Research Progress of CRISPR/Cas9 Application in Rice Cultivation

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Abstract. Rice, being one of the world's most important staple crops, plays an important role in global food security and sustainable agriculture. The CRISPR/Cas9 gene editing technology has attracted widespread attention globally. With its precision and simplicity, rice breeding and research now heavily rely on the CRISPR gene editing method. The rice genome has been fully sequenced and intensively studied. Genome editing in rice holds great potential for variety improvement and agricultural production. In recent years, many studies have reported successful cases of gene knockout, point mutations, and targeted genome insertions using CRISPR-mediated genetic improvement strategies in rice. Using CRISPR gene editing technology to introduce new beneficial traits in rice, such as increased quality and nutritional value, disease resistance, stress tolerance, and greater control of rice growth and development has shown great potential. However, despite the broad prospects of CRISPR technology in rice, there are still many challenges and technical limitations to overcome, and issues regarding precision and safety remain unresolved. This review intended to provide an overview of the CRISPR technology's principles, methods, and researched progress in rice, as well as to analyze its possible benefits and difficulties in rice breeding while also providing information on its potential future applications.

Keywords: CRISPR/Cas9; rice; genetic improvement; gene editing.

1. Introduction

Rice, as one of the most staple crops globally, takes significant part in global food security and sustainable agriculture. Since China is the country which with the largest demand for rice around the world, so it needs to produce rice in large quantities [1]. Through the efforts of generations of scientists, rice breeding technology has rapidly developed, leading to increased yields. However, with economic development and population growth, countries worldwide have raised higher demands for rice quality and productivity [2]. These new requirements pose new problems and challenges to rice breeding techniques, such as non-biological stresses like drought, salinity, and high temperatures, the uncertainties and long breeding cycles associated with traditional breeding, as well as the issue of rice blast disease. These limitations significantly restrict the improvement of rice yield and quality.

Gene editing is a tool used to precisely edit the genome of organisms [3]. This technique enables accurate editing and alteration of genetic information in organisms to achieve specific purposes. The CRISPR/Cas9 is one of the most widely used technologies in the field of gene editing nowadays, known for its efficiency, flexibility, precision, and low cost [4]. CRISPR/Cas9 was first applied to plant genome editing in 2013 and was recognized as one of the top ten scientific breakthroughs by Science that year [5]. It has provided new avenues for improving rice varieties. By utilizing the CRISPR/Cas9 system, researchers have efficiently and precisely edited the rice genome through gene knockout, targeted mutations, and genome insertions, effectively improving traits such as disease resistance, stress tolerance, yield, and quality. The increasing application of CRISPR/Cas9 technology in rice has made positive contributions to global food security and sustainable agriculture. Against this backdrop, this revie introduced CRISPR/Cas9 technology in rice and summarized the

achievements and challenges encountered. It also discussed the role of CRISPR in the improvement of rice biological characteristics, and explore the possibility of future research through the study of related literature.

2. The principle and working mechanism of CRISPR technology

Currently, CRISPR-Cas9 technology has become one of the most effective, straightforward, affordable, and simple to use technologies in existing gene editing and gene modification [1-3], and is currently the most common gene editing system.

Three stages can be used to comprehend how CRISPR-Cas9 works.

Phase I: CRISPR spacer zone acquisition using highly variable spacers

When a foreign phage or a short piece of plasmid DNA sequence is incorporated into the genome of the host bacterium, the highly variable spacer of CRISPR is created and this is the location where the highly variable spacer is formed. Three steps make up the acquisition of the new interval sequence: The PAM region will be recognized by the proteins to scan the invasive DNA, and the DNA sequence next to the PAM will be used as a potential prototype spacer sequence. After that a new spacer sequence is inserted to the genome's CRISPR sequence after the DNA is repaired, closing the open double-strand gap.

Phase II: CRIPSR locus expression

Pre-crRNA is produced by the transcription of the CRISPR sequence, together with complementary tracrRNA. Through base complementary pairing, pre-crRNA and tracrRNA create double-stranded RNA and come together to form a complex with the Cas9-encoded protein. It will choose the appropriate spacer RNA, cut this "ID card" and then produce a short crRNA. The finished complex, prepared for the subsequent cleavage step, is composed of crRNA, Cas9, and tracrRNA.

Phase Ⅲ: targeted interference

The final complex functions as a precision guided missile that can attack an intruder's DNA. The original spacer sequence that completes the crRNA will be found by scanning the full foreign DNA sequence with this complex. The DNA duplex will now be unraveled, generating an R-Loop, and the complex will be confined to the PAM/protospacer sequence area. The complementary strand will hybridize with the crRNA, leaving the other strand free.

