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# CRISPR Interference (CRISPRi) for Functional Genomics in Rice

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**Abstract** Functional genomics is an important approach to analyzing the gene functions and complex biological processes of rice. CRISPR interference (CRISPRi) technology has become an efficient, reversible and highly specific gene regulatory tool by using Cas9 (dCas9), which lacks cutting activity, to target and bind to DNA with single-guide RNA (sgRNA), thereby inhibiting gene transcription without causing double-strand breaks. This study systematically introduces the principle and composition of the CRISPRi system, including the dCas9 variant, the design strategy of sgRNA and its transcriptional inhibition mechanism. It reviews the latest progress of CRISPRi in the construction and optimization of rice platforms, and focuses on discussing its application in gene silencing, systematic research of gene families, and combined transcriptome and metabolome analysis. Meanwhile, through typical cases such as flowering time regulation, grain quality improvement and stress resistance enhancement, the application value of CRISPRi in the research of important traits of rice was demonstrated. This study also analyzed the advantages of this technology over the traditional CRISPR-Cas9, as well as the current technical bottlenecks (such as off-target effects, differences in silencing efficiency) and possible directions for improvement. This study aims to comprehensively summarize the research strategies of rice functional genomics based on CRISPRi, identify key challenges, and propose future development directions to accelerate molecular breeding and precise trait improvement of rice.

**Keywords** CRISPRi; Rice; Functional genomics; Gene silencing; Molecular breeding

## 1 Introduction

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population. Its genetic improvement is of great significance to global food security. Functional genomics is a systematic approach to studying the functions and interactions of genes, which can identify genes that control important agronomic traits, such as yield, stress resistance and rice quality. Traditional breeding and early genetic tools have driven the improvement of rice, but due to the complexity of the rice genome and the current need for more precise and higher-throughput gene function analysis, more powerful methods are required.

Functional genomics can analyze the gene networks behind key traits, helping to identify candidate genes for targeted breeding more quickly. The emergence of genome editing technology, especially the CRISPR system, has made the gene modification of rice more precise and efficient, and enabled the simultaneous regulation of multiple genes. This has greatly accelerated the improvement in terms of output, quality and stress resistance (Miao et al., 2013; Zegeye et al., 2022).

CRISPR interference (CRISPRi) is an improvement of the CRISPR/Cas9 system. It uses catalytically inactivated Cas9 (dCas9) that fuses with transcriptional repressors. Under the guidance of sgRNA, dCas9 binds to the promoter of the target gene, blocking transcription without cutting DNA. This method can achieve reversible and tunable simultaneous inhibition of multiple genes, so it is very useful in research that requires gene knockdown rather than knockout. Compared with RNA interference and traditional genome editing, CRISPRi has higher specificity, lower off-target rate, and can also target non-coding regulatory regions (Fiaz et al., 2019; Zhang et al., 2025).

This study will review the principles and latest progress of CRISPRi in rice functional genomics, with a focus on its application in analyzing gene functions and regulatory networks. It will also explore its potential in

accelerating rice genetic improvement and the future prospects of integrating CRISPRi into the breeding process. This study aims to provide an expandable, high-precision and low-risk regulatory platform for the research of rice gene functions, promote the development of functional genomics, and offer theoretical and technical support for rice breeding.

## 2 Components and Mechanism of the CRISPRi System

### 2.1 dCas9 and its mutated characteristics (loss of nuclease activity)

The CRISPRi system uses an inactivated Cas9 protein called dCas9. It has undergone a mutation on Cas9, which has deprived it of the cutting function of endonuclease. Unlike normal Cas9, dCas9 can find and bind to target DNA without causing double-strand breaks. So it doesn't modify the DNA sequence but reduces gene expression by blocking transcription. It is precisely because it does not cut DNA that this method can achieve controllable and reversible gene suppression (Ma, 2024).

