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A Current Status and Prospects of CRISPR/Cas9 Technology in Rice Genetic Improvement

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Abstract. Food security is a fundamental strategic issue concerning the survival and development of the global population. With the continuous growth of the population, the continuous reduction of cultivated land resources, the frequent occurrence of extreme climates, and the rampant spread of pests and diseases, various factors restricting food production have become increasingly prominent, and the urgency of rice genetic improvement has also grown more intense. As the most promising gene editing technology at present, CRISPR/Cas9 technology has obvious advantages such as simple operation, high specificity and low cost. It has achieved breakthrough results in many fields of rice genetic improvement, including yield improvement, quality improvement, stress resistance enhancement, and research related to apomixis. This paper systematically sorts out the application status of CRISPR/Cas9 technology in rice genetic improvement, carefully analyzes the problems and challenges existing in the practical application of this technology, such as off-target effects and heterogeneity of editing efficiency, and also discusses its future development directions in technical optimization and integrated application. The purpose of this paper is to provide theoretical reference and practical guidance for the further promotion and application of this technology in rice molecular breeding, promote the innovative development of rice genetic improvement technology, and ultimately help alleviate the contradiction between food supply and demand and ensure global food security.

Keywords: CRISPR/Cas9, Rice, Genetic Improvement, Gene Editing, Molecular Breeding, Trait Improvement

1. Introduction

Food security is a fundamental strategic issue concerning the survival and development of the global population. In recent years, the global population has been growing continuously, while cultivated land resources have been shrinking continuously. Coupled with frequent extreme climates and rampant pests and diseases, the contradiction between food supply and demand has become increasingly prominent, which has put forward higher requirements for the yield, quality and stress resistance of food crops [1]. As one of the most important food crops in the world, rice is widely planted, and about 3.5 billion people in the world take rice as their staple food, accounting for more than half of the world's total population [2]. Therefore, the production status of rice directly affects the global food supply balance and the stability of food security. At present, rice production is still

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facing many restrictive factors, which further highlights the urgency of rice genetic improvement. Therefore, accelerating the process of rice genetic improvement and breeding new varieties with high quality, high yield and multiple resistances has become one of the core tasks of current agricultural scientific research.

Traditional breeding technology has been widely used and has solved some problems of rice trait improvement to a certain extent, but it has obvious defects such as long breeding cycle, high cost and unstable genetic traits. Although molecular marker-assisted breeding has also been applied in rice blast resistance breeding and rice aroma quality improvement, it still has problems such as linkage disequilibrium between markers and target genes, high selection cost and narrow adaptation range [3].

The rise of gene editing technology has provided a brand-new technical path for crop genetic improvement and broken the bottleneck of traditional breeding. Because it can perform accurate and efficient site-specific genome editing in animals and plants, this technology has been widely used in many aspects such as gene function research, gene therapy and genetic improvement [4]. The so-called gene editing technology refers to the site-specific modification of the genome of an organism through artificially designed nucleases, so as to achieve precise operations such as gene knockout, knock-in, single base substitution, fragment insertion or deletion. Its core advantage lies in the ability to directionally and efficiently transform target genes and significantly shorten the breeding cycle. In the development process of gene editing technology, zinc finger nucleases (ZFNs) technology, transcription activator-like effector nucleases (TALEN) technology, etc. have appeared successively [5]. However, both of these two technologies have obvious shortcomings: ZFNs technology is complex in design, poor in specificity, and requires redesign of zinc finger structures for different targets, with relatively high cost; although TALEN technology has better specificity than ZFNs, its construction process is very cumbersome, time-consuming and laborious, and it is difficult to achieve high-throughput editing, which also limits its wide application in crop breeding.

The emergence of the clustered regularly interspaced short palindromic repeats and its associated protein (CRISPR/Cas9) system has completely changed the pattern of the gene editing field and has become the most promising gene editing technology at present [6]. Compared with ZFNs and TALENs technologies, the CRISPR/Cas9 system has significant advantages such as simple operation, high specificity, high editing efficiency, low cost and strong universality. As long as a guide RNA (sgRNA) complementary to the target gene is designed, it can guide the Cas9 nuclease to precisely cut the target genome site, thereby achieving directional modification of the gene [7]. After years of optimization and improvement, the CRISPR/Cas9 system has achieved a leap from "site-specific cleavage" to "precise editing at the nucleotide level", and has derived a variety of improved technologies such as base editing and prime editing, which further improve the precision and flexibility of editing. It can achieve precise modification of almost any gene in the rice genome, and can obtain stably inherited excellent traits without introducing foreign genes.