To generate a flat-end product, the Cas9 protein's precise flat-end cleavage site is three nucleotide positions upstream of PAM. The Cas9 protein's HNH domain cuts one complementary DNA strand, whereas the protein's RuvC domain cuts another non-complementary DNA strand. Cas9 finally silences the DNA double-strand break (DSB), which in turn silences foreign DNA expression.

3. Application of CRISPR/Cas9 in rice cultivation

3.1. Enhancement of rice's disease resistance

Rice blast is the major cause of annual rice yield loss [2]. Improving blast resistance of rice is essential for breeding, stable yield and increasing yield. Over 100 blast resistant sites have so far been found, and more than 37 of those have been cloned [9, 10]. Rice blast resistance can be modified in a specific direction by CRISPR/Cas9 technology.

In addition to conventional breeding and marker-assisted selection breeding, genetic engineering breeding is one of the most direct methods for targeted improvement of rice blast [11]. Pib is the first major anti-plague gene to be cloned and Pi21 is the first partial resistance gene to be cloned, located on chromosomes 2 and 4 respectively [12, 13]. Zhao and coworkers discovered the broad-spectrum resistance gene ptr, whose spectral resistance is independent of pi-ta while pi-ta is dependent on ptr. These genes are not equally distributed throughout the chromosomes, and chromosomes 6 and 11 make up the majority of the important blast resistance genes that have been cloned [14]. CRISPR can introduce resistance genes that rice does not have into rice, and breed new resistant plants, which provides the possibility for disease resistance breeding. The five indica rice varieties were genetically

modified by Pan Sujun and colleagues using an agrobacterium-mediated method to introduce broad-spectrum blast resistance genes. The experiment showed that the modified plants had strong blast resistance, and that resistance rose significantly with increased breeding and passage [15].

3.2. Improvement of stress resistance and yield of rice

Using CRISPR/Cas9, genes can be knocked out, introduced and replaced to enable organisms to have specific biological functions. Abiotic stress, including high temperature, salt and drought, has an impact on plant height, yield and quality and reduces the spike rate of rice. Excessive abiotic stress may lead to the death of leaves or even the whole rice plant. The International Rice Research Institute divides the salinity tolerance of rice into six levels according to the degree of growth of rice by measuring the mortality rate of leaves. Saline-alkali stress had significant effects on rice yield (including panicle number, effective panicle number, thousand grain weight and seed setting rate), and rice yield decreased with the increase of saline-alkali stress [4].

Kumar and coworkers edited the DST gene of indica rice cultivars using CRISPR/Cas9 [16]. They used gRNA to create allelic mutations and obtained a homozygous dst mutant with 366bp deletion dstΔ184-305 (this mutant had wide leaves and low stomatal density, which greatly enhanced leaf water retention, resulting in strong tolerance to saline-alkali stress). Meanwhile, to enhance the saline-alkali tolerance of rice, Zhan and others constructed a Cas9-OsRR22-gRNA vector with rice OsRR22 gene as the target and obtained 6 mutant types at the target sites which could be stably inherited [17]. Studies indicated that the seedlings of T2 homozygous mutant showed no significant differences in agronomic traits, however, their salt tolerance is much higher than conventionally bred rice, which consistently demonstrated the mutant rice's survival and passage under saline-alkali stress [18].

Studies have found that there are few genes regulating rice yield under saline-alkali stress, including OsGATA8, SiMYB19, OsPQT3, etc [19]. OsPQT3 negatively regulated the salt tolerance, while OsGATA8 positively regulated the salt tolerance. Rice's ability to tolerate salt can be improved, and overexpression of OsGATA8 can result in a 46% increase in yield, mainly by improving the photosynthetic efficiency, grain length, panicle number and panicle number of rice and by increasing drought resistance. OsGATA8 improves the survival rate and yield of rice in high salinity environments by increasing photosynthetic rate and active oxygen scavenging capacity. In addition, the saline-alkali stress related genes can be knocked out to improve rice yield under saline-alkali conditions. TIAN and others used CRISPR/Cas9 to obtain the OsPQT3 gene knockout mutant ospqt3 [20]. Due to the increased expression levels of OsGPX1, OsAPX1 and OsSOD1 genes, the salt tolerance of the mutant improved and its yield was increased by 30% under saline-alkali stress.