### 2.2 Design principles of guide RNA (sgRNA) and target site selection

CRISPRi requires the use of single guide RNA (sgRNA) to deliver dCas9 to a specific DNA location. Whether sgRNA is well designed or not directly affects its specificity and efficiency. The PAM sequence and the 3' end seed sequence of sgRNA are important in identifying and binding to target sites (Larson et al., 2013). If there is an incomplete match in the seed region, off-target situations may occur. Therefore, special attention should be paid when designing and validating Sgrnas (Rohatgi et al., 2024). In rice research, computational tools and phylogenetic analysis can be combined to select more suitable target sites and also reduce the interference of gene family redundancy (Hong et al., 2020).

### 2.3 Molecular mechanism of transcriptional blockage and gene expression regulation

When the dCas9-sgRNA complex binds to the DNA target, it physically blocks the advancement of RNA polymerase, thereby preventing the initiation or extension of transcription (Larson et al., 2013; Vercauteren et al., 2024). This method does not modify the DNA sequence, but it can effectively and tunably inhibit gene expression. Because it is modular, it is very suitable for high-throughput research on gene functions and regulatory elements, and is very useful for functional genomics studies of rice and other organisms.

## 3 Construction and Optimization of the Rice CRISPRi System

### 3.1 Vector construction and promoter selection

In rice, the CRISPRi vector typically needs to simultaneously express catalytically inactivated Cas9 (dCas9) and one-way guide RNA (sgRNA). The common approach is to create a binary carrier and assemble these two components together. The selection of the promoter is very important. dCas9 often employs potent constitutive promoters, such as the rice ubiquitin promoter (OsUbi), which ensures its stable expression in various tissues. sgRNA typically uses RNA polymerase III promoters, such as OsU3 or OsU6, because they can efficiently transcrib small Rnas and the starting nucleotides can be set as needed to achieve the best effect of sgRNA. Studies have shown that such promoters can enhance the editing and regulation efficiency of rice (Figure 1) (Butt et al., 2018; Fiaz et al., 2019; Zegeye et al., 2022).

### 3.2 Efficient sgRNA expression and screening

To achieve efficient expression of sgRNA, one can optimize the sgRNA scaffold and select an appropriate promoter. When designing, it is necessary to ensure high targeting activity as much as possible while minimizing off-target activities. This can predict the specificity and efficiency of sgRNA using computational tools (Zegeye et al., 2022). When screening for effective Sgrnas, computer prediction is usually conducted first, followed by experimental verification, such as using rapid detection platforms or observing phenotypic changes to determine the inhibitory effect. There are now some more convenient detection methods, such as instrumentless detection based on CRISPR/SpRY, which can identify CRISPRi events in rice more quickly (Su et al., 2024).

