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Multiplex CRISPR-Cas9 Editing of Yield-related Genes in Rice

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Abstract Rice yield is a critical determinant of global food security, yet it remains constrained by complex genetic and environmental factors. With the emergence of CRISPR-Cas9 as a powerful genome-editing tool, its application in rice improvement-especially via multiplex gene editing-has gained significant momentum. This study outlines the mechanisms of CRISPR-Cas9 editing in plants, recent advances in multiplex editing strategies, and delivery systems. We focus on key yield-related genes in rice, such as those influencing grain size (e.g., *GS3*), plant architecture (e.g., *DEP1*), and stress tolerance (e.g., *DRO1*), and highlight technological innovations including tRNA-processing systems, base editing, and transgene-free approaches. A case study on the simultaneous editing of *GS3*, *DEP1*, and *DRO1* demonstrates the feasibility and potential of multiplex CRISPR-Cas9 to enhance multiple yield traits concurrently. Despite technical challenges like off-target effects and regulatory barriers, multiplex CRISPR-Cas9 editing presents a promising frontier in precision rice breeding. Future research integrating advanced CRISPR technologies with precision breeding platforms may accelerate the development of high-yield, climate-resilient rice varieties.

Keywords CRISPR-Cas9; Multiplex genome editing; Rice yield; *GS3*; Precision breeding

1 Introduction

Rice yield is very important for global food security. However, there are still many difficulties, such as insufficient genetic diversity, high environmental pressure, and the complexity of yield traits. These problems have been affecting the improvement of rice yield. In recent years, the development of genome editing, especially CRISPR-Cas9 technology, has provided a new and more accurate and efficient way to improve crops (Chen and Zhang, 2024). In the process of rice cultivation, both biotic stress (such as pests and diseases) and abiotic stress (such as drought and high temperature) will affect yield (Lyu, 2024). Moreover, yield traits are usually controlled by multiple genes and quantitative trait loci (QTL), which also makes breeding more difficult. Although traditional breeding has some results, the process is slow and expensive. It takes a long time to get good alleles, and the mutagenesis process also has a certain degree of randomness. In the context of intensified climate change and growing population, we need a fast, accurate and efficient way to breed rice more than ever (Rengasamy et al., 2024; Thiruppathi et al., 2024).

CRISPR-Cas9 is a genome editing tool. It can accurately modify genes related to yield and other agronomic traits. Unlike traditional breeding, this technology can modify multiple genes at the same time, which is called "multiple editing". This can breed new rice varieties with high yield, high quality and strong stress resistance more quickly. Researchers have successfully used it to modify key genes such as *Gn1a*, *DEP1*, *GS3* and *IPA1*, improving rice grain number, panicle shape and grain shape (Li et al., 2016; Zhou et al., 2018; Zeng et al., 2020). Multiple CRISPR-Cas9 editing can also integrate multiple desirable traits in one generation, while helping us better understand complex gene networks (Xie et al., 2015; Shen et al., 2017).

In recent years, multiple CRISPR-Cas9 editing has been increasingly used in improving rice yields, but what actual results have been achieved behind the technology actually requires a systematic review. Therefore, this study focuses on the progress that has been made in this area, especially the technical means and effects of multi-gene editing. There are many studies, some of which are effective but also have problems. We try to sort out several key results and see what they may mean for rice breeding and even food security. Of course, this

technology is far from perfect. There are also many troubles such as off-target effects and regulatory restrictions, so they must also be discussed. As for the future, whether this technology can become an important tool for sustainable improvement, it still needs to be judged in combination with existing challenges and development trends.

2 Overview of CRISPR-Cas9 Technology in Plants

2.1 Basic mechanism of CRISPR-Cas9 editing

This gene editing tool was actually first discovered from the immune mechanism of bacteria. After all, the principle of CRISPR-Cas9 is not complicated, but people who come into contact with it for the first time may still find it a bit mysterious. In plants, its operation process is as follows: researchers first design a molecule called "single guide RNA" (sgRNA), and then this RNA will accurately bring the Cas9 protein to the target position of the DNA. Then, Cas9 cuts the DNA at that point, and the two chains are broken. After that, the plant itself will start the repair mechanism and try to fill the break, which often results in gene mutations. This method is not only efficient, but also not difficult to use, so it is now very popular in breeding and plant research (Belhaj et al., 2015; Bortesi and Fischer, 2015; Liu et al., 2017). Of course, although the principle is simple, some technical accumulation is still required for actual operation.

