

Sculpting the future of rice: CRISPR/Cas9 gene precision

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Abstract

RNA-guided genome editing technique known as the CRISPR/Cas9 (CC9) system is comprised of a Cas9 nuclease and a single-guide RNA, also known as sgRNA. This invention has completely transformed the field of genome editing, emerging as an essential tool for altering the genetic makeup of different crops, such as rice. This article comprehensively reviews the CC9 system, its operating mechanisms, assembling methodologies, and techniques for identifying mutants with modified genes. We conducted a thorough analysis of the CC9 system in rice, focusing on the design and implementation. Our study explored techniques for accurate gene insertion and replacement, approaches for multiplex editing, and the processes influencing targeted and base editing, which control gene expression. Additionally, recent research was assessed to highlight the significance of specific genes in determining rice yields, quality, and tolerance to environmental stresses. Moreover, the article presents recent use cases of CC9 for rice development, while examining the challenges and prospects of employing this system-in rice breeding.

Keywords: abiotic stress tolerance; base editing; crop breeding; genome editing; mutant screening

Received: 17 Nov 2024. Received in revised form: 27 Jun 2024. Accepted: 27 Jun 2025. Published online: 30 Jun 2025.

From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Introduction

Rice (*Oryza sativa* L.) is a significant grain crop. Around 6.6 billion tons were generated in Asia in 2016, with the United States of America producing 36 million tons and Africa producing 32 million tonnes (Bin Rahman and Zhang, 2023). Rice can grow in many conditions, even in unfavorable ones. However, climate change has increased biotic stress and harsh climatic conditions worldwide, including drought, salt, and severe temperatures, reducing rice production and imposing huge losses. Consequently, rice production has decreased gradually in recent years (Rasheed *et al.*, 2022b; Gull *et al.*, 2023). By 2050, the human population is expected to increase by 34%, reaching 9 billion people. Therefore, worldwide rice consumption is anticipated to increase by 45% over the next thirty years, from 450 million tonnes in 2020 to 650 million tonnes by 2050 (Bahar *et al.*, 2020). Improving rice's tolerance to various stress conditions and increasing production are essential to addressing recent challenges.

Rice is very susceptible to climate change impacts since it requires ideal conditions of temperature and irrigation to thrive. A 1 °C increase in temperature may reduce rice yields by 3.44%, while an increase in precipitation might reduce overall production by 0.12% and crop harvest by 0.21% (Xie *et al.*, 2023). It has been stated that the granary region in Malaysia may experience a temperature increase from 0.3 °C to 0.5 °C, and precipitation may increase from 133 to 200 millimeters (Mohidem *et al.*, 2022). In Cambodia the temperature and precipitation are projected to increase by 2.5% and 8.3%, respectively, resulting in humid conditions during the rainy season and reduced humidity throughout the year's dry season (Ameen *et al.*, 2023a). Because of this, the dynamic climatic situation has become a severe challenge for the entire agricultural production system, especially rice, in maintaining both current and future global food security.

In recent decades, progress in breeding methodologies, specifically forward genetic approaches, has been vital for grasping the molecular mechanisms regulating advantageous agricultural traits in rice. The use of physical irradiation, such as heavy ion beams or gamma rays, chemical mutagenization (ethyl methane sulfonate-EMS), and bacterial genetic insertion (transposons and T-DNAs) has historically been employed in forward genetic approaches to rice mutations (Romero and Gatica-Arias, 2019). Regardless of this, these methods have limitations. For example, forward genetic screening cannot identify desirable phenotypes in the M1 generation, which is attributable to the high frequency of mosaicism induced by irradiation and EMS. Assessing the mutant phenotype induced by T-DNA/transposon is similarly necessary at the T1 generation due to the heterozygosity present in the T0 generation (Ul Haq *et al.*, 2022). Nevertheless, identifying causative mutations for mutant phenotypes requires only one generation via RNA interference-based screens. Despite this, the primary drawbacks of mutagenesis utilizing RNA interference are off-target effects and instability.

Various approaches have been implemented to increase rice grain yields and quality. Recent years have seen only modest increases in crop yields even though conventional breeding techniques have been enhanced (Rasheed *et al.*, 2021b; Sandhu *et al.*, 2021). Additionally, rice has been engineered genetically, but the lack of consumer access to these commodities is primarily attributable to political and public resistance. Because it is possible to carry out precise alterations of DNA sequences *in vivo*, genome engineering technologies ultimately provide more significant potential for crop enhancement. Various methods have been developed to accomplish specific goals, for example, prime editing, base editing, CRISPR/Cas9, TALEN, and Agrobacterium-mediated site-specific mutagenesis. CC9 has transformed genome engineering by introducing a new technique for DNA editing (Ameen *et al.*, 2023b; Rasheed *et al.*, 2021a). The development of this evolution has extended over multiple decades.

CRISPR/Cas9 is a cost-effective method for multiplex genome editing that is characterized by its low complexity, high accuracy, and efficiency. Multiplex genome editing is a significant approach because it permits the alteration of many genes in various genomic areas (Dixit *et al.*, 2023). CC9 enables the specific targeting of genes and the induction of double-strand breaks at exact locations in the genome (Rasheed *et al.*, 2022a). In order to repair double-strand breaks (DSBs), the repair machinery of the host makes use of either homology-directed repair (HDR) or non-homologous end joining (NHEJ). At the end, this process results in the insertion

of genes, the deletion of genes, and modifications to the genome. On the other hand, whereas HDR necessitates utilizing a donor template for the purpose of repairing the double-strand break, NHEJ removes the need for a donor template throughout the repair process. Instead, the host is responsible for performing the repair by the donor template.

The use of HDR in plants is associated with an array of complexities because HDR is a less common phenomenon than NHEJ (Rozov *et al.*, 2019; Janik *et al.*, 2020). The CC9 technology is extensively used for altering plant genomes, particularly in rice, and is an increasingly growing area for study (Figure 1).