At present, CRISPR/Cas9 technology has been widely applied to various fields of rice genetic improvement, and has achieved a series of breakthrough results in yield improvement, quality improvement, stress resistance enhancement, disease and insect resistance improvement, etc., providing an efficient and convenient technical means for rice breeding. This paper systematically reviews the application status of CRISPR/Cas9 technology in rice genetic improvement, analyzes the problems and challenges existing in the application process of this technology, and also discusses its future development direction and application prospects. The purpose is to provide theoretical reference and practical guidance for the further promotion and application of

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CRISPR/Cas9 technology in rice molecular breeding, and promote the innovative development of rice genetic improvement technology.

2. Visualization analysis of CRISPR/Cas9 technology in rice genetic improvement based on VOSviewer

To systematically sort out the research hotspots, thematic clustering and evolution trends of CRISPR/Cas9 technology in the field of rice genetic improvement, VOSviewer software was used, with the Web of Science Core Collection as the data source, to retrieve English literatures with the topics of "CRISPR/Cas9", "rice", "genome editing" and "genetic improvement" (the retrieval time range is 2020-2023). After deduplication and screening, 1680 valid literatures were finally obtained. The software parameters were set as follows: the analysis type was keyword co-occurrence, the counting method adopted full counting, and the association strength was constructed based on co-occurrence frequency. The keyword co-occurrence and time evolution maps were generated to intuitively present the research pattern and development context of this field.

The keyword co-occurrence map (Figure 1) shows that the core keyword clusters in this field are clear, forming a main cluster with "rice" and "CRISPR/Cas9" as the core, radiating secondary cluster nodes such as "genome editing", "gene expression", "yield" and "resistance". On the whole, there are four major thematic clusters: first, the technology optimization cluster represented by "CRISPR/Cas9", "genome editing" and "off-target", focusing on the off-target prevention and control of gene editing tools, base editing and other technological iterations; second, the molecular regulation cluster represented by "gene expression", "transcription factor" and "protein", focusing on rice gene expression and stress response mechanisms; third, the agronomic trait improvement cluster represented by "yield", "grain yield" and "natural variation", focusing on the directional editing of yield-related traits; fourth, the stress resistance and nutrition fortification cluster represented by "tolerance", "biofortification" and "cadmium", focusing on heavy metal tolerance and nutritional quality improvement. The time evolution of node colors shows that from 2020 to 2021, the research focused on the basic construction and functional verification of CRISPR technology, and the keywords were mainly blue; from 2022 to 2023, the frequency of yellow-green keywords such as "yield", "biofortification" and "drought stress" increased significantly, indicating that application-oriented directions such as yield improvement, abiotic stress improvement and nutrition fortification have become the research frontiers in recent years.

In conclusion, VOSviewer visualization analysis clearly presents the thematic distribution and temporal evolution characteristics of CRISPR/Cas9 technology in the field of rice genetic improvement, reveals the research path from the development of technical tools to the implementation of application scenarios, and provides data support and reference for the selection of subsequent research directions.

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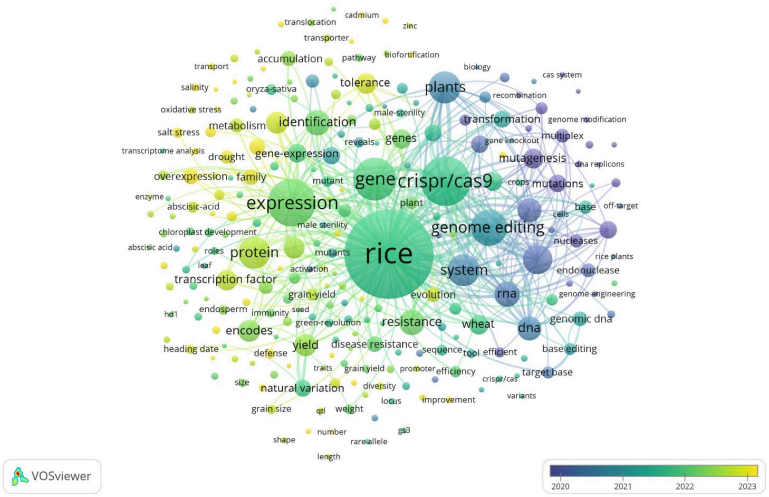