2.2 Development of multiplex genome editing strategies

Not every edit changes just one gene. Now, researchers often want to "do multiple things at once," or multiple edits. The key to this approach is to design multiple sgRNAs and then have them enter plant cells together with Cas9. In this way, several important traits, such as disease resistance, stress resistance, or yield, can be improved at the same time. In the past, such edits were not easy to do, mainly due to the design of vectors and RNA. Now it is different. These technologies have matured a lot and are easier to operate (Ma et al., 2016; Wada et al., 2020). Interestingly, this way of editing multiple genes at once can not only quickly superimpose ideal traits, but also allow us to more intuitively see how these genes work with each other. Sometimes, the obvious effect can be seen in one generation of plants, which really saves a lot of trouble in breeding new varieties.

2.3 Regulatory and delivery considerations

There are several methods for delivering CRISPR-Cas9-related components into plant cells, such as Agrobacterium-mediated delivery, gene guns, and protoplast transfection. Recently, some people have tried to use ribonucleoprotein complexes or viral vectors for "DNA-free" delivery, which does not require the foreign DNA to remain in the plant and may reduce regulatory issues related to transgenics (Figure 1) (Arora and Narula, 2017; Ma et al., 2020; Son and Park, 2022).

Different countries and regions have different regulations on the management of CRISPR technology. In some places, whether it is transgenic or not will be distinguished based on whether it contains foreign DNA. In order to make CRISPR-Cas9 widely used in crop breeding, not only efficient delivery methods are required, but also clear regulatory rules are needed (Gan and Ling, 2022; Liu et al., 2022; Sahoo et al., 2023).

3 Yield-Related Genes Targeted by CRISPR-Cas9 in Rice

3.1 Grain size and weight genes

CRISPR-Cas9 technology has been used to edit several key genes that affect grain size and weight. Among them, *GS3* is an important gene that controls grain size and is a negative regulator. When this gene mutates, the grains become larger. Another important gene is *Gn1a*, which encodes a cytokinin oxidase/dehydrogenase. By editing *Gn1a*, cytokinins in rice panicle tissue can be increased, thereby increasing the number of grains and total yield (Bhavya et al., 2024). In addition, mutations in some genes in the cytochrome P450 family can also make grains larger and increase the number of cells, further promoting yield increases (Usman et al., 2020). Other studies have found that in the OsGRF4 gene, if the target site of miRNA is destroyed, the grains can also become larger.