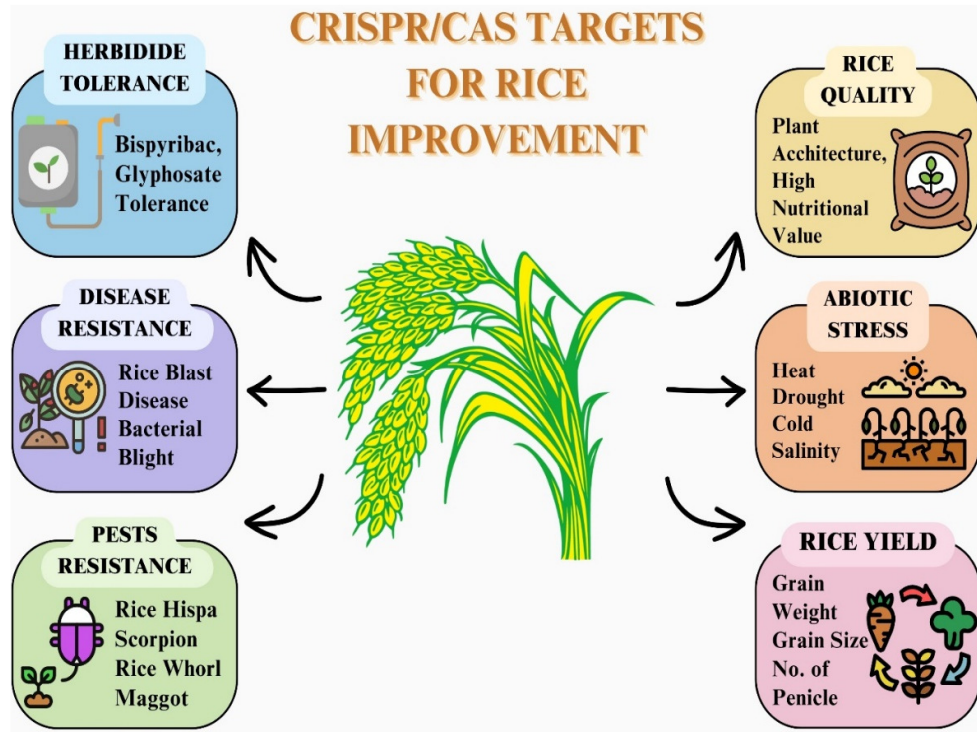


Figure 1. CRIPSER/Cas9 targets for Rice improvements

Rice is a suitable plant for studying functional genomics since it has a compact genome and has considerable genetic similarities with other cereal crops. This review article aims to investigate and clarify the revolutionary potential of precision gene editing using CC9 to influence rice agriculture's future. We aim to thoroughly analyze several CC9 technology elements concerning modifying the rice genome. To provide a strong basis for future discussions, we first delve into the fundamental knowledge by going over the discovery and concept of the CRISPR/Cas system. We then go into detail about how CC9 is used explicitly in rice genome editing, emphasizing methods to reduce off-target effects such as using Cas9 orthologues and variants, adjusting CRISPR component expression, changing sgRNA, using heterologous nucleases, and investigating other novel techniques. In addition, we look at numerous uses and case studies of CRISPR-Cas9 in rice, including boosting herbicide tolerance, nutrient use efficiency, and rice quality and yield, as well as giving resistance against abiotic stressors, diseases, and pests. Despite the positive progress, we also discuss the challenges and moral issues surrounding CC9 technology, highlighting the significance of its responsible and ethical use.

Discovery and principle of CRISPR/Cas system

CRISPR was first discovered by Ishino *et al.* (1987) when they started performing genetic sequencing of the *iap* gene inside the genome of *Escherichia coli*. Researchers found a unique pattern of 29-nucleotide DNA sequences repeated in tandem, alternating with 32-nucleotide spacer sequences (Hamdan *et al.*, 2022). The sequence was situated in the downstream region of the gene. Because repetitions lacked any sequence homology with any other sequences known at the time, their biological purpose was initially unknown. Mojica *et al.* (1995) found long sequences of repeating DNA throughout the *Haloflex mediterranei* genome while sequencing various fragments. This incident signified the initial identification of direct repeats in archaea. Interspaced repeat sequences were defined by Mojica *et al.* (2005) as short, regularly spaced repetitions. Jansen *et al.* (2002) and colleagues classified the CRISPR-associated genes (Cas), Cas1 through Cas4. CRISPRs, which stands for clustered regularly interspaced short palindromic repeats, were the designations that they gave to the sequences in order to avoid misconceptions in future research.

The working mechanism of Cas9 nuclease

Modifications may be made using targeted genome editing, in contrast to random mutagenesis techniques such as EMS mutagenesis and radiation to be introduced in certain sections of the genome that are very effective and targeted to the requirements of the individual. To achieve sequence specificity, the CRISPR/Cas9 system requires using the Cas9 protein structure and conformation (Bravo *et al.*, 2022). The Helix bridge connects a prominent recognition (REC) lobe to a nuclease (NUC) lobe in the Cas9 protein, with a conserved core and a bi-lobed design. Both lobes are responsible for generating nucleases. The arrangement in consideration consists of two nucleic acid binding grooves and two active sites next to one another. In conjunction with a protospacer-adjacent motif (PAM)-interacting domain (PI), the nuclease domains that makeup NUC are referred to as RuvC and HNH. The Cas9-specific function, on the other hand, is the responsibility of the REC mechanism (Jacob *et al.*, 2022). Cas9 remains dormant in its original condition. Activation proceeds at the REC lobe upon combination with the sgRNA. Cas9-sgRNA complexes to analyze a DNA double-strand for PAMs (the trinucleotide NGG) utilizing Watson–Crick pairing with single-stranded RNA (sgRNA) and specific DNA (Miglani *et al.*, 2020). RuvC cleaves the complementary strand to induce a double-strand break (DSB), while the HNH nuclease domain produces a DSB by cleaving the RNA–DNA hybrid after it has adhered to the relevant PAMs (Wang *et al.*, 2023).

Endogenous methods for repairing double-strand breaks (DSBs) are present in both prokaryotic and eukaryotic organisms. These processes are referred to as homology-directed repair (HDR) and non-homologous end joining (NHEJ) (Figure 2).