Figure 1. Keyword co-occurrence map of CRISPR/Cas9 technology in rice genetic improvement (2020-2023)

3. Mechanism and optimization of CRISPR/Cas9 technology in rice

The CRISPR/Cas9 system mainly recognizes the target sequence through guide RNA (sgRNA), and under the mediation of the protospacer adjacent motif (PAM), the Cas9 nuclease induces DNA double-strand breaks. Cells mainly achieve gene knockout through non-homologous end joining repair, and can also achieve gene knock-in or base substitution through homologous directed repair. This technology has now been widely applied to rice genetic improvement, and optimization strategies around vector construction, transformation methods and off-target detection are also making continuous progress.

The efficient application of the CRISPR/Cas9 system in rice is inseparable from the continuous optimization of the vector construction system, and the core of vector construction is the efficient expression and delivery of Cas9 protein and sgRNA. At present, the commonly used vector strategies in rice are mainly divided into two types: single vector and binary vector, each with its own advantages, which can be flexibly selected according to breeding needs [1]. The single vector (also known as the mono-vector) integrates the Cas9 protein expression cassette and the sgRNA expression cassette into the same plasmid, and introduces them into rice cells at one time through Agrobacterium-mediated transformation or biolistic method to complete the delivery of the editing system. This method is simple to operate and takes a short time, and does not need to establish rice lines that stably express Cas9. It is the most commonly used vector strategy in current rice genetic improvement, especially suitable for single gene editing and rapid creation of mutants. The binary vector constructs the Cas9 protein expression cassette and the sgRNA expression cassette on two different vectors respectively. It is necessary to first obtain a rice transgenic line that stably expresses Cas9 protein through genetic transformation, and then introduce the vector carrying sgRNA into this line. The advantage of this method is that the design is flexible, and different target genes can be edited by replacing the sgRNA vector, and the continuous expression of Cas9 protein helps to improve the editing efficiency, but the breeding cycle is relatively long, and it is more suitable for multi-gene editing and long-term genetic improvement research. In recent years, the research focus of vector optimization has been concentrated on three aspects: first, improving editing efficiency, by optimizing the codon preference of Cas9 protein to make it more in line with

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the codon usage habit of rice and enhance the expression level of Cas9 protein; at the same time, optimizing the backbone structure of sgRNA to improve its binding efficiency with Cas9 protein and target recognition ability, and reduce off-target risk. Second, simplifying the screening process, developing a visual screening system, coupling fluorescent protein genes (such as GFP, RFP) or color indicator genes (such as β-glucosidase gene GUS) with CRISPR editing elements, and successfully edited rice individuals can be directly identified by fluorescence or phenotypic color without molecular detection, which significantly reduces the screening cost and workload. Third, enhancing the safety of editing, constructing a vector system carrying a "suicide switch", which can efficiently remove the exogenous transgenic fragments in the vector through specific induction conditions or progeny segregation after editing is completed, avoiding the residual of exogenous genes in the rice genome, which not only reduces the off-target effect, but also improves the biosafety of gene-edited rice [8]. In addition, important progress has been made in the research and development of multi-gene editing vectors. By connecting multiple sgRNA expression cassettes in series, simultaneous editing of multiple target genes in rice can be achieved, providing an efficient tool for improving complex agronomic traits such as yield, quality and stress resistance, and further expanding the application scope of CRISPR/Cas9 technology in rice genetic improvement.