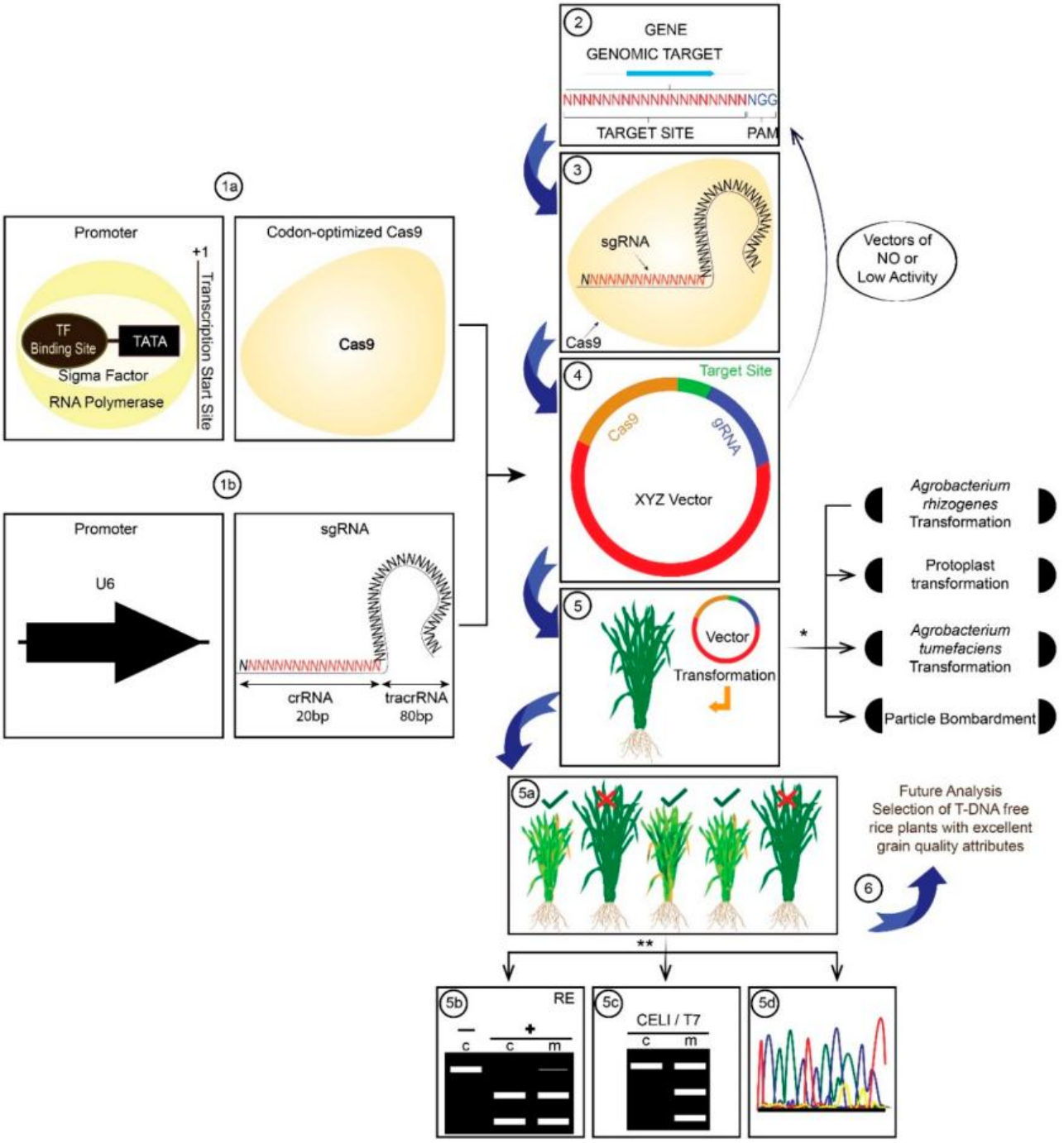


Figure 1 Basic flow chart of the CRISPR/Cas9 genome editing system. The engineered CRISPR/Cas9 system consist of two components; (1a) the Cas9 endonuclease and, (1b) a single-guide RNA (sgRNA). "The sgRNA contains a spacer sequence followed by 79 nt of an artificially fused tracrRNA and crRNA sequence",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

### 3.3 Transformation methods and rice material selection

In rice, the most commonly used method for introducing CRISPRi vectors into plants is Agrobacterium-mediated transformation. This method is highly efficient and transgenic can be stably integrated (Butt et al., 2018; Fiaz et al., 2019). The selection of materials is also very crucial. *Japonica* rice varieties like "Nihon Haru" are widely used because they are easy to transform and regenerate. Selecting healthy and uniformly growing seedlings and then optimizing the tissue culture conditions can further increase the success rate of transformation and also make subsequent analysis more accurate.