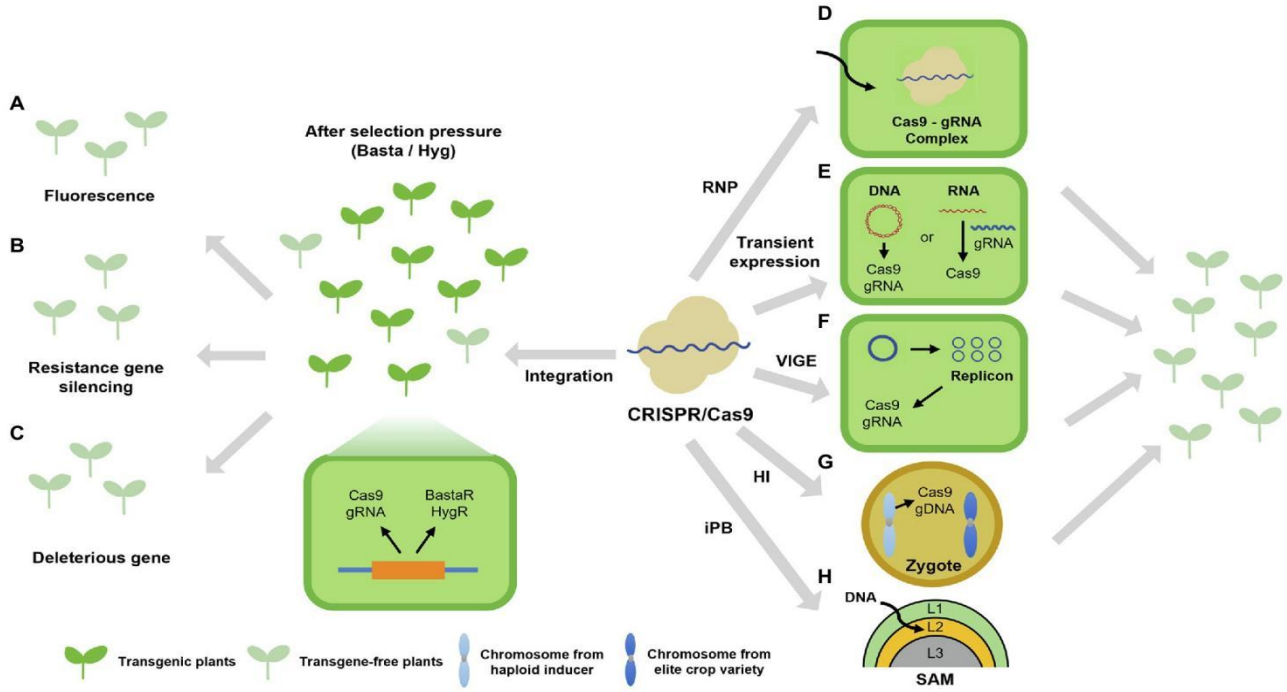

Figure 1 Schematic overview of strategies for the generation and isolation of transgene-free edited plants (Adapted from Son and Park, 2022)

Image caption: (A-C) Strategies for the isolation of transgene-free edited plants. (D-F) Strategies for the generation of edited plants without the stable integration of a transgene. Several CRISPR/Cas9 systems allow gene editing without requiring the stable integration of a transgene. (G) Transgene-free genome editing based on haploid induction. (H) The in planta genome editing based on in planta particle bombardment (iPB) method (Adapted from Son and Park, 2022)

3.2 Plant architecture genes

The appearance of rice is not only determined by leaves and height, but also by the shape of the panicle and the number of tillers, which are closely related to yield. The *DEP1* gene is related to the structure of the panicle. If it is edited with CRISPR, rice will usually grow more compact and upright panicles, and the plant height will be reduced (Wang et al., 2017). Such plants are more resistant to lodging and the harvest is often more stable. However, not all plant type modifications can go smoothly. Genes like *IPA1* have no problem regulating plant type, but their mutation effects are related to the target of OsmiR156, which sometimes increase the number of tillers and sometimes reduce them (Li et al., 2016). It depends on the specific situation. There is also OsFWL4, which is a member of the FW2.2 class of genes. It has a "suppressive" effect on tillering and yield. Studies have found that as long as this gene is knocked out, the number of tillers and yield will often increase (Gao et al., 2020).

3.3 Stress tolerance genes linked to yield

Unexpected events happen all the time, and rice is not always grown in an ideal environment. When saline-alkali land, drought, and adverse conditions come, the yield can easily decline. Therefore, whether the stress resistance is strong or not is also the key. CRISPR-Cas9 was used to knock out a gene called *OsRR22*. It was found that the salt tolerance of rice increased, but other agronomic traits were basically unaffected (Zhang et al., 2019). This kind of "improvement without side effects" editing result is actually rare, which is a bright spot. There is another idea, which is to target certain miRNAs and indirectly regulate functional factors such as OsGRF4 through them, which can also improve stress resistance and increase yield by the way (Yadav et al., 2023; Lin et al., 2021).