Through the use of DNA ligase IV, NHEJ can re-join the broken ends, which is a procedure that has the potential to generate insertion or deletion alterations (indels) (Agrawal *et al.*, 2023). In contrast, HDR repairs double-strand breaks (DSBs) using a homologous complementary template, frequently yielding a perfect repair. Many benefits are presented by the error-prone NHEJ in gene knock-out operations. The HDR technique is applied in plants for gene knock-in and gene replacement. ZFNs and TALENs use the nuclease domain of FokI endonucleases to specifically target and cut double-stranded DNA (Saraswat *et al.*, 2023). CRISPR/Cas9 exhibits greater ease of manipulation than TALENs and ZFN, thereby enabling its broader application. ZFN, on the other hand, comprises a collection of Cys2-His2 ZF domains, and each finger of this array binds to a different PAM. This results in a target sequence selection (Ichikawa *et al.*, 2023). In the process of execution, two ZFNs aggregate into a dimer, which identifies a typical DNA sequence of 18-24 base pairs (Chuang and Lin, 2021). However, this technique has disadvantages, including potential off-target effects, challenges in formulating modular DNA-binding proteins, and binding requirements that vary depending on the scenario.

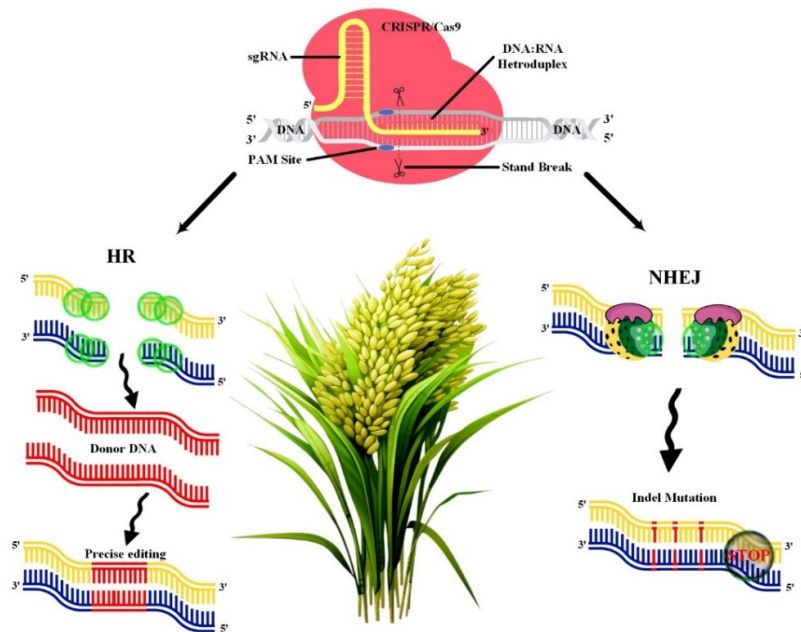


Figure 2. System components and pathways of CRISPR/Cas9 genome editing in rice. Non-homologous end-joining (NHEJ) repair pathway, Homologous recombination (HR) repair pathway

Rice genome editing by CRISPR/Cas9

Strategies to alleviate off-target effects

Within C3 plants, such as rice, modifications to the genome that CRISPR/Cas9 mediates have the potential to boost the productivity and efficiency of photosynthesis. According to Zheng *et al.* (2021), rice engineered to express the Cas9 gene targeting OsHXK1 exhibited notable enhancements in various physiological and yield-related traits, including stomatal conductance, photosynthetic products, light tolerance, light saturation points, and rice yields. The CC9 may also be used for crop biofortification, another exciting application (Mishra *et al.*, 2024). Staple crops provide nutritional value but fail to fulfill the requirements for micronutrients. Therefore, implementing biofortification techniques on staple cereals has been suggested as a potential approach to mitigate deficiencies in essential nutrients (Figure 3).

Conventional breeding for biofortification is challenging because of the complex genomic alterations required, making genome editing a promising option. Research on CRISPR-mediated genome editing applications in several grains was conducted by Manghwar *et al.* (2020). The CC9 system is vital for improving crops, but it is concerning because of its increased off-target activity.

Utilization of Cas9 orthologues and variants

Due to the requirement of a 5'-NGG-3' PAM sequence, the Cas9 targeting range has been restricted. Alternative Cas9 versions from other bacterial species may be used to address this particular issue, since several bacterial strains have Cas9 proteins capable of recognizing particular target PAM sequences (Chatterjee *et al.*, 2020). It was discovered that the Cas9 proteins derived from *Neisseria meningitidis* (NmeCas9) and *Staphylococcus aureus* (SaCas9) could identify the PAM sequence of 5'-NNNNGATT and 5'-NNGRRT, respectively (Bharathkumar *et al.*, 2022). The lack of interference between SaCas9 and Cas9, as demonstrated by Tadić *et al.* (2019), suggests that target regions could be edited utilizing distinct Cas9 orthologs. The NmeCas9 ortholog has substantially increased target specificity and decreased off-target cleavage by demonstrating a decreased tolerance for base mismatches and DNA bulges within mammalian cells. Müller *et al.* (2016) utilized Cas9 cassettes (St1Cas9 and St3Cas9) derived from *S. thermophilus* to modify the *PRKDC*

and *CARD11* genes. To increase the protein's compatibility with the target genomic loci and expand its targeting range, Asano *et al.* (2021) created *Streptococcus pyogenes* Cas9 (SpCas9)-NG, which is a variation of Cas9 that is capable of differentiating NG-PAM from NGG-PAM. Gene editing has also been accomplished using this method on *Arabidopsis* and rice.