## 4 Applications of CRISPRi in Rice Gene Function Analysis

### 4.1 Gene silencing and functional validation

CRISPRi can precisely target and inhibit the expression of specific genes. Researchers can use it to "silence" genes and then observe the changes in plants to verify the functions of the genes. This method is particularly suitable for rapid gene inhibition and can also be used for high-throughput screening. It is a powerful complement to gene knockout or RNA interference (Bendixen et al., 2023). Because it does not modify the DNA sequence, it can be used more safely and flexibly in functional annotations (Larson et al., 2013).

### 4.2 Systematic study of gene family functions

In rice, members of different gene families sometimes compensate for each other, resulting in knockout mutants not showing obvious phenotypes. Combining tools such as CRISPRi and CAFRI-Rice can simultaneously target multiple members within a gene family, thereby reducing this redundancy (Hong et al., 2020). This makes it easier to identify which genes are primary and which are standby, and also enables a more comprehensive mapping of the role of gene families in the growth and development of rice (Sanson et al., 2018).

### 4.3 Integration with transcriptomics and metabolomics analyses

The process by which CRISPRi inhibits genes is reversible and can also regulate strength, making it highly suitable for combined analysis with transcriptomics and metabolomics. Researchers can first suppress a certain gene using CRISPRi, and then conduct RNA sequencing and metabolite detection to see what downstream changes will be caused (Hong et al., 2020; Bendixen et al., 2023). This multi-omics combined approach can more clearly discover new gene functions and regulatory mechanisms, and also make functional genomics research more precise.

## 5 Case Studies: CRISPRi Applications in Key Rice Trait Research

### 5.1 Functional repression studies of flowering time regulation genes

CRISPR gene editing has been employed to study genes that control flowering time and plant type, such as IPA1 (ideal plant type 1). When the IPA1 gene mutates, rice will exhibit different tillering characteristics, which indicates that it plays a significant role in flowering and plant morphology regulation. These studies have shown that using CRISPR to inhibit or modify flower-related genes can reveal their multi-faceted effects on developmental and yield traits (Figure 2) (Li et al., 2016; Ricroch et al., 2017; Butt et al., 2018).

### 5.2 Functional analysis of genes related to grain quality regulation

CRISPR/Cas9 is widely used in the research and improvement of rice quality, including appearance, chalkiness and nutritional components. For instance, large-scale mutant libraries of seed-related genes have identified many candidate genes that affect amylose content, protein levels and starch viscosity. Knocking out specific genes, such as Chalk3, can explain the genetic basis of chalkiness in rice and its impact on endosperm structure and composition, which also provides a new target for quality improvement (Fiaz et al., 2019; Zhao et al., 2024).

### 5.3 CRISPRi functional studies of stress-resistance-related genes

CRISPR technology is also used to study genes related to stress resistance such as drought resistance, salt resistance and disease resistance. It can rapidly complete the identification and verification of stress-resistant genes, providing support for the breeding of rice varieties that are more resistant to environmental stress (Ricroch et al., 2017).

