination. Drought stress treatment; phenotypic changes at 20% soil moisture content. Phenotypes after 7 days of recovery achieved by watering (recovery from 20% soil moisture content). Phenotypes of 3,3-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining when the soil moisture content was 20%. After drought stress treatment, H_2O_2 was visualized as brown spots by DAB, and O_2^- as blue spots by NBT. The picture presented is the most representative of five independent biological experiments per each line. I: Ilmi. G: GeD_0 1–1. T: *OsSAP*-T_6. Different letters on columns represent significant ($p < 0.05$) difference between rice lines based on Duncan's test (Adopted from Park et al., 2022)

3.3 Case studies of yield improvement in rice

Several case studies have highlighted the successful application of CRISPR/Cas9 in improving rice yield. One notable example is the editing of the *GS3* gene, which resulted in rice mutants with a 31.39% increase in grain length and a 27.15% increase in 1 000-grain weight compared to wild-type plants. This study demonstrated the effectiveness of CRISPR/Cas9 in creating high-yielding rice varieties through precise genetic modifications (Usman et al., 2021). Another case study involved the editing of multiple genes, including *OsPIN5b*, *GS3*, and *OsMYB30*, to produce rice mutants with both high yield and improved cold tolerance. The resulting transgenic lines exhibited significant improvements in yield-related traits and stress resistance, showcasing the potential of CRISPR/Cas9 for comprehensive agronomic trait enhancement (Zeng et al., 2020). These case studies underscore the transformative impact of CRISPR/Cas9 technology on rice breeding and its role in addressing global food security challenges.

4 Yield Traits in Rice and Their Genetic Basis

4.1 Definition and importance of yield traits

Yield traits in rice are critical agronomic characteristics that directly influence the productivity and economic value of rice crops. These traits include grain size, grain number, and panicle length, among others. Grain size, for instance, is a major determinant of rice yield and is a vital trait for both domestication and breeding efforts (Usman et al., 2020; Usman et al., 2021). Grain number per panicle and panicle length also significantly contribute to the overall yield, with higher numbers generally correlating with increased productivity (Huang et al., 2018; Zeng et al., 2020). The importance of these traits lies in their direct impact on the quantity of rice produced per unit area, which is essential for meeting the food demands of a growing global population.

4.2 Major yield-related genes in rice

Several key genes have been identified that play crucial roles in determining yield traits in rice. For example, the Grain Size 3 (*GS3*) gene is known to regulate grain length, with mutations in this gene leading to increased grain size and weight (Zeng et al., 2020; Usman et al., 2021). Another important gene is Grain number 1a (*Gn1a*), which influences the number of grains per panicle. Mutations in *Gn1a* have been shown to enhance grain number, thereby boosting overall yield (Huang et al., 2018; Zhou et al., 2018). Additionally, the DENSE AND ERECT PANICLE1 (*DEP1*) gene is associated with panicle architecture, and its favorable alleles can lead to denser and more erect panicles, contributing to higher yield (Huang et al., 2018).

4.3 Genetic pathways influencing yield traits

The genetic pathways influencing yield traits in rice are complex and involve multiple genes and regulatory networks. For instance, the CLAVATA-WUSCHEL pathway, which is regulated by CLE peptide signals, plays a significant role in controlling meristem size and, consequently, yield-related traits (Liu et al., 2021a). The *OsSPL16*/*qGW8* gene, when edited using CRISPR/Cas9, has been shown to modulate the expression of pyruvate enzymes and cell cycle proteins, leading to increased grain size and yield (Usman et al., 2020). Furthermore, the *OsPIN5b* gene, which affects panicle length, and the *OsMYB30* gene, which is involved in cold tolerance, have been successfully edited to produce rice varieties with improved yield and stress resistance (Zeng et al., 2020). These genetic pathways highlight the intricate network of interactions that govern yield traits and the potential of CRISPR/Cas9 technology to enhance these traits through precise genome editing.

5 CRISPR/Cas9 Strategies for Yield Trait Improvement

5.1 Gene knockout approaches

Gene knockout strategies using CRISPR/Cas9 involve the targeted disruption of specific genes to enhance desirable traits in rice. This approach has been effectively utilized to improve various yield-related traits. For instance, the knockout of the *Os8N3* gene in rice has been shown to confer enhanced resistance to *Xanthomonas oryzae* pv. *oryzae*, a significant pathogen, without affecting other agronomic traits (Kim et al., 2019). Similarly, the editing of the *Oryza sativa* Senescence-associated protein (OsSAP) gene has demonstrated improved drought resistance, which is crucial for maintaining yield under stress conditions (Park et al., 2022). These examples highlight the potential of gene knockout strategies in improving yield traits by enhancing resistance to biotic and abiotic stresses.