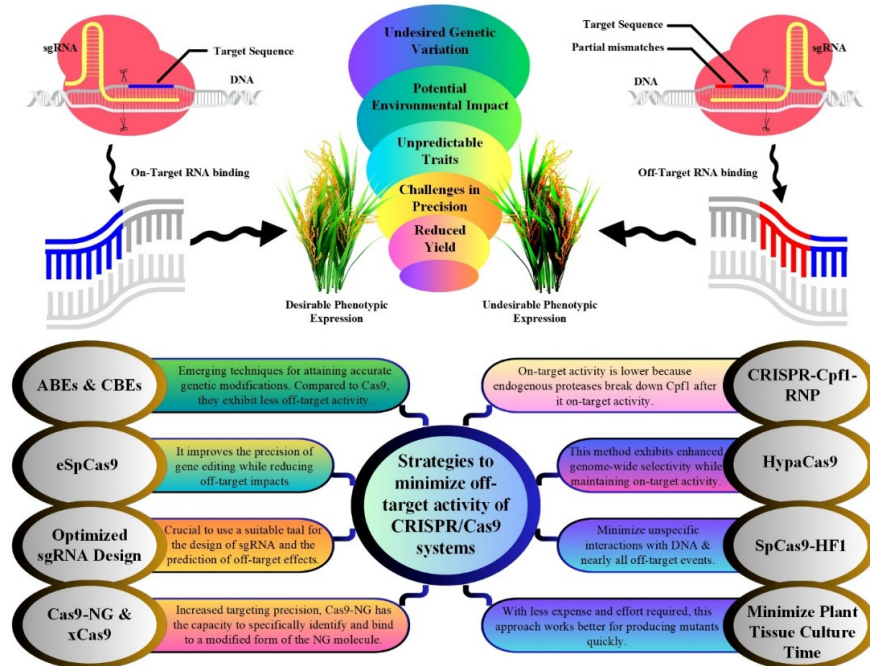


Figure 3. Major concerns about the consequences of off-targeting and several effective strategies have been developed to reduce the off-target actions by CRISPR/Cas9 systems

Among the modified Cas9 proteins that have been found, the enhanced-specificity eSpCas9 variation, the HypaCas9 variant, and the high-fidelity SpCas9-HF1 variant are some of the ones that have been identified (Allemailem *et al.*, 2023). It has been shown that these modified Cas9 proteins can prevent nonspecific DNA editing frequently (Modrzejewski *et al.*, 2020). *Campylobacter jejuni* CAS9 (CjCas9) was recently identified as the most smallest Cas9 ortholog (Kweon *et al.*, 2023). The off-target impact has also been significantly enhanced without compromising the activity of the Cas9 protein on its intended target.

Fine-tuning expression of CRISPR components

To control the off-target effects, cells must express CRISPR components like Cas9 and sgRNA. Certain conditions may significantly impact the selectivity and activity of the Cas9/sgRNA complex (Naeem *et al.*, 2020). An off-target implication will be less likely to occur in cells with a lower concentration of the Cas9/sgRNA complex; however, this may come at the expense of a reduction in efficiency at the spot where the Cas9/sgRNA complex is being used. Rose *et al.* (2020) successfully maintained on-target efficiency and controlled off-target effects by reducing the quantity of Cas9 and sgRNA expression plasmid in transfected cells. There is a possibility that the quick degradation of the CRISPR components inside the cell might mitigate off-target effects. Increasing the time the CRISPR components are allowed to incubate in cells may result in a higher probability of off-target binding and cleavage. An alternate method has been devised for directly delivering CRISPR components into cells, whereas the Cas9/sgRNA complex is typically administered by plasmid transfection or viral vector integration. This technique aims to reduce the duration of exposure to the Cas9/sgRNA complex inside the cell (Fajrial *et al.*, 2020). By delivering the Cas9 protein and the sgRNA that were synthesized in vitro as distinct molecules or purified complexes known as ribonucleoproteins (RNPs), we

were able to reduce the amount of off-target effects that occurred in the cells. Jena *et al.* (2022) discovered that RNPs targeting the CCR5 gene degraded quickly, leading to less off-target mutations, unlike plasmid transfection.

Modification of sgRNA

The sgRNA is a crucial element in the CRISPR method. The design of sgRNA is critical in mitigating off-target mutations, given its role as a guide for Cas9. Studies have shown that sgRNAs with a high GC content (40-60%) may effectively increase the expression of target genes in wheat (Wang *et al.*, 2020). Improving on-target gene editing efficiency may be accomplished by placing a high GC percentage closer to the PAM site. Regarding unwanted mutations, the length of sgRNA is another critical consideration. In an investigation, Naeem and Alkhnabashi (2023) observed that optimizing the on-target accuracy while reducing the size of sgRNA to 17 or 18 base pairs (from 20 to 17 or 18 base pairs) caused a decrease in the number of off-target occurrences by a factor of 500. Recent research has shown that a strategy known as dead RNA off-target suppression (dOTS), which uses dead truncated small guide RNA, may significantly improve on-target activity while simultaneously lowering off-target effects by an average of 40. To alleviate off-target effects, sgRNAs may also undergo chemical modification by incorporating substances into the ribose-phosphate backbone, such as 2'-O-methyl-3'-phosphonoacetate (Ryan *et al.*, 2017). By implementing this improvement, off-target cleavage was decreased by a factor of 120, but the performance on the target was unchanged. In addition to that, alterations such as site-specific inclusion of 2'-O-methyl, 2'-4' bridged nucleic acid, 2'-4' bridged nucleic acids, and phosphorothioate links, as well as partial replacement of crRNAs with DNA, are also possible alternatives (O'Reilly *et al.*, 2018).

Application of heterologous nucleases

Alterations have been made to the configuration of Cas9 in order to mitigate the adverse consequences of the off-target effect. For example, it has been demonstrated that the off-target rate of the D10A Cas9 nickase (nCas9), a Cas9 mutant, is reduced due to structural modifications in its binding region (Lee *et al.*, 2023). The nCas9 gene-editing technique does not effectively induce double-strand breaks (DSB), but instead creates a fragment or single-stranded break at the target site. The associated binding of nCas9 on the complementary strand enables the creation of double-strand breaks (DSB) with enhanced specificity and decreased potential for off-target effects. This may be achieved by duplicating the recognition site of the gene that is being targeted. This paired nicking approach, creating 5' overhangs, may lead to a more significant occurrence of indels. One may fuse the FokI nuclease domain with nCas9 or dCas9 to enhance gene targeting precision and minimize off-target effects. The RNA-guided FokI-Cas9 nuclease, comparable to nCas9, requires dimerization for optimum functionality (Kondrateva *et al.*, 2021). In comparison to Cas9, it has been established that this technique successfully reduces off-target activity by 40%.

