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Development of CRISPR-Cas9 Multiple Editing System for Genetic Improvement of Rice

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Abstract The CRISPR-Cas9 multiple editing system has become an important tool in the field of rice genetic improvement. This review aims to outline the principles and applications of the system, emphasizing its cutting-edge position in rice breeding. The advantage of a multiple editing system is that it can simultaneously edit multiple loci to achieve precise improvement of rice yield, resistance, and quality traits. This review also discusses in detail the working principle, development process, and widespread application of multiple editing systems, briefly introduces CRISPR-Cas9 technology, explains how multiple editing systems can achieve efficient multi gene editing, and delves into the specific applications of multiple editing systems in rice genetic improvement, including increasing yield, increasing resistance, and improving quality. These applications have enriched the genetic resources of rice and provided new avenues for food security and sustainable agricultural development.
Keywords CRISPR Cas9; Multiple editing system; Rice breeding; Increased production; Enhanced disease resistance

Rice (*Oryza sativa*) is one of the most important food crops in the world, providing a major source of food for many people worldwide. With the growth of global population and the threat of climate change, ensuring the improvement of rice yield, quality, and resistance has become particularly urgent. In the past few decades, traditional breeding methods have made significant progress, but their speed and efficiency are still limited. Fortunately, breakthroughs in modern molecular biology and gene editing technology have opened up new avenues for genetic improvement in rice.

CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR associated protein 9) is a revolutionary gene editing technique that allows scientists to modify genes at specific locations in rice and other plants. However, the initial CRISPR-Cas9 technology had some limitations, one of which was that only one gene could be edited. Over time, researchers have developed the CRISPR-Cas9 multi editing system, and the introduction of this technology has changed the game rules of rice genetic improvement (Romero et al., 2019).

The purpose of this review is to comprehensively explore the application of CRISPR-Cas9 multiple editing system in rice genetic improvement. This review will focus on introducing the working principle, development history, and cutting-edge position of multiple editing system in rice breeding. The uniqueness of a multi editing system lies in its ability to simultaneously edit multiple gene loci, providing new opportunities for precise shaping of rice quality and traits.

This review briefly reviews the basic principles of CRISPR-Cas9 technology to ensure readers have a basic understanding of this technology, and delves into the construction and working methods of multiple editing systems, explaining how to achieve efficient multi gene editing. In addition, this review will also discuss in detail the specific applications of multiple editing systems in rice genetic improvement, including how to increase rice yield, increase resistance, and improve quality. These applications not only enrich the genetic resources of rice, but also provide new avenues for global food security and sustainable agricultural development.

Through in-depth exploration of the application of CRISPR-Cas9 multi editing system, this review aims to stimulate more research on rice genetic improvement and provide more possibilities for food production. We hope that readers can gain a deeper understanding of this exciting field and provide new ideas and methods for future research and practice of rice genetic improvement.

1 The basic principles of CRISPR-Cas9 technology

1.1 Overview of multiple editing systems

The multiple editing system is a further development of CRISPR-Cas9 technology, allowing for simultaneous editing of multiple genes or DNA loci to achieve more complex genetic improvements. The core of a multi editing system is to design multiple RNA guidance sequences, each sequence used to guide Cas9 protein to different target sites. These RNA guidance sequences typically complement and pair with different target genes or DNA sequences. Therefore, multiple editing systems require scientists to carefully design multiple different RNA guidance sequences to meet the needs of editing multiple loci.

In a multi editing system, each designed RNA guide sequence typically binds to a Cas9 protein. This means that multiple Cas9 proteins simultaneously act on different target sites. These Cas9 proteins can be the same or different variants of Cas9, depending on the research needs (Xu et al., 2022) (Figure 1).