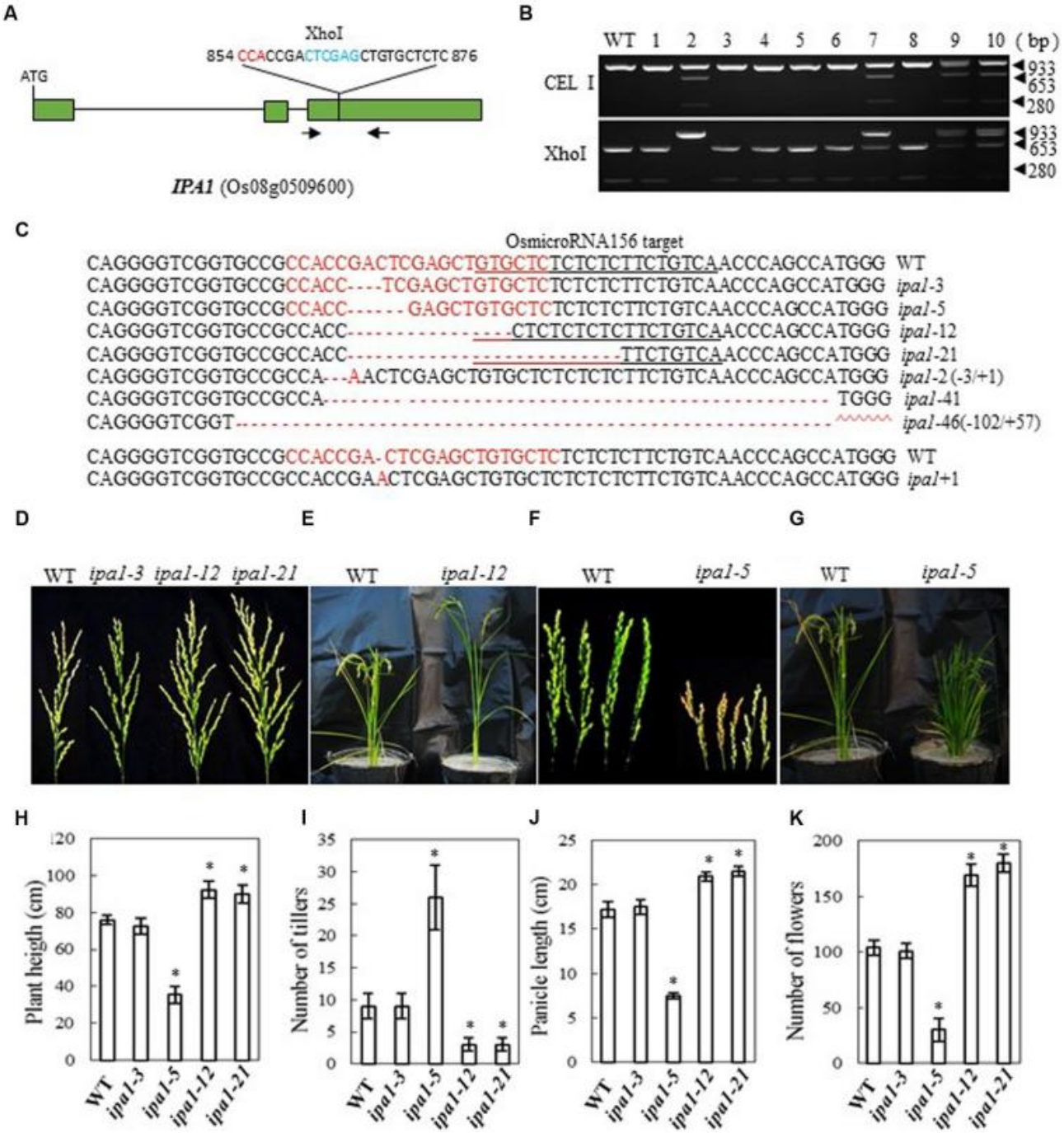


Figure 2 CRISPR/Cas9-induced ipa1 mutant plants and phenotype analysis (Adopted from Li et al., 2016)

Image caption: (A) Schematic map of the genomic region of IPA1 and the sgRNA target site; arrows show the positions of PCR primers used for mutation detection; The PAM motif (NGG) is shown in red; Restriction site is shown in blue; (B) Gel electrophoresis of PCR products amplified from the mutated region digested with CEL I (upper panel) or XhoI (lower panel); WT and 1–10 are DNA samples from wild type and different transgenic plants. Arrows show the expected band sizes after CEL I or XhoI 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 of wild type plants and mutants with the IPA phenotype; (E) Wild type and mutant plant with IPA phenotype grown in a greenhouse; (F) Panicle morphology of wild type plants and ipa1-5 mutant plants with a frame-shift in the coding region; (G) Phenotype of wild type, and ipa1-5 mutant plants with a frame-shift in the coding region, in the greenhouse; statistics for plant height (H), number of tillers (I), panicle length (J), and number of flowers (K) 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 controls (Adopted from Li et al., 2016)