Other approaches

A novel technique known as base editing has been devised in conjunction with the modification of nCas9. This method eliminates the need for DSBs when directly converting a target DNA base to another. It is possible to convert A-T to G-C using CRISPR-mediated base editing, accomplished with the combination of nCas9 and adenine base editors (Eghbalsaid *et al.*, 2024). Using cytosine base editors, it is possible to convert a C-G base pair into a T-A base pair. By inserting a C287T mutation into acetolactate synthase, Shimatani *et al.* (2017) successfully engineered herbicide-resistant rice plants via base editing. Rice plants resist imazamox, a broad-spectrum herbicide from the imidazolinone family that targets acetohydroxyacid synthase (AHAS), due to a mutation called C287T, which causes an amino acid substitution of A96V (Yarra and Sahoo, 2021).

The CC9 DNA base-editing technology has shown tremendous effectiveness in DNA editing. Nevertheless, its ability to generate precise base edits is restricted to just the four transition mutations. Prime editing, a novel approach that does not include double-strand breaks (DSBs), has been recently created to

address these restrictions. The approach involves the use of a Prime Editing Guide RNA (pegRNA) that is specifically engineered and linked to nCas9, along with a reverse transcriptase enzyme (Grünewald *et al.*, 2023). In addition to a guide sequence, a reverse transcriptase template that also encodes the necessary genetic alterations that makes it easier to identify the target location are two distinguishing characteristics that set pegRNAs apart from small guide RNAs (sgRNAs). Using prominent editors modified by Lin *et al.* (2020), insertions, point mutations, and deletions were recently introduced into rice. In addition to the many base substitutions, insertions, and deletions, there are a total of 12 different types of base-to-base replacements used that were successfully and accurately identified by this method. The authors disclosed that the exceptional editor was responsible for a frequency of prime editing that was as high as 21.8% of the total. Many other researchers have recorded outcomes equivalent to those (Li *et al.*, 2022; Xu *et al.*, 2022). While prime editing offers numerous benefits over alternative methods, such as the ability to delete, add precisely, and substitute sequences, it is currently in its early phases of development. Additional research is necessary to ascertain the specificity of this technology and its potential for unintended alterations.

Using CRISPR-based epigenetic engineering, epigenetic factors such as methyltransferases and histones could be targeted. By combining epigenetic modulators with dCas9, the chromatin markers in the DNA region could be characterized and mapped (Josipović *et al.*, 2019). A brief overview of the most recent CRISPR technologies for modifying the epigenome was provided by Chiarella *et al.* (2020). Despite this, there is a possibility that this practice may result in some degree of activity that was not anticipated. The fusion protein that exhibits DNA methyltransferase activity could be responsible for the off-target methylation. Zhu *et al.* (2024) discovered that decreasing the catalytic activity of the fusion protein and preventing the multimerization of DNA methyltransferase might be used to lessen the consequences of an unintended outcome.

Applications and case studies of CRISPR-Cas9 in rice

Rice quality

New cultivars with higher nutritional and economic advantages are one of the main objectives of rice improvement. Making changes to the starch and amylose amount is especially significant. Amylose-rich cereal grains include resistant starch, which travels straight to the large intestine without being digested in the stomach or small intestine. Resistant starch improves health and reduces the risk of non-infectious illness (Adebowale *et al.*, 2019). Changing the starch branching enzymes (SBEs) can raise the amounts of amylose and resistant starch. SBEIIb was selected in the capacity of a target gene for the CRISPR/Cas mutation because it primarily occurs in rice grains (Aqib *et al.*, 2022). Sun *et al.* (2017) observed that there are 22-30 transgenic plants that reveal mutations at the predicted site after *Agrobacterium* transforms rice (Kitaake) calli. The frequencies of mutations for bi-allelic, homozygous, and heterozygous lines are as follows: 36.6%, 26.7%, and 6.7%, respectively. However, in the predicted sites, no off-target impacts are seen. After segregating transgenic-free mutant lines, the best mutants may contain up to 25.0% amylose and 9.8% resistant starch. For cooking and consuming rice, amylose content is the most essential quality indicator. Due to their high amylose level, some types of rice, especially indica hybrids, are seen as low quality in some areas (Ishfaq *et al.*, 2023). Getting sticky rice by lowering the amylose content is a very desired trait in this case. Rice amylose is controlled by one dominant gene called *Waxy*. Therefore, Zhang *et al.* (2018) synthesized a specific kind of RNA called short guide RNA (sgRNA) that was designed to target the first section of the *Waxy* gene. This was done in order to produce a mutant organism that lacks the normal function of the gene. The CRISPR/Cas system was used in this process. The transformation of calli by *Agrobacterium* altered the genetic makeup of two crops, namely Xiushui 134 and Wuyunjing 7. The T0 generations showed a high mutagenesis rate (82% to 87%). There were homozygous mutants in about 4% to 15% of the cases (Figure 4). Surprisingly, none of the mutants that were examined exhibited any off-target modifications.