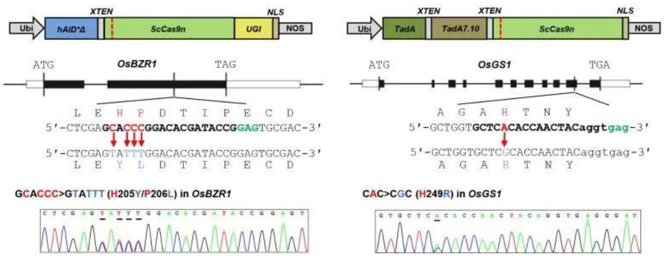

Figure 1 Mutants of Cas proteins and different species of Cas proteins can to some extent expand the targeting range of CRISPR tools (Xu et al., 2022)

By using a multiple editing system, scientists can simultaneously edit multiple DNA sites. This allows them to achieve more complex genetic improvements, such as multi gene regulation, optimization of complex metabolic pathways, and more functional enhancements. Multi editing systems have wide applications in fields such as agriculture, medicine, biological research, and biotechnology. For example, it can be used to develop multi resistant crop varieties, treat polygenic genetic diseases, or achieve regulation of multiple cytokines. Multiple editing systems provide more possibilities for solving complex biological problems (Xin et al., 2018).

Despite the enormous potential of multiple editing systems, there are also challenges and limitations. This includes complex RNA guided sequence design, interference between multiple Cas9 proteins, and potential safety and ethical issues. In addition, editing multiple loci may lead to unwanted side effects.The introduction of multiple editing systems has expanded the application scope of CRISPR-Cas9 technology, enabling scientists to perform gene editing and genetic improvement more comprehensively. However, it requires careful planning and precise experimental design to ensure the desired editing effect and minimize unwanted impacts.

1.2 Mechanism of Cas9 protein and RNA guided action

The core mechanism of CRISPR-Cas9 technology lies in the synergistic effect guided by Cas9 protein and RNA to achieve precise gene editing. Cas9 is an endonuclease of the CRISPR-Cas9 system, responsible for cleaving target DNA. The Cas9 protein has two main functional domains: RuvC and HNH. These two functional domains work together to bind Cas9 protein to target DNA and cause DNA double strand breaks.

In the CRISPR-Cas9 system, the RNA guide sequence is designed by scientists to complement and pair with the DNA sequence of the target gene. This RNA guidance sequence is merged into the CRISPR-Cas9 system to guide Cas9 protein binding to specific DNA sites. Accurately designed RNA guidance sequences are key to achieving specific gene editing.

The Cas9 protein forms a Cas9 RNA complex through complementary pairing with RNA guiding sequences. This complex has high specificity, allowing it to recognize and bind to the target DNA. Once the Cas9 RNA complex binds to the target DNA, it forms a DNA-RNA double helix structure that locates the Cas9 protein at a specific location of the target gene.

Once the Cas9 protein and RNA guide recognition and binding to the target DNA, it will guide the Cas9 protein to cleave DNA, resulting in DNA double strand breaks. This rupture activates the DNA repair mechanisms of cells, including non homologous end junctions (NHEJ) and homologous recombination (HDR). NHEJ typically causes small fragment insertions or deletions, while HDR allows for more precise DNA repair (Zhang et al., 2019).

Ultimately, DNA double strand breaks and repair processes can lead to gene editing. If the goal of scientists is to knock out genes, the NHEJ repair process may result in inserted or missing bases, thereby disrupting gene function. If the goal is to repair genes or add new DNA sequences, HDR can be used to achieve more precise editing. This approach enables scientists to modify specific genes to achieve the desired genetic improvement (Zhang et al., 2016).

The mechanism guided by Cas9 protein and RNA is the core of CRISPR-Cas9 technology, allowing scientists to achieve precise gene editing. The understanding of this mechanism is of crucial importance for the study of genetic improvement and reproductive organ development in rice.

1.3 Basic process of targeted gene editing

Targeted gene editing is one of the core applications of CRISPR-Cas9 technology, allowing scientists to accurately modify the DNA sequence of organisms. Firstly, scientists need to determine the target genes they want to edit. This can be genes related to the development of specific reproductive organs, or other genes of interest. The selection of target genes depends on the research purpose and the required genetic improvements.

Once the target gene is identified, scientists need to design an RNA guidance sequence, which typically consists of 20 nucleotides and complements the DNA sequence of the target gene. A well-designed RNA guidance sequence is key to achieving precise editing, as it determines where the Cas9 protein will cleave DNA.

The designed RNA guide sequence is merged into the CRISPR-Cas9 system and used in conjunction with the Cas9 protein. This Cas9 RNA complex forms a highly specific complex through complementary pairing with the RNA guide sequence. This complex guides the Cas9 protein to a specific location of the target gene (Romero and Gatica Arias, 2019).