With its precise gene targeting modification ability, CRISPR/Cas9 technology is not only the core tool for the directional improvement of rice agronomic traits, but also an efficient research means for analyzing the key molecular regulatory pathways of rice growth and development, environmental response and quality formation, and mining unknown functional genes. It has realized the closed loop of "gene editing-mechanism analysis-trait improvement" and provided a solid support for rice whole-genome design breeding. Compared with traditional methods such as map-based cloning and reverse genetics screening, CRISPR/Cas9 technology can quickly clarify the upstream and downstream relationships and biological functions of genes in regulatory pathways by directional knockout and site-specific modification of target genes, greatly shortening the analysis cycle of rice molecular regulatory networks, and at the same time providing new target resources for rice genetic improvement, breaking the inefficient mode of "phenotypic screening-gene mapping" in traditional breeding. In the field of plant hormone signaling pathway analysis, the team led by Duan Zhenchun from Huazhong Agricultural University used CRISPR/Cas9 technology to directionally knockout CRL1, the core gene of rice lateral root development, combined with transcriptome sequencing, yeast two-hybrid and other genetic interaction verification methods, systematically analyzed the auxin signal transduction pathway regulated by the OsIAA10-OsARF5/21-CRL1 molecular module in rice callus induction, and clarified the upstream and downstream regulatory relationships of key genes in this pathway, which not only enriched the molecular mechanism of rice auxin signal transduction, but also provided important theoretical support for optimizing the rice genetic transformation system and improving the callus induction efficiency of difficult-to-transform varieties [9]; in the analysis of the molecular mechanism of rice reproductive development and heterosis fixation, the team led by Wang Kejian, including Hu Fengyue and Liu Chaolei from China National Rice Research Institute, constructed a targeted knockout vector using CRISPR/Cas9 technology to directionally mutate the endogenous OsPLDα2 gene of rice, systematically verified the feasibility of this gene for haploid induction in hybrid rice, and at the same time, combined with the simultaneous editing of three key meiosis genes PAIR1, REC8 and OSD1, successfully established an apomixis system with no significant difference in seed setting rate from the wild type, clearly analyzed the core molecular pathways of rice gametophyte development and haploid induction, and provided brand-new gene resources and technical paths for the permanent fixation of heterosis in hybrid rice [10]; in the field of analyzing the molecular mechanism of broad-spectrum

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disease resistance in rice, rice blast is the primary fungal disease restricting the safe production of rice worldwide, which seriously affects the yield and quality of rice. The team led by Ning Yuese from Chinese Academy of Agricultural Sciences used CRISPR/Cas9 technology to directionally knockout OsERF922, a rice blast susceptibility gene, combined with genetic interaction verification and transcriptional regulation analysis, clarified that OsERF922 inhibits the broad-spectrum resistance of rice to rice blast fungus by negatively regulating the salicylic acid and jasmonic acid-mediated disease resistance signaling pathways, further improved the molecular mechanism of rice innate immune network, and provided key target resources for rice broad-spectrum disease resistance molecular breeding [11]. In addition, this technology has also been widely applied to the analysis of molecular mechanisms of important agronomic traits such as rice photosynthetic efficiency, nutrient absorption and heading date. By precisely editing key genes and clarifying their biological functions, it provides rich target selection and theoretical basis for rice genetic improvement. These studies fully confirm that CRISPR/Cas9 technology has broken through the technical barrier between rice molecular mechanism analysis and directional genetic improvement, provided a whole-chain solution from target mining to trait creation for rice whole-genome design breeding, and promoted the transformation of rice genetic improvement from "experience-based" to "precision-based".

4. Application of CRISPR/Cas9 technology in the improvement of important rice traits

Rice is not only the staple food of nearly half of the world's population, but also a model plant for gramineous crops. Compared with wheat, rice has a simpler genome and a more efficient tissue culture and genetic transformation system [8]. Nowadays, CRISPR/Cas9 gene editing technology has been widely applied to rice genetic improvement and plays an important role in the improvement of multiple important traits.

4.1. Improvement of yield and quality-related traits

Rice yield is usually affected by multiple indicators such as the number of effective panicles, the total number of grains per panicle and the 1000-grain weight, while the appearance, processing and nutritional quality are the core evaluation indicators of commercial rice. With the advantages of precise editing and efficient creation of new alleles, CRISPR/Cas9 technology has become a key means to synergistically improve rice yield and quality, and has made a series of breakthroughs in multiple directions such as grain weight regulation, eating quality improvement and nutrition fortification.