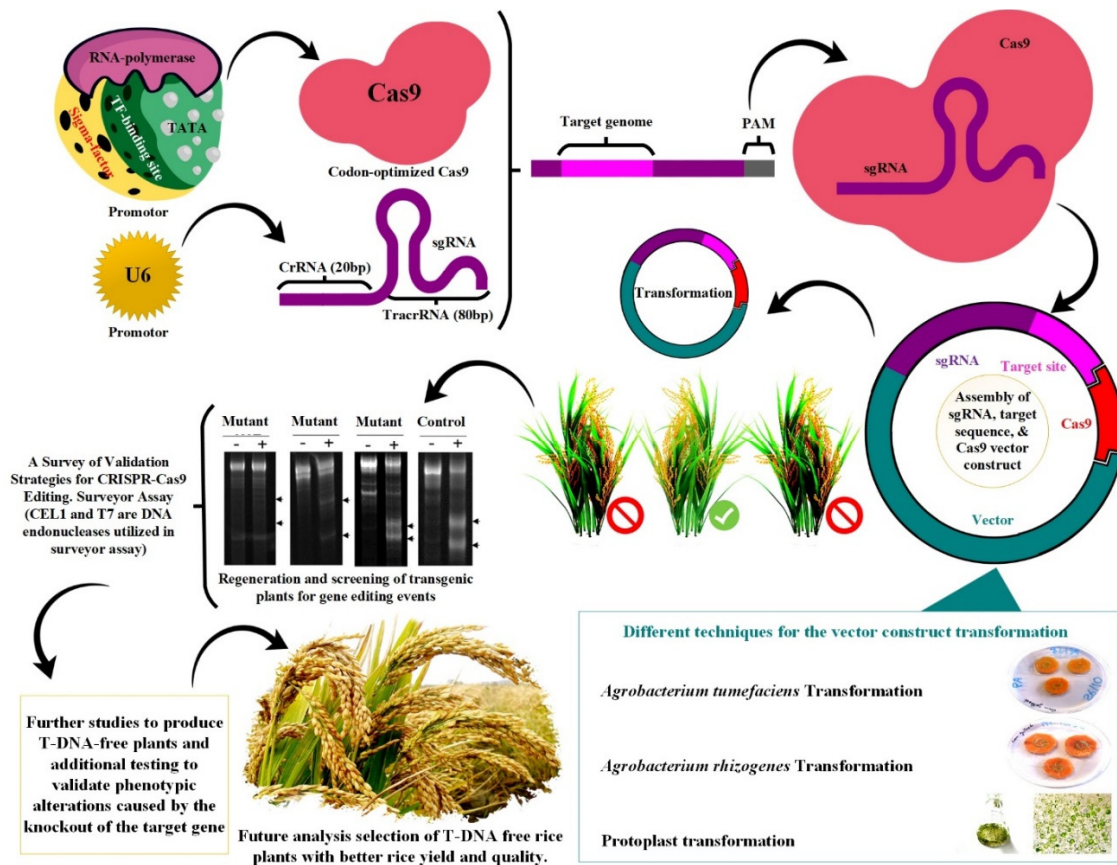


Figure 4. Basic flow chart of the CRISPR/Cas9 genome editing system to improve rice yield and quality

Several Asian nations cultivate rice bran oil (RBO), a commercial product generated from rice, and use it. One factor contributing to the prevention of chronic illnesses is oleic acid, one of the components of RBO, which has critical beneficial characteristics. Increased oleic acid and RBO might result from fatty acid production pathway modifications. Oleic acid is converted into linoleic acid with the help of an enzyme called fatty acid desaturase 2 (FAD2) (Zhiguo *et al.*, 2019). Rice (Nipponbare) calli transformed by *Agrobacterium* generated six mutant lines (Abe *et al.*, 2018). The T2 line fatty acid profiles were examined after validating the mutation in the progeny. Fad2-1 homozygous mutants exhibited twice as much oleic acid and zero linoleic acid as wild type.

Cadmium (Cd), a heavy metal, is very hazardous to the health of both plants and animals. When rice grains contain excessive cadmium, those who use rice as their primary source of nutrition are in significant danger. Several health problems could result from persistent intake of Cd. It is extremely essential to develop improved rice types that do not accumulate an excessive amount of cadmium (Chen *et al.*, 2018). This low-Cd indica rice strain was developed by Chen *et al.* (2023) by implementing CRISPR/Cas9 technology to induce a mutation in the *NRAMP5* metal transporter gene. Mutating this gene significantly decreased Cd concentration because this transporter controls root intake. There is an 82.4% mutation rate for Huazhan and an 80% mutation rate for Longke 6385 when *Agrobacterium* modifies embryogenic calli while off-target effects are absent. It is feasible to produce five homozygous knockout lines without transgenes in the same generation through segregation. Three of these lines come from Huazhan, and the other two come from Longke 6385 (Tang *et al.*, 2017). When cultivated on Cd-contaminated paddy fields, *nramp5* lines accumulate 0.05 mg/kg less Cd than the wild type. Compared to wild-type plants, mutant plants had similar grain production, straw weight, and grain quality.

Rice yield

The production of grains is affected by several variables, such as agronomic methods and quantitative trait loci (QTL). The most frequent plant breeding method involves the generation of QTL combinations and the selection of the most advantageous ones. Researchers utilizing CRISPR/Cas9 to create higher-yield rice lines have chosen several QTLs as target genes (Table 1).

Table 1. Various genetic applications and Strategies of CRISPR in rice crop improvement

Application	Targeted gene	Strategies	Reference
Cadmium toxicity	<i>Nramp5</i>	NHEJ	(Vidya and Arun, 2023)
Bacterial blight response	<i>SWEET11, SWEET13, SWEET14</i>	NHEJ	(Sree <i>et al.</i> , 2023)
Bacterial leaf streak resistance	Os09g29100	TALENs	(Chattopadhyay <i>et al.</i> , 2022)
Bacterial resistance blight	OsSWEET13	TALENs	(Duy <i>et al.</i> , 2021)
Blast disease resistance	OsERF922	CRISPR/Cas 9	(Távora, 2021)
Cold stress response	<i>OsPRP1</i>	NHEJ	(Nawaz <i>et al.</i> , 2019)
Comparison among <i>Cpf1</i> and <i>Cas9</i>	<i>EPFL9</i>	NHEJ	(Yin <i>et al.</i> , 2019)
Control death and senescence	OsCDC48	Base editing	(Yarra and Sahoo, 2021)
Controls leaf stomatal density	OsEPFL9	CRISPR/Cas 9 and CRISPR/Cpf1	(Bhat <i>et al.</i> , 2024)
Drought stress response	<i>SAPK2</i>	NHEJ	(Aslam <i>et al.</i> , 2022)
Drought tolerance	OsSAPK2	CRISPR/Cas 9	(Tripathy <i>et al.</i> , 2021)
Early maturity	Hd2, Hd4, Hd5	CRISPR/Cas 9	(Kumari and Begum, 2023)
Enhanced quality	<i>OsAAP6, OsAAP10</i>	NHEJ	(Tabassum <i>et al.</i> , 2021)
Fatty acid metabolism	<i>FAD2-1</i>	NHEJ	(Le <i>et al.</i> , 2022)
Flowering period	<i>Hd2, Hd4, Hd5</i>	NHEJ	(Massel <i>et al.</i> , 2021)
Genome wide mutant Library	12 802 genes, 34 234 genes	NHEJ	(Romero and Gatica-Arias, 2019)
Glyphosate resistant	OsEPSPS	CRISPR/Cas 9	(Tabassum <i>et al.</i> , 2021)
Grain number and weight	<i>OsPAO5</i>	NHEJ	(Zhao <i>et al.</i> , 2022)
Grain number, size, and panicle architecture	<i>GS3, Gn1a, DEPI, IPA1</i>	CRISPR/Cas 9	(Singh <i>et al.</i> , 2024)
Grain numbers and yield	<i>OsPYL1, OsPYL12</i>	NHEJ	(Roy and Soni, 2021)
Grain size	<i>OsGS3, OsGW6</i>	NHEJ	(Le <i>et al.</i> , 2022)
Grain size, yield, and cold tolerance	<i>OsPIN5b, OsMYB30</i>	NHEJ	(Srikanth <i>et al.</i> , 2021)
Grain weight	GW2, GW5, TGW6	CRISPR/Cas 9	(Chen <i>et al.</i> , 2021)
Grain weight, size, and yield	<i>OsGW2, qGW8/OsSPL16</i>	NHEJ	(Zeb, 2022)
Grain yield	<i>OsIPA1, OsGn1a, OsGW2</i>	NHEJ	(Yan <i>et al.</i> , 2022)
Grain yield and size	<i>OsBADH2</i>	NHEJ	(Usman <i>et al.</i> , 2021)
Growth and yield	PYLs	CRISPR/Cas 9	(Mehta <i>et al.</i> , 2020)
Herbicide resistant	<i>ALS, BEL</i>	HR, CRISPR/Cas 9	(Kumari and Begum, 2023)
Herbicide resistant	<i>EPSPS</i>	Exon replacement by NHEJ	(Zhao <i>et al.</i> , 2022)
Herbicide resistant	OsALS	TALENs	(Gosavi <i>et al.</i> , 2022)
Herbicide resistant	C287	Base editing	(Seraj <i>et al.</i> , 2020)
Improve nitrogen use efficiency	NRT1.1B gene	Base editing	(Yang <i>et al.</i> , 2023)
Improved fragrance	<i>OsBADH2</i>	NHEJ, TALENs	(Ashokkumar <i>et al.</i> , 2020; Imran <i>et al.</i> , 2023)