Once the Cas9 RNA complex binds to the target DNA, the Cas9 protein will cleave the DNA, causing DNA double strand breaks. This rupture will activate the cell's DNA repair mechanism. There are two main repair mechanisms, non homologous terminal junction (NHEJ) and homologous recombination (HDR).

NHEJ often leads to small fragment insertion or deletion, as it is a relatively imprecise repair process. This method is commonly used for gene knockout as it can cause loss of gene function. HDR allows for more precise DNA repair as it uses exogenous DNA templates for repair. This method is commonly used to repair damaged genes or add new DNA sequences.

Scientists need to analyze the effectiveness of editing. This can be achieved through PCR, sequencing, or other molecular biology techniques. The analysis of editing effects helps to determine whether the required genetic improvements have been successfully achieved. This provides a precise method for modifying the DNA of living organisms in the basic process of gene editing, which is very useful for genetic improvement and reproductive organ development research in rice. The success of this process depends on the careful design of RNA guided sequences and the analysis of editing effects.

2 The Demand for Genetic Improvement in Rice

2.1 Challenges faced by genetic improvement of rice

Although the CRISPR-Cas9 multi editing system provides new possibilities for genetic improvement in rice, this field still faces numerous challenges. These challenges involve ethics, regulations, technology, and the practical application of genetic improvement. Ethical and regulatory issues have always been one of the key challenges in the field of rice genetic improvement. How to ensure that gene editing does not pose potential environmental and health risks, as well as how to address potential ethical and social issues, are issues that need to be carefully considered. The regulations for gene editing vary in different countries, so researchers need to comply with local regulations to ensure that their work is legal and safe.

Genetic improvement of rice involves complex biological processes, including gene interactions and expression regulation. When designing a multi editing system, it is necessary to have a deep understanding of the interactions between multiple target genes to avoid unwanted effects. In addition, how to efficiently deliver editing tools to rice plants and ensure the accuracy of editing are also technical challenges (Elena and Estela, 2021).

Another challenge is how to successfully apply basic research to practical breeding projects. From the laboratory to the field, it is necessary to cross multiple stages to ensure that the performance of edited rice varieties is consistent with expectations. This requires a significant amount of time and resources, and involves collaboration with the agricultural sector, growers, and communities. Genetic improvement of rice still needs to address climate change, pest and disease pressures, and ongoing agricultural sustainability challenges. Therefore, rice varieties need to have more stress resistance, higher yield, and better quality. Multiple editing systems provide potential solutions in these areas, but effectively introducing these varieties into the market and ensuring their widespread application remains a complex challenge.

Despite facing these challenges, the development of multiple editing systems still brings hope for genetic improvement in rice. With the continuous advancement of technology and deeper research, we can look forward to seeing more innovative solutions in the future, thereby accelerating the development of rice breeding and improving the global food security level.

2.2 Potential applications of genetic improvement

As one of the world's major food crops, the yield of rice has always been of great concern. Multiple editing systems can be used to improve the growth characteristics of rice, enhance photosynthetic efficiency, and increase biomass accumulation, thereby achieving higher yields, which is crucial for meeting the growing global food demand. Rice is threatened by various biotic and abiotic stresses, including pests and diseases, drought, saline alkali soil, etc. Multiple editing systems can be used to enhance the resistance of rice by editing genes related to resistance to diseases, pests, and stress, which will help reduce pesticide use and improve crop survival rates.

In addition to quantity, the quality of rice is also a key factor. Multiple editing systems can be used to improve the taste, flavor, appearance, and storage characteristics of rice. This is crucial for meeting the needs of different markets and improving the market competitiveness of rice (Mohammad et al., 2021). In some regions, a lack of specific nutrients in the diet is a significant cause of health problems. Multiple editing systems can be used to increase the content of specific nutrients in rice, such as vitamins, minerals, or antioxidants, thereby improving people's dietary quality.