4. Challenges and Limitations of CRISPR/Cas9 Application in Rice:

4.1. Limited availability of PAM sequences

The PAM (Protospacer Adjacent Motif) sequence is a necessary element in the CRISPR/Cas9 system for targeting DNA sequences [21, 22]. Cas9 protein requires the presence of a PAM sequence to recognize and cleave the targeted DNA. Different Cas9 proteins have specific PAM sequence requirements [23]. The reliance on PAM sequences for designing and selecting target sites in CRISPR/Cas9 editing limits the flexibility in choosing knockout sites and makes it difficult to achieve gene editing at arbitrary sites, which is a major limiting factor of CRISPR/Cas9 gene editing technology [24].

4.2. Complexity and size of the rice genome

The rice genome is approximately 466 Mb in length and contains 46,022-55,015 genes [25]. The complexity of the rice genome increases the difficulty of finding suitable target sites and may result in off-target effects and unintended edits in non-target genes when applying the CRISPR/Cas9 system in rice.

4.3. Low efficiency of tissue regeneration

Genome editing in rice is often achieved through the transformation of callus tissue [26]. However, the efficiency of tissue regeneration in rice is relatively low. This necessitates multiple transformation events to obtain edited plants, leading to reduced efficiency and precision during the application of CRISPR/Cas9.

5. Conclusion

Gene editing is a technology capable of making targeted modifications to the target genes. Gene editing technology has evolved into an effective tool for examining and modifying the function of the target genome.

The widespread application of CRISPR/Cas9 gene editing technology in rice has brought significant progress in rice breeding, production and even life sciences. The application of CRISPR/Cas9 gene editing technology to target edit genes related to various traits in rice can achieve targeted improvement of disease resistance, quality, fertility, agronomic traits and abiotic stress in rice, thus enabling rice to possess stable and more excellent traits, and providing higher quality conditions for rice breeding and production.

However, the off-target rate and specificity issues of CRISPR/Cas9 gene editing technology still need our attention and more in-depth research. In this review, we believe that the optimisation of sgRNA, PAM site modification and crRNA can further improve the editing range and specificity of CRISPR/Cas9 gene editing, as well as reduce the off-target rate and greatly increase the efficiency of the technology.

We believe that as the above problems are continuously solved, CRISPR/Cas9 gene editing technology will continue to progress and improve, and the technology will have a sustained and extensive application in rice breeding and trait improvement, and the efficiency of rice breeding will be greatly improved and more excellent traits will be obtained. The application of CRISPR/Cas9 gene editing technology in rice will have a brighter future.

Authors contributions

All the authors contributed equally and their names were listed in alphabetical order.

References

[1] Cheng, S. H. (2021). Development and prospect of 100 years of rice breeding in China. China Rice, 27(04), 1-6.

[2] Li, W. L., Luan, X., Zhang, Q., Yu, N., Feng, X. M., & Liu, Z. X. (2022). Research progress on targeted improvement of rice based on CRISPR/Cas9 gene editing technology. Guangdong Agricultural Sciences, 49(09), 114-124.

[3] Li, J., Zhang, Y., Chen, K. L., Shan, Q. W., Wang, Y. P., Liang, Z., ... & Gao, C. X. (2013). CRISPR/Cas systems: RNA-targeting gene editing technology. Hereditas, 35(11), 1265-1273.

[4] Leng, C. X., Yan, P., Xu, Z. H., Sun, Z. Y., Zhang, S. L., Wu, H. T., & Wu, L. C. (2022). Application of CRISPR/Cas gene editing system in salt-alkali tolerance research of rice. Heilongjiang Agricultural Sciences, (08), 52-56.

[5] Liu, J. J., Xiao, N., Wu, Y. Y., Cai, Y., Pan, C. H., Shi, W., ... & Zhang, X. X. (2023). Research progress of CRISPR/Cas gene editing system in rice. Jiangsu Agricultural Sciences, 51(11), 1-10.

[6] Mussolino, C, Cattomen T. RNA-guided genome engineering [J]. Nature Biotechnology, 2013, 31(3): 208-209

[7] Yaoguang Liu, Zhisi Li, Yaling Zhang, et al.Research progress of CRISPR/Cas plant genome editing technology[J].Journal of South China Agricultural University,2019,40(5):38-49.) LIU Y G, LI G S,

ZHANG Y L,et al.Research Progress of Plant CRISPR/Cas Genome Editing Technology[J]. Journal of South China Agricultural University,2019,40(5):38-49.