Improved fragrance	<i>BADH2</i>	CRISPR/Cas 9	(Imran <i>et al.</i> , 2023)
Improved storage tolerance	<i>LOX3</i>	TALENs	(Tiwari and Lata, 2019)
Increase amylose level	<i>OsBEI</i> , <i>SBEIIb</i> , <i>SBEI</i>	NHEJ, CRISPR/Cas 9	(Tabassum <i>et al.</i> , 2021)
Lower cadmium content	<i>OsNRAMP5</i> , <i>OsLCD</i> , <i>OsNramp5</i> , <i>OsLCT1</i>	CRISPR/Cas 9	(Chen <i>et al.</i> , 2023; Songmei <i>et al.</i> , 2019)
Number of tillers and plant architecture	<i>OsFC1/OsTB1</i>	NHEJ	(Le <i>et al.</i> , 2022)
Plant architecture	<i>DEP1</i> , <i>Gn1a</i> , <i>GS3</i> , <i>IPA1</i>	CRISPR/Cas 9	(Li <i>et al.</i> , 2016; Singh <i>et al.</i> , 2024)
Plant architecture and yield	<i>OsFWL4</i> , <i>OsGA20ox2</i>	NHEJ	(Le <i>et al.</i> , 2022)
Reduces amylose level	<i>OsISAI</i> , <i>OsWaxy</i>	NHEJ	(Le <i>et al.</i> , 2022)
Salinity stress response	<i>RAV2</i>	NHEJ	(Sedeek <i>et al.</i> , 2019)
Starch metabolism	<i>SBEIIb</i> , <i>SBEI</i> , <i>WAXY</i>	NHEJ	(Abdelrahman and Zhao, 2020)
Tiller number	<i>CCD7</i> , <i>OsAAP3</i>	CRISPR/Cas 9	(Zegeye <i>et al.</i> , 2022)
Yield enhancement	<i>Gn1a</i> , <i>DEP1</i> , <i>GS3</i> , <i>IPA1</i>	NHEJ	(Zeb, 2022)
Yield enhancement	<i>GW2</i> , <i>GW5</i> , <i>TGW6</i> , <i>TMS5</i>	NHEJ	(Zegeye <i>et al.</i> , 2022)

To regulate grain size, grain quantity, panicle design, and plant architecture in rice (Zhonghua 11), Li *et al.* (2022) altered the genes *DEP1*, *IPA1*, *Gn1a*, and *GS3*. The miniature guide RNAs are directed at the first exon of *GS3* and *Gn1a* and the third exon of *IPA1* and *DEP1*. This is because modifications to these regions have been shown to trigger the desirable trait (better yield). Furthermore, T0 transgenic lines include 42.5% of *Gn1a* mutations, 67.5% of *DEP1* mutations, 57.5% of *GS3* mutations, and 27.5% of *IPA1* mutations from genetic material. Genetically modified *GS3* lines have husks with longer awns and bigger grains. The *Gn1a* gene makes the plant taller, the panicles bigger, and the flowers on each panicle about 90% more than the control variants (Parida *et al.*, 2022). Compared to the control group, the *dep1* mutants have shorter plants and panicles (about 20% shorter); however, they have around 50% more flowers per panicle than the control group. In conclusion, alterations to *IPA1* have the potential to display three distinct phenotypes, which are determined by the kind of mutation. Controlling the position of the miR156 target is the objective of the small guide RNA (sgRNA). According to Gupta *et al.* (2023), the phenotype exhibited was similar to the wild type, indicating a modification of the miR156 target site. However, the plant height, panicle length, and flower abundance were all enhanced. The mutant plants were smaller and had a more significant number of tillers due to the mutation that produced a change in the protein frame. Three of every four sgRNAs within the expected genetic regions cause off-target activities. Frequencies of off-target mutations are 67% for *Gn1a*'s sgRNA, 47.5% for *IPA1*, and 2.5% for *DEP1* (Ahmad *et al.*, 2024). These results support that sgRNAs must be developed carefully to prevent unwanted genomic modifications. Several bioinformatics techniques have been created to synthesize sgRNA that is exceptionally efficient and specific to increase the effectiveness of mutations and decrease the impact of off-target mutations.