The multi editing system provides flexibility for the development of new rice varieties, allowing researchers to accurately edit target genes and introduce new traits in rice, such as resistance, growth characteristics, or improved production efficiency. This provides new opportunities for creating more innovative and competitive varieties. Climate change will have profound impacts on global agriculture. Multiple editing systems can be used to breed rice varieties that are more adaptable to different climate conditions. This helps to ensure the sustainability of food production, maintaining high yields even in constantly changing environments.

These applications demonstrate the diversity and potential of multiple editing systems in rice genetic improvement. With the continuous development of research and technology, we can expect more innovative methods and solutions to accelerate the progress of rice breeding, promote global food security and sustainable agricultural development.

2.3 Application of CRISPR-Cas9 in the rice field

CRISPR-Cas9 gene editing technology has become a revolutionary tool in the field of rice genetic improvement. It provides researchers with a fast, accurate, and efficient method to edit rice genes to improve yield, resistance, quality, and other agronomic traits. CRISPR-Cas9 has a wide range of applications in the field of rice.

CRISPR-Cas9 technology can be used to selectively knock out unwanted or unwanted genes in rice. This helps researchers understand the function of specific genes or improve rice by removing unfavorable traits. Through CRISPR-Cas9, targeted editing or repair of rice genes can be achieved. This provides possibilities for improving the traits of rice, including increasing yield, improving disease resistance, and improving quality. Multiple editing systems allow researchers to simultaneously edit multiple genes, thereby better achieving improvements in multiple traits. This strategy is very useful for creating rice varieties with stronger comprehensive performance. CRISPR-Cas9 technology can be used to increase the resistance of rice to various pests and diseases. By editing genes related to resistance, pesticide use can be reduced and crop survival rates can be improved. Rice grows under various environmental conditions, including drought, saline alkali soil, and high temperature. CRISPR-Cas9 enables researchers to edit genes related to stress resistance to make rice more adaptable to these environments (Zhang et al., 2019).

CRISPR-Cas9 technology provides an opportunity to create new rice varieties. Researchers can combine the beneficial traits of different varieties to create more competitive and high-yield new varieties. CRISPR-Cas9 technology helps to enhance the genetic resources of rice, including improving the traits of varieties to cope with constantly changing demands and environmental conditions.

With the development of various aspects, CRISPR-Cas9 technology has greatly accelerated the process of genetic improvement in rice, providing a fast, accurate, and multifunctional method to improve this key food crop. These application areas demonstrate the widespread use of CRISPR-Cas9 in the rice field, which is expected to provide important support for global food security and sustainable agricultural development.

3 Application of CRISPR-Cas9 Multi Editing System in Rice

3.1 Single gene editing

Single gene editing is one of the core applications of CRISPR-Cas9 technology in rice genetic improvement. This method enables scientists to improve specific traits, increase rice yield, disease resistance, and quality by selectively editing or repairing individual loci in rice genes. CRISPR-Cas9 technology is known for its highly precise and efficient characteristics, which can accurately guide Cas9 nucleases to the desired gene loci for editing or repair. This greatly reduces non-specific gene editing and improves the accuracy of editing.

Single gene editing can be used to improve traits related to rice yield. Scientists can edit genes that control grain size, quantity, and distribution to achieve high-yield rice cultivation. By editing genes related to pathogen resistance, rice's resistance to pathogens can be enhanced, which helps reduce the harm of diseases to rice and reduce pesticide use. Single gene editing can be used to improve the taste, storage performance, stress resistance, and other quality characteristics of rice. This helps to meet the needs of different markets and consumers. Through single gene editing, scientists can improve the traits of different rice varieties, thereby enriching the genetic resources of rice. This is crucial for improving global food security. Single gene editing technology provides a faster breeding method, accelerating the development and commercialization process of new varieties (Mishra et al., 2018).

With the support of CRISPR-Cas9 technology, the application of single gene editing in rice genetic improvement provides scientists with opportunities for improvement and innovation. The advantage of this method lies in its highly precise editing and efficient performance, paving the way for cultivating more competitive and stress resistant rice varieties. Single gene editing is a key tool in the field of rice genetic improvement, and is expected to continue promoting the improvement of rice yield, quality, and sustainability in the future.