## 6 Advantages, Limitations, and Improvement Directions of CRISPRi Technology

### 6.1 Unique advantages over CRISPR-Cas9

CRISPRi uses an inactivated Cas9 protein (dCas9). It can bind to the target DNA but will not cut through the double strands of DNA, so it will not cause permanent genetic changes. There are several benefits here. CRISPRi's gene silencing is reversible and tunable, allowing for the control of gene expression at different times and the study of essential genes without destroying them (Mandegar et al., 2016; Khan et al., 2024). Because it does not cut DNA, the risk is lower, and it is less likely to have unexpected mutations, chromosomal rearrangements or cytotoxicity, so it is safer and suitable for functional genomics and high-throughput screening. In addition, CRISPRi can specifically target multiple genes at one time, which is used for complex genetic circuit design and system function analysis (Chen and Zhang, 2024; Vercauteren et al., 2024).

### 6.2 Technical bottlenecks in current applications

Although CRISPRi has a promising future, there are still some problems:

Off-target effect: If sgRNA is poorly designed or dCas9 binding is not precise enough, it may inhibit non-target genes and reduce specificity (Karlson et al., 2021; Vercauteren et al., 2024).

Silencing efficiency differences: The inhibitory effect is influenced by the sgRNA sequence, the accessibility of target sites, and the expression levels of dCas9 and sgRNA. There may be significant differences among different targets or cell types (Replogle et al., 2022; Qiao et al., 2023).

The challenge of transmission and expression: In plant systems, efficiently and stably delivering CRISPRi elements into and expressing them remains a difficulty (Karlson et al., 2021).

### 6.3 Strategies to improve specificity and efficiency

To solve these problems, researchers have proposed some solutions:

Optimizing sgRNA design: Selecting highly active and low-off-target Sgrnas using computational tools, while taking into account GC content and distance from the transcription start site (Karlson et al., 2021).

Double sgRNA and effector optimization: By using a double sgRNA library along with modified dCas9 effectors (such as Zim3-dCas9), the inhibitory intensity and consistency can be enhanced (Replogle et al., 2022).

Antisense RNA and feedback control: The addition of antisense RNA or feedback mechanisms can reduce background suppression and make the system more stable (Specht et al., 2022; Van Hove et al., 2023).

Improving delivery methods: Developing more efficient and less toxic delivery systems (such as RNP complexes or optimized vectors) is the focus of the future (Bendixen et al., 2023).

## 7 Conclusions and Perspectives

CRISPR interference technology (CRISPRi) is transforming the research of functional genomics in rice. It can achieve high-throughput, precise and simultaneous regulation of multiple genes, accelerating the speed of gene discovery, trait improvement and molecular breeding. CRISPRi can systematically and on a large scale silence genes, which can solve the problems caused by functional redundancy, as these problems often mask the phenotypes of knockout mutants. Tools like CAFRI-Rice can help researchers identify and locate gene families with similar functions, simplify the functional annotation of thousands of rice genes, and also efficiently conduct large-scale gene function screening. This is very useful for analyzing complex traits and advancing research on the rice genome.

The function of CRISPRi can also be combined with CRISPR activation technology (CRISPRa) and other gene regulation methods. This enables the simultaneous inhibition and activation of different genes in the same experiment, facilitating more comprehensive functional research and also contributing to the construction of complex genetic regulatory networks. This combined approach makes it more possible for us to study gene networks and regulatory pathways in rice and other crops.

CRISPRi can regulate gene expression without altering the DNA sequence, so it is of great value in precision molecular breeding. Through this method, key genes can be reversibly and tunable controlled. When applied to the breeding process, it can fine-tune agronomic traits, solve the problem of gene function redundancy, and also accelerate the breeding of superior varieties. As this technology becomes more and more mature, its application in precision breeding will also increase, and it is expected to support the sustainable improvement of crops.

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## Conflict of Interest Disclosure

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

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