5.2 Gene knock-in strategies

Gene knock-in strategies involve the precise insertion of beneficial genes into specific genomic locations to enhance yield traits. This method allows for the introduction of new functions or the enhancement of existing ones. For example, the insertion of genes that regulate grain size, such as the Grain Size 3 (*GS3*) gene, has resulted in rice mutants with significantly increased grain length and weight (Usman et al., 2021). This approach not only improves yield but also ensures the stable inheritance of these traits across generations. The ability to introduce specific genes with known beneficial effects makes gene knock-in strategies a powerful tool for yield improvement in rice breeding.

5.3 Promoter engineering for enhanced expression

Promoter engineering using CRISPR/Cas9 focuses on modifying the regulatory regions of genes to enhance their expression levels, thereby improving yield traits. This strategy has been successfully applied in maize, where weak promoter alleles of *CLE* genes were engineered to increase meristem size, leading to improved grain yield-related traits (Liu et al., 2021a). In rice, similar approaches can be employed to enhance the expression of genes involved in grain quality and yield. For instance, the modulation of promoter regions to increase the expression of genes related to grain quality has shown promising results in improving both yield and quality traits (Bandyopadhyay et al., 2018; Fiaz et al., 2019). Promoter engineering thus offers a targeted approach to fine-tune gene expression for optimal yield improvement.

6 Challenges and Limitations of CRISPR/Cas9 in Rice Breeding

6.1 Off-target effects and mitigation strategies

One of the primary challenges of using CRISPR/Cas9 in rice breeding is the potential for off-target effects, where the Cas9 enzyme cuts DNA at unintended locations. This can lead to unintended mutations, which may affect the plant's phenotype and overall health. Various strategies have been developed to mitigate these off-target effects. For instance, the use of high-fidelity Cas9 variants and the careful design of guide RNAs (gRNAs) can significantly reduce off-target activity (Chen et al., 2019; Ahmad et al., 2020; Rao and Wang, 2021). Additionally, employing bioinformatics tools to predict and avoid potential off-target sites can further enhance the specificity of CRISPR/Cas9 editing (Zegeye et al., 2022).

6.2 Regulatory and biosafety issues

The regulatory landscape for genetically edited crops, including those modified using CRISPR/Cas9, is complex and varies significantly across different countries. Some nations have stringent regulations that classify CRISPR-edited crops similarly to genetically modified organisms (GMOs), which can hinder their commercial adoption (Ahmad et al., 2020; Rao and Wang, 2021). Biosafety concerns also arise from the potential ecological impacts of releasing CRISPR-edited plants into the environment. These concerns necessitate thorough risk assessments and the development of robust regulatory frameworks to ensure the safe use of CRISPR/Cas9 technology in agriculture (Zegeye et al., 2022).

6.3 Technical challenges and solutions

Despite its precision, CRISPR/Cas9 technology faces several technical challenges in rice breeding. One significant issue is the delivery of the CRISPR/Cas9 components into rice cells. Traditional methods like Agrobacterium-mediated transformation and biolistics can be inefficient and may cause tissue damage (Ren et al., 2019; Rao and Wang, 2021). Recent advancements in delivery methods, such as the use of nanotechnology and virus particle-based systems, have shown promise in overcoming these challenges (Rao and Wang, 2021). Another technical hurdle is the efficient repair of DNA double-strand breaks (DSBs) induced by Cas9. Homology-directed repair (HDR), which is often desired for precise editing, occurs at a low frequency in plants. Researchers are exploring alternative strategies, such as base editing and prime editing, to achieve more efficient and precise genome modifications (Chen et al., 2019; Ren et al., 2019).

7 Future Perspectives

7.1 Emerging trends in CRISPR/Cas9 technology

The CRISPR/Cas9 technology has rapidly evolved, with several emerging trends poised to further enhance its application in rice breeding. One significant trend is the development of base-editing tools that enable precise nucleotide substitutions without inducing double-strand breaks, thereby reducing off-target effects and increasing editing efficiency (Chen et al., 2019; Rao and Wang, 2021). Additionally, advancements in delivery systems, such as DNA-free methods and nanotechnology-based delivery, are making genome editing more efficient and less reliant on traditional transformation techniques (Chen et al., 2019; Rao and Wang, 2021). The use of high-throughput mutant libraries and multiplex gene editing is also gaining traction, allowing for the simultaneous editing of multiple genes, which can accelerate the breeding of rice varieties with improved yield traits (Chen et al., 2019; Rao and Wang, 2021; Liu et al., 2022).