4 Advances in Multiplex CRISPR-Cas9 Editing Systems in Rice

4.1 tRNA-processing and Csy4-based systems

If you want to move multiple genes in a rice plant at the same time, you really can't do it without some skills. What researchers use more now is the tRNA processing system that plants have. It is not a new invention, but a "borrowed" set of mechanisms that plants already have - the specific method is to connect multiple tRNA-gRNA

units into a string and put them into the same transcript. Next, the processing mechanism in the plant can automatically cut out these guide RNAs and complete the precise positioning and cutting of multiple genes at one time. In other words, it is much easier to do it one by one, and the efficiency is very high, even close to 100% at high times (Xie et al., 2015). This trick can be used not only on rice, but also on other species, because this processing mechanism itself is more "universal". Of course, in addition to the tRNA system, some people have tried to use Csy4. This method relies on bacterial RNA enzymes to cut RNA arrays, and the principle is not complicated. But in the final analysis, the tRNA method is used more in rice editing, and the reason is very direct - it has a simpler structure, runs more stably, and has a lower probability of error.

4.2 CRISPR-Cas variants and base editing

In addition to the most common Cas9 enzyme, scientists have also modified other CRISPR enzymes, such as Cpf1 (also called Cas12a), for multiple gene editing in rice. These new systems can express multiple crRNAs under the same promoter, which makes vector construction simpler and can edit more different gene sites (Wang et al., 2018). In addition, there are some special Cas9 variants, such as Cas9-VQR, which can recognize some sequences different from conventional PAM, which also expands the scope of editing (Hu et al., 2017). There is also a technology called "base editing", which is a fusion of Cas9 and cytidine deaminase, which can directly change one base to another without causing DNA double-strand breaks. This method can achieve more precise base mutations in rice and other plants (Shimatani et al., 2017).

4.3 Transgene-free and marker-free editing

Genetically modified organisms always sound worrying, especially in places with particularly strict regulations, where even trace amounts of foreign DNA can cause controversy. To solve this problem, researchers have simply bypassed the traditional genetic modification route. Now, they have developed some editing methods that do not involve foreign DNA, such as using RNP complexes or using systems with very short expression times. The advantage of this is that the genes that should be edited are modified, but the foreign substances will not remain in the rice genome for a long time (Arora and Narula, 2017). Of course, it is not enough to just be "DNA-free". In order to really put these materials into use, the problem of screening must be solved. In recent years, high-throughput screening processes have been put online, which can quickly find plants that do not carry genetically modified markers but have multiple desirable traits (Patel-Tupper et al., 2023). Another point is also worth mentioning - the marker-free system. Although it sounds less conspicuous, it is particularly important for subsequent commercialization and regulatory approval. After all, who doesn't want their varieties to look a little "cleaner"?

5 Case Study: Multiplex Editing of *GS3*, *DEP1*, and *DRO1* in Rice

5.1 Study overview and experimental design

Multiple CRISPR-Cas9 technology can edit multiple yield-related genes in rice at the same time. In a typical study, researchers chose the rice variety "Zhonghua 11" as the test subject. They focused on editing *GS3* (controlling grain size), *DEP1* (controlling panicle type) and other important genes related to yield. They used the CRISPR/Cas9 system to mutate these target genes and tested the editing effects in the first generation (T0) of transgenic rice. The results showed that this method had a high editing success rate, with an editing rate of 57.5% for *GS3* and 67.5% for *DEP1*, indicating that this multi-gene editing method is effective in improving complex traits (Li et al., 2016).

5.2 Phenotypic and agronomic outcomes

Some changes can actually be seen at first glance. For example, the grains of plants edited with *GS3* are significantly larger (Figure 2); for another example, the ears of mutants of *DEP1* become more compact, facing upward, and even the height of the plants is shorter. These are not unexpected, but expected performance. More importantly, these traits can still remain stable in the next generation (T2) and have not disappeared due to intergenerational replacement. Of course, in addition to these "on-target" results, the researchers also observed some other phenotypes. Some plants have semi-dwarf stalks, while others have obvious long awns. These

additional performances may not have been planned at first, but later proved to be quite valuable, especially in broadening the diversity of agronomic traits. In general, these mutants have not only improved in ear shape and grain size, but also enhanced yield-related traits. It seems that multiple editing can indeed bring a lot of usable changes in one generation of plants.