[8] GUPTA R M, MUSUNURU K. Extended Gene Editing Kit: ZFN, TALENs and CRISPR-Cas9[J]. Journal of Clinical Research, 2014, 124(10): 4154-4161

[9] SKAMNIOTI P, GUURR S J. Against the grain: safeguarding rice from rice blast disease [J]. Trends in Biotechnology. 2009.27 (3):141-150. The DOl:10.1016 / j. t. ibtech. 2008.12.002.

[10] YANG Dewei, WANG Mo, HAN Libo, et al. Progress of cloning and breeding application of blast resistance genes in rice and avirulence genes in blast fungi[J]. Chin Bull Bot,2019,54(2):265–276.

[11] HAN Xueqin, Shen Wenjuan, ZHANG Zhenhai, Tian Lei, LUO Chengke, Yang Shuqin, LI Peifu, ZHANG Yinxia. Research progress of blast resistance gene in rice resistance breeding [J]. Xinjiang Agricultural Sciences, 2019,58(03):483-492.

[12] Wang Z, Yano M, Yomanouchi U. The Pib gene for rice blast resistance belongs to the nucleotide binding and leucine rich repeat class of plant resistance genes[J]. Plant Journal,1999,19:55-64.

[13] Fukuoka S,Saka N,Koga H. Loss of function of a prolinecontaining protein confers durable disease resistance inrice[J].Science,2009,325:998-1001

[14] PAN Sujun. Studies on application and evolution of the broadspectrum blast resistance gene pi9 in rice[D]. Changsha: Hunan Agricultural University 2006.

[15] Yang Yakun, Zhao Fei, Liu Jian, et al. Research progress on effects of salt and alkali stress on rice and its related mechanisms [J]. Molecular Plant Breeding, 2021:1-17 [2022-05-20].

[16] KUMAR V V S, VERMA R K, YADAV S K, et al. CRISPR-Cas9mediated genome editing of drought and salt tolerance (ODST)gene in indica mega rice cultivar MTU1010[J].Physiology and Molecular Biology of Plants,2020,26(6):1099-1110.

[17] ZHANG A, LIU Y, WANG F, et al. Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the OSRR22 gene[J]. Molecular Breeding, 2019,39:47.

[18] Duan Junzhi, Yang Cuiping, Wang Nan et al. Research progress of improving rice yield under abiotic stress by genetic engineering technology[J]. China Rice, 2023, 29(03):15-23.

[19] TIAN Q X, SHEN L K, LUAN J X, et al. Rice shaker potassium channel OsAKT2 positively regulates salt tolerance and grain yield by mediating K+ redistribution[J]. Plant, Cell&Environment, 2021,44(9):2 951 - 2 965.

[20] Ahmad, S., & Hasanuzzaman, M. (2012). Integrated effect of plant density, N rates and irrigation regimes on the biomass production, N content, PAR use efficiencies and water productivity of rice under irrigated semiarid environment. Not Bot Horti Agrobot Cluj Napoca, 40(1), 201-211.

[21] Ahmad, S., Wei, X., Sheng, Z., Hu, P., & Tang, S. (2020). CRISPR/Cas9 for development of disease resistance in plants: recent progress, limitations and future prospects. Brief Funct Genomics, 19, 26-39.

[22] Chatterjee, P., Jakimo, N., Lee, J., et al. (2020). An engineered ScCas9 with broad PAM range and high specificity and activity. Nat Biotechnol, 38(10), 1154.

[23] Li, W., Luan, X., Zhang, Q., Yu, N., Feng, X., & Liu, Z. (2022). Research progress of rice targeted improvement based on CRISPR/Cas9 gene editing technology. Guangdong Agricultural Sciences, 49(9), 114-124.

[24] Cong, L., Li, W., & Yang, H. (2002). Introduction to the study of rice (Oryza sativa L.) whole genome framework sequence. Bulletin of Botanical Research, (3), 380-381.

[25] Ding, L., Li, S., Gao, J., Wang, Y., Yang, G., & He, G. (2009). Optimization of Agrobacterium-mediated transformation conditions in mature embryos of elite wheat. Molecular Biology Reports, 36, 29-36.

[26] Ali, J., Nicolas, K. L. C., Akther, S., Torabi, A., Ebadi, A. A., Marfori-Nazarea, C. M., & Mahender, A. (2021). Improved anther culture media for enhanced callus formation and plant regeneration in rice (Oryza sativa L.). Plants, 10, 839.