3.2 Multi gene editing

Multi gene editing is one of the cutting-edge fields of CRISPR-Cas9 technology in rice genetic improvement, providing powerful tools for improving rice traits and quality. A multi gene editing system allows scientists to simultaneously edit multiple rice genes at the same time. This is crucial for improving multiple traits, such as increasing yield, disease resistance, and quality.

The traits of rice are usually regulated by multiple genes, and through multi gene editing, scientists can better understand and adjust complex traits such as drought resistance and stress resistance. A multi gene editing system can achieve efficient and accurate editing, reduce non-specific changes, and improve editing accuracy. This method helps to enrich the genetic resources of rice. Scientists can edit multiple genes in different varieties to create more new variants. By simultaneously editing multiple genes related to yield and quality, multi gene editing has the potential to cultivate more high-yielding and high-quality rice varieties. Multi gene editing systems can be used to improve the stress resistance of rice, making it more adaptable to climate change and adverse environmental conditions. Multi gene editing can help improve rice yield and quality, which is expected to contribute to global food security and sustainable agricultural development (Li et al., 2019).

The application of multi gene editing systems has brought more possibilities for genetic improvement in rice, enabling scientists to comprehensively improve the traits of rice. This technology will continue to drive the forefront of rice breeding in the future, providing new opportunities for improving yield, quality, and sustainability.

3.3 Gene stacking and innovation

Gene stacking and innovative editing represent two key aspects of the CRISPR-Cas9 multi gene editing system, which are of great significance for genetic improvement in rice. Gene stacking refers to embedding multiple beneficial genes into rice plants to simultaneously improve multiple traits. This is a key application of multi gene editing systems. Through gene stacking, scientists can introduce multiple beneficial traits, such as increased yield, improved quality, and increased resistance. For example, multiple genes can be combined to enhance the resistance of rice to various biotic and abiotic stresses, thereby creating more survivable rice varieties. This is expected to improve the productivity of rice, reduce crop damage, and thus enhance food safety (Zhou et al., 2014).

Innovative editing involves precise and innovative editing of rice genes to achieve new traits or improve existing traits. This editing method can be achieved by adding, deleting, or changing specific gene sequences. Scientists can selectively edit rice genes to better adapt to different climates, soils, and growth conditions. This is crucial for adapting to constantly changing environments. In addition, innovative editing can also be used to improve the taste, preservation, and other quality characteristics of rice.

Gene stacking and innovative editing jointly promote innovation in rice genetic improvement. Through these methods, scientists can introduce new traits into rice, improve yield and quality, and increase its stress resistance. This will help address the growing global demand for food, improve the sustainability of agriculture, and adapt to challenges such as climate change. Therefore, gene stacking and innovative editing have enormous potential in rice genetic improvement and will continue to lead the forefront of rice breeding.

4 Outlook and Future Development

The CRISPR-Cas9 multiple editing system represents the forefront of rice genetic improvement, however, there are still many potential applications and future development directions worth further exploration. With the continuous improvement and accuracy of the CRISPR-Cas9 multi editing system, we can look forward to a wider range of application areas. In addition to the already involved improvements in yield, quality, and resistance, this technology can also be used for rice drug production, ecological restoration, as well as adaptive and evolutionary research. In the future, we may see more examples of gene stacking and innovative editing to achieve more complex trait improvement (Mehta et al., 2020).

With the continuous growth of the global population, the demand for food is also increasing. Rice is one of the most important food crops in the world, therefore, its genetic improvement holds an important strategic position. The CRISPR-Cas9 multi editing system provides new opportunities for the improvement of rice varieties, which can meet future food needs. In the future, we are expected to see more customized rice varieties that can adapt to different climate and soil conditions, improving agricultural productivity.

As the continuous progress of science, the field of rice genetic improvement will also benefit from emerging technologies and research directions. For example, researchers can use artificial intelligence and big data analysis to better understand the rice genome and quickly screen for potential editing targets. In addition, the application of emerging fields such as synthetic biology, metabolic engineering, and nanotechnology will bring more opportunities for genetic improvement in rice.

Therefore, the CRISPR-Cas9 multiple editing system is an important milestone in the field of rice genetic improvement, but its future applications, prospects for rice improvement, and emerging technologies and research directions all require further in-depth research and exploration. These efforts will help improve rice production and quality, thereby promoting global food security and sustainable agricultural development.

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