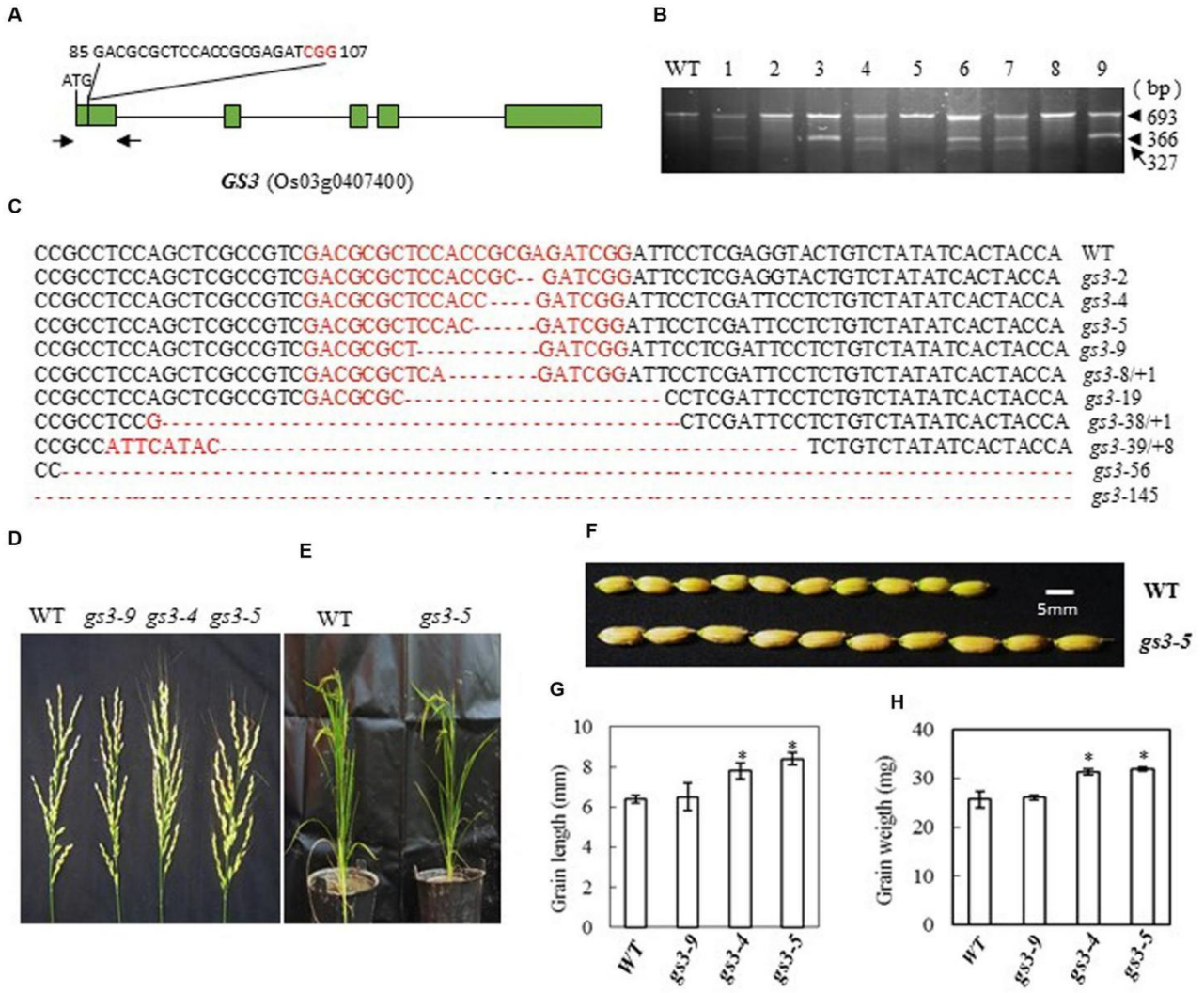

Figure 2 CRISPR/Cas9-induced *gs3* mutant plants and phenotype analysis. (A) Schematic map of the genomic region of *GS3* and the sgRNA target site; arrows show the positions of PCR primers used for mutation detection; The PAM motif (NGG) is shown in red; (B) Gel electrophoresis of PCR products amplified from the mutated region digested with CEL I; WT and 1-9 are DNA samples from wild type and different transgenic plants. Arrows show the expected band sizes after CEL I digestion. (C) Sequence alignment of the sgRNA target region showing altered bases in different mutant lines; (D) Representative pictures showing the morphology of the main panicle; (E) Phenotype of the mutant plants grown in a greenhouse; (F) Comparison of the grain size of mutant *gs3-5* plants with that of WT plants; statistics for the average grain length (G) and average grain weight (H) of representative mutant plants. Data were collected from 10 to 15 plants per mutant line. * indicates a significant difference (P<0.05) in comparison to WT counterparts (Adopted from Li et al., 2016)