In terms of grain weight and yield improvement, TGW6 is a core gene regulating 1000-grain weight, which encodes indole-3-acetic acid-glucose hydrolase. Once the function of this gene is lost, it can increase the number of cells and grain weight by increasing the auxin level in endosperm, thereby improving rice yield. Wang Jiafeng et al. used CRISPR-Cas9 technology to target the exons of rice TGW6 gene, and the 1000-grain weight of the obtained mutant lines increased by 5% [12], which also confirmed the high efficiency of this technology in high-yield breeding. In addition, the editing of grain type genes such as GW2 and GS3 can simultaneously optimize grain width and grain length, and combined with the multi-target co-editing strategy, the number of grains and grain weight can be increased simultaneously in a single rice plant, providing a feasible technical path for "synergistic improvement of high yield". In addition, Wang Haiming et al. from Hunan Agricultural University also proved that knocking out the rice NRR gene can promote the development of rice roots and enhance the absorption capacity of rice for nutrients [13]; at the same time, CRISPR/Cas9

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gene editing technology can directionally create mutants, which can be used to efficiently screen and detect rice panicle development genes and regulate the development of rice panicle type [12].

In the field of quality improvement, OsBadh2 is a key gene in aromatic rice breeding. The loss of function of this gene will lead to the accumulation of 2-acetyl-1-pyrroline (2-AP), which is the main source of rice aroma. Researchers knocked out the OsBadh2 gene in different genetic backgrounds and obtained aromatic rice materials with significantly increased 2-AP content, and the agronomic traits of these aromatic rice materials showed no obvious defects, truly realizing the rapid and precise breeding of high-quality aromatic rice [14]. Amylose content determines the cooking and eating quality of rice. The Wx gene encodes granule-bound starch synthase. Huang Lichun et al. edited the C-terminal exons of the Wx gene in japonica rice "Nipponbare" and obtained 8 homozygous mutants without transgenic tags. The amylose content of these mutants was significantly reduced, and the eating quality was also improved simultaneously [4].

In terms of nutrition fortification, relevant research mainly focuses on the enrichment of trace elements such as iron and zinc and resistant starch. By editing the OsNAS family genes, the transport and accumulation of iron ions can be promoted, thereby significantly increasing the iron content in rice; at the same time, editing the OsSSIIIa gene can reduce the degradation of resistant starch, which has a certain improvement effect on human intestinal health. The team from Guangxi Academy of Agricultural Sciences also achieved synergistic improvement of 1000-grain weight and nutritional quality by editing new genes such as OsAP2σ, providing new targets for functional rice breeding. At present, CRISPR/Cas9 technology has been successfully applied to the editing of multiple rice yield and quality-related genes. The editing effects and application values of different genes are shown in Table 1.

Table 1. CRISPR/Cas9-mediated gene editing of yield- and quality-related genes in rice

Target Gene	Gene Function	Editing Method	Editing Effect
TGW6	Regulates auxin in rice endosperm and affects 1000-grain weight	Targeted editing of exons to achieve loss-of-function mutation	1000-grain weight increased by 5%, yield improved, no abnormal agronomic traits
NRR	Regulates the growth and development of rice roots	Gene knockout, loss of gene function	Accelerated root growth, enhanced nutrient absorption capacity
OsBadh2	Degrades the precursor of rice aroma substances	Gene knockout, promotes the accumulation of aroma substances	Increased 2-AP content, rice aroma enhanced, no defects in agronomic traits
Wx	Regulates amylose synthesis in rice	Edited C-terminal exons to obtain transgene-free homozygous mutants	Reduced amylose content, improved eating quality
OsAP2σ	Regulates 1000-grain weight and nutrient metabolism	Precise modification of functional regions to regulate gene expression	Increased 1000-grain weight, improved nutritional components, achieved synergistic improvement

In conclusion, CRISPR/Cas9 technology can simultaneously break through the genetic bottlenecks of rice yield and quality through single-gene precise editing, multi-gene co-modification and site-specific transformation, and also provides an efficient and convenient technical tool for breeding new rice varieties with "high yield, high quality and multiple resistances".