7.2 Integration with other breeding techniques

The integration of CRISPR/Cas9 with other breeding techniques holds great promise for the future of rice breeding. Combining CRISPR/Cas9 with traditional breeding methods, such as marker-assisted selection and hybrid breeding, can enhance the precision and efficiency of developing new rice varieties (Fiaz et al., 2019; Rao and Wang, 2021). Moreover, the use of CRISPR/Cas9 in conjunction with genomic selection can facilitate the identification and manipulation of key genes associated with yield traits, thereby accelerating the breeding process (Fiaz et al., 2019; Rao and Wang, 2021). The potential for CRISPR/Cas9 to be used in plant synthetic biology and domestication also opens new avenues for creating rice varieties with novel traits that are tailored to specific environmental conditions and consumer preferences (Chen et al., 2019; Rao and Wang, 2021).

7.3 Potential impact on global rice production

The application of CRISPR/Cas9 technology in rice breeding has the potential to significantly impact global rice production. By enabling the rapid development of rice varieties with enhanced yield, quality, and stress resistance, CRISPR/Cas9 can contribute to meeting the growing food demands of an increasing global population (Liu et al., 2022; Zegeye et al., 2022). For instance, the successful editing of genes related to drought resistance and grain quality has already demonstrated the potential of CRISPR/Cas9 to improve rice yield and resilience under adverse environmental conditions (Fiaz et al., 2019; Park et al., 2022; Zegeye et al., 2022). Furthermore, the development of transgene-free edited plants, which are more likely to gain regulatory approval and public acceptance, can facilitate the widespread adoption of CRISPR/Cas9 technology in rice breeding programs worldwide (Ahmad et al., 2020; Zegeye et al., 2022). Overall, the continued advancement and integration of CRISPR/Cas9 technology in rice breeding are expected to play a crucial role in ensuring global food security and sustainable agricultural production.

8 Concluding Remarks

The application of CRISPR/Cas9 technology in rice breeding has shown significant promise in enhancing yield traits and overall crop quality. This advanced genetic tool enables precise genome editing, facilitating the modification of specific genes linked to desirable traits. In terms of grain quality improvement, CRISPR/Cas9 has been effectively utilized to target and edit genes that influence various quality attributes. Additionally, the technology has been used to boost yield-related traits by altering genes that govern plant architecture, stress resistance, and other yield determinants. It has also played a crucial role in developing rice varieties with enhanced tolerance to abiotic stresses such as drought, which is essential for sustaining yield in adverse environmental conditions. The simplicity and precision of CRISPR/Cas9 not only ensure its versatility as a tool for plant genome editing but also enable the rapid development of new rice cultivars with improved traits.

The future of CRISPR/Cas9 in rice breeding appears highly promising, with numerous potential advancements and applications on the horizon. Future research could increasingly focus on multiplex genome editing, which involves editing multiple genes simultaneously to achieve complex trait improvements within a single generation Additionally, the development of base and prime editing technologies could significantly enhance the precision of genome editing by allowing for single nucleotide changes without causing double-strand breaks. Efforts are also underway to develop non-genetically modified (Non-GMO) crops using CRISPR/Cas9, which is crucial for gaining public acceptance and regulatory approval; this involves the creation of transgene-free plants through precise editing and subsequent breeding strategies. Moreover, expanding the use of CRISPR/Cas9 for functional genomics studies will facilitate the identification and validation of new target genes for yield and quality improvement, thereby providing a deeper understanding of the genetic basis of these traits.

To fully harness the potential of CRISPR/Cas9 in rice breeding, sustained research and robust collaboration are vital. Effective integration of CRISPR/Cas9 technology into practical breeding programs requires interdisciplinary collaboration among geneticists, molecular biologists, agronomists, and breeders. Additionally, the establishment of global research networks and the sharing of resources, such as gene-editing tools and germplasm, are essential to accelerate the development and dissemination of improved rice varieties. Furthermore, the development of clear and science-based regulatory frameworks for genome-edited crops is crucial to facilitate the adoption of

CRISPR/Cas9 technology in agriculture, ensuring safety and public trust. Engaging with the public and stakeholders to communicate the benefits and safety of CRISPR/Cas9 technology is also critical for its acceptance and widespread use in rice breeding.

In conclusion, CRISPR/Cas9 technology holds immense potential for advancing rice breeding and addressing global food security challenges. Continued research, collaboration, and effective communication will be key to harnessing this technology's full potential for sustainable agriculture.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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