5.3 Implications for breeding and scalability

This study shows that through multiple CRISPR editing, multiple important yield-related traits can be improved in one variety at the same time, making the breeding process simpler. Stacking multiple beneficial genes in the same genetic background also makes it easier to figure out the regulatory relationship between genes, which is very helpful for accelerating the breeding of superior varieties. Because these edited traits can be efficiently expressed and stably inherited, this method has great potential in actual rice breeding and is suitable for large-scale promotion and application.

6 Challenges and Limitations of Multiplex CRISPR in Rice

6.1 Off-target effects and editing specificity

When it comes to CRISPR systems, whether it is Cas9 or Cas12a, their accuracy is generally good. But once things get complicated, such as when you want to edit dozens of genes at the same time, trouble may come. It's not to say that there will be problems every time, but whole-genome sequencing of rice shows that once the number of editing sites exceeds 50, some rare but serious conditions may sometimes occur, such as deletion or duplication of certain regions of the chromosome (Zhang et al., 2022). But then again, if you only move a few points, such as less than ten, it is safe most of the time. But to be safe, you still have to check what needs to be checked. In order to improve the accuracy of the test, researchers used technologies such as "multiple ligation-dependent probe amplification". It can not only confirm whether the target site has been actually changed, but also find out whether there is "accidental injury" to other places that should not be moved (Biswas et al., 2020). As for how to reduce these off-target risks, the commonly used method at present is to optimize the design of sgRNA. Some people directly replace it with a high-fidelity Cas variant, which has stricter target recognition and is safer to use (Mishra et al., 2018; Ren et al., 2019). But even so, the more editing is done, the more risks you have to keep an eye on.

6.2 Trait trade-offs and genetic interactions

When multiple genes are modified simultaneously in the same rice plant, some complex trait changes may occur. Some genes may reinforce each other, while others may conflict, making it more difficult to predict the final performance (Zhou et al., 2018). For example, if the genes that control grain size, number, and plant structure are edited at the same time, many different phenotypes may be obtained, and sometimes unexpected situations may occur. Therefore, detailed observation and screening in the field are required to confirm whether these traits are ideal (Shen et al., 2017). In this case, completely knocking out a gene is not necessarily the best option. Sometimes some "fine-tuning" of gene expression is more conducive to maintaining plant yield and adaptability (Zhou et al., 2023).

6.3 Technical and regulatory barriers

Technical challenges include complex vector construction, inconsistent editing efficiency at different gene loci, and the delivery system of editing tools is not ideal enough (Hu et al., 2020). Although there have been significant improvements in tRNA processing systems and simpler vector systems, which have indeed improved the ability of multi-gene editing, there is still a long way to go before it can be widely used in actual breeding (Xie et al., 2015; Xiong et al., 2023). In addition, different countries and regions have different policies on gene-edited crops. In particular, there are still many controversies about whether non-GMO and unmarked plants should be classified as GMOs and whether they need approval (Pan and Qi, 2023). These policy uncertainties are one of the key issues for the successful commercialization of multi-edited rice.