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4.2. Improvement of stress resistance

In the process of rice production, improving the stress resistance of crops is another important research direction and focus. Specifically, it can be divided into two aspects: biotic stress (such as rice blast, bacterial blight, etc.) and abiotic stress (such as drought, salt damage, high temperature, etc.). Traditional breeding technology has a long cycle and insufficient specificity, while CRISPR/Cas9 technology, with its advantages of precise targeting and efficient creation of resistant materials, has become the core technology of stress resistance breeding, and has made breakthrough progress in disease resistance, abiotic stress tolerance and other directions.

In terms of biotic stress resistance improvement, CRISPR/Cas9 technology has achieved broad-spectrum resistance to a variety of rice diseases by editing susceptibility genes. The team led by Ning Yuese from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, used CRISPR/Cas9 technology to simultaneously knockout the susceptibility factors Pi21, Bsrd1 and Xa5 in breeding materials carrying the disease resistance gene Piz-t, and successfully created the BSR line. This material showed significantly enhanced broad-spectrum resistance to both rice blast and bacterial blight, and the main agronomic traits were not negatively affected, realizing the resistance complementary strategy of disease resistance genes and susceptibility factors [15]. He Ziwen et al. from Sichuan Agricultural University used CRISPR/Cas9 technology to edit rice YH208, and the obtained mutants could produce lesion mimics and enhanced resistance to rice blast [16]. Relevant studies have shown that the CRISPR/Cas9-mediated susceptibility gene knockout strategy has significant control effects on bacterial diseases (such as bacterial blight, bacterial leaf streak), fungal diseases (such as rice blast, sheath blight) and viral diseases, and in most cases, will not affect the important agronomic traits of rice.

In terms of abiotic stress tolerance improvement, high temperature stress has become a key factor restricting rice yield and quality, and the CRISPR/Cas9 system has also been widely used in the research of analyzing the functions of heat stress-related genes. The heat tolerance of rice can be enhanced by knocking out heat-sensitive genes or regulating the target genes of heat shock transcription factors. Studies have found that under heat stress conditions, the accumulation of reactive oxygen species will cause oxidative damage to rice plants, while the antioxidant enzyme system (such as APX, CAT, SOD, etc.), osmotic adjustment substances (such as proline, soluble sugar, etc.) and heat shock proteins together constitute the heat tolerance mechanism of plants; by editing related regulatory genes, the redox homeostasis of rice plants can be maintained and the membrane stability can be enhanced, thereby alleviating the adverse effects of high temperature on pollen viability, grain filling and grain quality. In addition, CRISPR/Cas9 technology has also made certain progress in the improvement of rice drought and salt tolerance. By regulating the expression of abscisic acid signaling pathway genes, ion transporters and root development-related genes, the survival ability and yield stability of rice under water deficit and saline-alkali stress can be improved. It can be seen that using CRISPR/Cas9 technology to edit rice genes is an effective method to improve rice yield and stress resistance.

4.3. Apomixis

The utilization of heterosis is the core of hybrid rice yield increase, but due to the segregation of traits in the process of sexual reproduction, hybrid offspring cannot stably maintain excellent traits, so farmers need to repurchase seeds and carry out seed production every year, which is relatively high in cost. Apomixis is an asexual reproduction method in which plants produce clonal seeds without the fusion of male and female gametes. Introducing this reproductive method into hybrid

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rice can fix heterosis and realize "one-line method" seed production, which is known as the ultimate goal of hybrid rice breeding. The emergence of CRISPR/Cas9 technology has made this goal possible.

In 2019, the team led by Wang Kejian from China National Rice Research Institute used CRISPR/Cas9 technology to simultaneously knockout three meiosis-related genes PAIR1, REC8 and OSD1 in the indica-japonica hybrid rice "Chunyou 84", and successfully constructed the MiMe material. This material can replace meiosis with mitosis in the gametogenesis process, thereby producing diploid gametes with the same genotype as somatic cells; subsequently, the team further knocked out the haploid induction gene MTL and created the Fix material that can produce clonal seeds, establishing an apomixis system in hybrid rice for the first time [17]. Since then, the team has continuously optimized this system, and the Fix2 system constructed by introducing the parthenogenesis induction gene BBM4 has increased the seed setting rate to more than 80%. In 2025, the team identified a new haploid induction gene OsPLDα2, and established the Fix4 system by simultaneously mutating the MiMe gene and this gene in hybrid rice. The seed setting rate of this system is completely normal compared with the wild type, and it only relies on gene editing to knockout endogenous genes without the transformation of exogenous elements; in the same year, the team also excavated the haploid induction gene OspPLAIIκ and constructed the Fix7 system, further enriching the gene resources for fixing heterosis [18].