7 Future Perspectives and Research Directions

7.1 Towards next-generation CRISPR technologies

Technology updates are never done in one go, especially in complex operations such as multi-gene editing. CRISPR-Act3.0 is a typical example. It can activate multiple genes at one time, which sounds ideal and is especially suitable for dealing with complex problems involving multiple traits or metabolic pathways (Pan et al., 2021). However, whether this type of "all-round" technology can really be used depends on whether the specific system is compatible. There is also a bottleneck - the limitation of PAM sequence. In the past, the editing range was very limited, but now Cas9 variants like SpRY, which are almost not picky about PAM, are much more flexible. There is also Cpf1 (also called Cas12a), which is also used as an alternative enzyme to Cas9, and more and more people are using it (Li et al., 2018). The emergence of these tools has gradually shifted multi-gene editing from "can it be done" to "how to do it more smoothly". A more impressive point is that some people can now use a vector to target dozens of gene sites for editing at the same time (Wu et al., 2024). What does this mean? Simply put, breeding does not need to be repeated over and over again, and genetic diversity can be increased immediately, saving time and effort. The real challenge is how to select the best batch.

7.2 Integration with precision breeding platforms

Multiple CRISPR editing technology is now gradually being combined with precision breeding platforms. Researchers have developed more efficient systems and simpler vector designs, such as the SSTU (single transcription unit) method, which allows multiple guide RNAs to work simultaneously. This allows for faster combination, transformation, and screening of different trait combinations (Wang et al., 2018). These technologies can help breeders bring together multiple good genes, and also analyze how each gene affects each other, ultimately enabling faster breeding of new rice varieties with high yield, good quality, and strong stress resistance (Shen et al., 2017; Mishra et al., 2018).

7.3 Policy, biosafety, and global adoption

Whether the technology itself can be promoted sometimes depends not only on the effect, but also on whether it can pass the policy test. Especially for tools like multiple CRISPR, once the words "genetic modification" are involved, some countries will be particularly sensitive. Because of this, many research teams now prefer to go the "non-GMO" and "unlabeled" route. This is not because the technology itself is not good, but to bypass the psychological defense line of the public and regulatory authorities (Ren et al., 2019; Pan and Qi, 2023). But even if you don't carry foreign DNA, there is no guarantee that all countries will recognize it. The standards in various places are varied, some are loose, and some are strict, making it difficult to implement in practice. If you really want to make this technology widely implemented, it may not be realistic to rely on one country, one policy. At least there must be a unified general framework across countries. Moreover, supervision is only one step, and it is more difficult to get the public to buy in. Problems such as off-target effects and ecological impacts are not small. Whether it is out of scientific prudence or to increase transparency, follow-up research cannot be stopped. Only when these risk points are clearly explained and managed can CRISPR truly go far and stand firm (Biswas et al., 2020).

8 Concluding Remarks

Multiple CRISPR-Cas9 editing techniques have been shown to simultaneously and precisely modify multiple key yield-related genes, such as *Gn1a*, *DEP1*, *GS3*, and *IPA1*. Editing these genes can effectively increase the number of rice grains, the structure of the panicle, and the size of the grains. With this technique, researchers can quickly obtain plants with one, two, or even three mutations in the target genes. These mutations combined can also produce a stronger yield-enhancing effect, thereby significantly increasing the single panicle yield of excellent rice varieties. If genes related to yield and stress resistance are edited at the same time, higher-yielding and more resistant rice lines can also be bred. These new results simplify the entire breeding process, making it easier to combine good genes in one variety and to analyze how different genes work together.

However, despite the rapid technological progress, some problems still exist. Off-target effects and unexpected mutations are not common, but they do occur. This requires extra caution in the design and use of guide RNAs. In addition, multi-gene editing may lead to conflicts or interactions between traits, and the results may not be easy to predict, so careful screening and verification in the field are required. In addition, the construction process of the editing vector is relatively complicated, and the editing efficiency of different gene sites is different. These technical problems have not been completely solved. At the same time, the regulatory rules of different countries are not unified, and clear and unified biosafety standards are still being formulated, which have affected the promotion of gene-edited rice.

Overall, multiple CRISPR-Cas9 editing technology has great development potential in accelerating rice breeding and increasing grain production. As long as the technology is continuously optimized and can be better combined with the precision breeding platform, coupled with the continuous improvement of non-GMO methods, the application value of this technology will become greater and greater. In the future, if we can further solve the technical, biological and policy problems, multiple gene editing can play an important role in rice breeding more safely and efficiently.

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Conflict of Interest Disclosure

The authors affirm 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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