The above series of breakthroughs mark that apomictic rice has moved from conceptual verification to a substantive stage of production application. With the continuous improvement of the precision of CRISPR/Cas9 technology and its derivative tools (such as base editors, prime editors), it is expected to carry out precise modification on more key regulatory nodes of meiosis and embryogenesis in the future, and further optimize the seed setting rate and genetic stability of clonal seeds. It can be seen that CRISPR/Cas9 technology is not only a powerful tool for analyzing the mechanism of rice reproductive development at present, but also the core driving force for promoting the reform of hybrid rice seed production technology and realizing the permanent fixation of heterosis. Its application prospect in rice genetic improvement will become broader with the increasing maturity of the apomixis system.

5. Existing technical problems and challenges

5.1. Off-target effects and gene dependence

Although CRISPR/Cas9 technology has achieved remarkable results in rice genetic improvement, its large-scale application still faces many limitations. Among them, off-target effects and gene dependence are still the two core bottlenecks restricting its precision and application scope, and also bring non-negligible challenges to the precise creation and stable inheritance of target traits.

Off-target effect is one of the most concerned safety issues of CRISPR/Cas9 technology, and its influence cannot be ignored when editing plant genes. The so-called off-target effect refers to that the CRISPR/Cas9 system will also cut non-target fragments with high homology to the target site in addition to normally cutting the DNA double-strand at the target site, thereby causing mutations in non-target genes [19]. Since the recognition of sgRNA and target sequence allows a certain degree of base mismatch, when the similarity between non-target sites and sgRNA sequence is high, Cas9 nuclease may cause cleavage at unexpected positions in the genome, leading to uncontrollable genetic variation. Although plant genomes have relatively high tolerance to mutations, off-target effects will not only interfere with the accurate analysis of the function of target genes, but also introduce uncontrollable background mutations into the rice genome. These unexpected genetic

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variations are often difficult to be completely eliminated by conventional field phenotypic screening in the breeding process, which may not only affect key agronomic traits such as yield and quality, but also produce unpredictable phenotypic defects in the genetic segregation of offspring, which increases the breeding screening burden and safety evaluation difficulty.

5.2. Heterogeneity of editing efficiency

The editing blind area caused by gene dependence is another major technical challenge faced by CRISPR/Cas9 technology. This dependence is mainly reflected in two aspects: first, the editability of gene loci itself is different. The heterogeneity of different target genes in chromatin conformation, GC content and epigenetic modification status directly leads to drastic fluctuations in editing efficiency. Some key agronomic trait regulatory genes are often faced with extremely low editing efficiency or even complete failure due to being in highly condensed heterochromatin regions or lacking appropriate PAM sequences, forming the so-called "editing blind area", which limits the full-coverage application of this technology in the improvement of complex regulatory network traits. Second, the problem of lethal editing of essential genes. There are a large number of core genes regulating growth and development, cell cycle and stress response in the rice genome. When using the conventional CRISPR/Cas9 system to knockout these essential genes, due to the continuous expression and cleavage activity of Cas9 protein, homozygous mutants with complete loss of function are easily produced in the T0 generation, which directly leads to plant death or sterility, making it difficult for researchers to obtain mutant materials that can be stably inherited, which seriously restricts the functional mining and genetic operation space of key nodes in important biological processes of rice.

5.3. Main challenges and countermeasures for the application of CRISPR/Cas9 technology in rice

In view of the core problems existing in the current application of CRISPR/Cas9 technology in rice genetic improvement, combined with the existing research progress, the main challenges and corresponding countermeasures are shown in Table 2 below.

Table 2. Major challenges and countermeasures for CRISPR/Cas9 technology application in rice

Core Challenge	Specific Performance	Countermeasure Direction
Off-target Effects	Non-specific cleavage caused by homology between sgRNA and non-target sites, producing unexpected genetic variation; the variation is difficult to be completely eliminated by field phenotypic screening, which may affect the core agronomic traits of rice and increase the difficulty of breeding screening and safety evaluation	Develop high-fidelity Cas9 variants to reduce non-specific binding; optimize sgRNA design to reduce homology; strengthen off-target detection through whole-genome sequencing and screen off-target-free editing materials
Heterogeneity of Editing Efficiency	The editing efficiency of different target genes varies significantly due to chromatin conformation, GC content, epigenetic modification and PAM sequence distribution; some key regulatory genes have "editing blind areas" with extremely low or even ineffective editing efficiency, limiting the full-coverage application of the technology	Optimize sgRNA backbone structure to improve binding efficiency and targeting; mine novel CRISPR nucleases to expand the applicable range of PAM; develop efficient delivery systems to overcome the "editing blind areas" of difficult-to-edit genes

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Table 2. (continued)

Lethal Editing of Essential Genes	When the conventional CRISPR/Cas9 system knocks out essential genes such as rice growth and development, the continuous expression of Cas9 will lead to the death/sterility of T0 generation homozygous mutant plants, and no stably inheritable mutant materials can be obtained	Adopt conditional editing systems, regulate the expression timing of Cas9 through inducible promoters to avoid complete gene deletion in key growth stages; realize fine-tuning of gene function through base editing to obtain stably inheritable materials

The future optimization of CRISPR/Cas9 technology will focus on three directions: "higher precision, higher efficiency and wider coverage". In terms of improving editing precision, the development and application of high-fidelity Cas9 variants will continue to reduce off-target risks. The fine regulation of the editing window of base editors and prime editors, as well as the improvement of deaminase performance, will further enhance the reliability of precise modification at the nucleotide level. In terms of breaking through the efficiency bottleneck, by optimizing sgRNA backbone design, mining novel CRISPR-related nucleases, and developing species-genotype-independent delivery systems, it is expected to significantly improve the efficiency of HDR-mediated knock-in and overcome the traditional "editing blind areas". In addition, the innovation of multiplex editing technology will enable simultaneous modification of dozens of targets, which also provides strong technical support for the directional remodeling of complex quantitative traits and de novo domestication of crops. CRISPR/Cas9 technology has a very wide range of applications. Through in-depth integration with synthetic biology, genomic selection and phenomics, a new paradigm of intelligent design breeding for rice can be designed. On the one hand, in response to high temperature, drought and saline-alkali stress caused by intensified climate change, by editing key hub genes regulating stress perception and signal transduction, the environmental adaptation network of rice can be systematically reshaped, and the breeding of "climate-smart" rice varieties can be accelerated. On the other hand, the continuous iteration and optimization of apomixis technology is expected to completely revolutionize the hybrid rice seed production system. By fixing heterosis to realize "one-line method" seed production, the production cost of hybrid rice can be greatly reduced, and the safety of seed use can also be guaranteed. Furthermore, combined with the excellent allelic variations discovered by genome-wide association analysis, gene editing technology can be used to precisely modify the key domestication genes in wild rice or local varieties, realize the rapid improvement and de novo domestication of germplasm resources, and reserve rich genetic resources for coping with future food security challenges.

6. Conclusion

As the third-generation gene editing technology, CRISPR/Cas9 technology has a very broad application prospect with its high efficiency and extremely low cost. It is undoubtedly a major discovery in the field of life sciences and has very important research value in multiple aspects such as rice quality improvement and stress resistance improvement. Through site-specific editing and knockout of unfavorable genes in crops, the plant genome can be precisely transformed, thereby breeding new rice varieties with high quality, high yield and multiple resistances, effectively alleviating the contradiction between food supply and demand and ensuring food security. Although the technology still has problems such as off-target effects and heterogeneity of editing efficiency in rice genetic improvement at present, with the continuous optimization and innovation of the technology and the in-depth integration with other disciplines, its application in rice genetic

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improvement will be more extensive and in-depth, and it will surely inject new vitality into the development of rice breeding.

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