The CRISPR/Cas9-mediated multiplex genome editing system is an essential component of this technology, particularly advantageous for improving rice yield by modifying quantitative trait loci (QTLs). Kabange *et al.* (2023) modified three specific genetic regions, known as quantitative trait loci (QTLs), that are linked to the weight of grains: *GW2*, *GW5*, and *TGW6*. There is an extensive understanding of the roles of *GW5*, *GW2*, and *TGW6*, and the absence of these genes leads to significant growth in grain weight. Granules are more conspicuous in the *gw5tgw6* and *gw2gw5tgw6* mutants compared to the wild type. The double mutant species exhibits an increase of around 12% in grain length, 8% in grain width, and 13% in weight per thousand grains. The triple mutants exhibit a respective rise of around 25%, 20%, and 28% in grain length, width, and weight per thousand grains. The findings indicate that this system is highly compatible with rice's expeditious production and pyramiding of advantageous alleles.

Utilizing the capability of CRISPR/Cas to delete a specific gene region has also resulted in increased yield. The DEP1 gene deletion of 625 bp leads to the development of compact, upright panicles with increased grain production and reduced plant height, in comparison to the wild type (Sun *et al.*, 2018). Wang *et al.* (2017) formulated four single-guide RNAs (S1–S4) to induce a consistent deletion in indica rice, especially in the inbred line IR58025B rice. There are four separate constructions that each include one of the four single guide RNAs (sgRNAs): S1, S2, S3, and S4. Additionally, there are three combinations of constructions: S1 with S4, S1 with S3, and S2 with S4. Researchers has shown that among 96 T0 events per construct, the deletion frequency may reach up to 21% for a target of 430 base pairs and 9% for a target of 10 kb. The *dep1* mutants produced via the use of CRISPR/Cas exhibit the intended phenotype, characterized by dense and upright panicles and a reduction in the height of the plant.

Hybrid rice has a yield that is 10-20% more than ordinary rice, making it widely used in rice cultivation globally. The development of hybrid lines is feasible via the use of both the two-line and three-line hybrid breeding techniques. Thermo-sensitive genic male-sterile (TGMS) or photoperiod-sensitive genic male-sterile (PGMS) lines are used in the process of two-line reproduction. These lines are either sterility lines or maintainer lines, depending on whether they are grown under restricted or permissive circumstances, respectively (Prasad *et al.*, 2017). Accelerating and improving the breeding process would be possible by establishing new P/TGMS lines. Romero and Gatica-Arias generated A new TGMS line was generated through the CRISPR/Cas modification of the TMS5 gene by Romero and Gatica-Arias (2019). *Agrobacterium* transforms rice calli (Zhonghua 11) using ten single-guide RNAs (sgRNAs) specifically developed to target this gene.

Resistance to abiotic stresses

The role of OsSAPK2 (osmotic stress/ABA-activated protein kinase 2) was clarified by Tabassum *et al.* (2021) who used CRISPR/Cas to generate loss-of-function mutations. The precise target location of a single guide RNA (sgRNA) is the third exon of the gene. Twenty transformed T0 lines are acquired via *Agrobacterium*-mediated transformation. Sequencing of the target region is used to characterize two homozygous T1 mutant plants obtained from these lines. Due to the ABA-insensitive phenotype and increased susceptibility to drought stress observed in *sapk2* mutants compared to the wild type, it is evident that *OsSAPK2* plays a crucial role in enabling the rice plant to acclimate to drought conditions. Nazir *et al.* (2022) investigated the promoter of the transcription factor *OsRAV2*, which has been associated with the saline stress response. This gene's salt induction is facilitated by a particular region of the promoter known as GT-1. The mutant lines' incapability confirms this region's significance in overexpressing the *OsRAV2* gene in high-salinity environments.

Que *et al.* (2023) examined the role of the rice annexin *OsANN5* in the response to cold stress. Here, the single guide RNA specifically targets the second exon of the gene. After being transformed by *Agrobacterium*, the specific regions of 19 transgenic T0 rice lines (Taipei 309) were subjected to sequencing in order to confirm the modifications. Six mutants have been found with four distinct forms of non-homologous end-joining (NHEJ) alterations. The alterations consist of 1-base pair insertion, 1-base pair deletion, 3-base pair deletion, and 4-base pair deletion. Three lines exhibit homozygous mutants, whereas one line has biallelic mutants. Three T1 lines, derived from T0 homozygous and biallelic mutants, exhibit increased vulnerability to cold stress in comparison to the wild type. The scientists showcased the precise accuracy of the CRISPR/Cas system in rice by examining off-target effects in other annexin family members and seeing no unexpected alterations. The HSA1 deletion mutant, generated using CRISPR/Cas9, exhibited greater heat sensitivity compared to the wild-type allele. Zhao *et al.* (2020) investigated the susceptibility of rice to salt stress and the involvement of the OsBBS1 gene in the onset of early leaf senescence Rice plants were susceptible to the cold when the *OsAnn3* gene was suppressed. *OsMIR528* has demonstrated positive regulation of salt stress via genome editing. Targeted mutations in plants have shown that the GT-1 element has a role as a regulatory component in the salt-induced production of OsRAV2. A modification was made to the OsNramp5 gene to

generate a low buildup of cadmium in rice without affecting yield. The presence of two rice SnRK2 genes, osmotic stress/ABA-activated protein kinases SAPK1 and SAPK2, was elucidated by Lou *et al.* (2018) via the production of loss-of-function mutants in salinity tolerance. Because of the CC9 technology (Table 2), many rice mutants were produced so that researchers could explore the activities of genes. Developing applications connected to chromosomal engineering and customized genome editing via the CRISPR system can potentially benefit both rice research and the agriculture sectors.

Table 2. Genome editing technique based on CRISPR to increase its resistance to abiotic stress in rice

Stress Tolerance	Target Gene	Cas9 Promoter	sgRNA Promoter	Improved Traits	References
Salinity Tolerance	<i>OsSAPK2</i>	Pubi-H	U3	Gene's function in scavenging reactive oxygen species, decreasing salinity, and enhancing tolerance to osmotic stress	(Le <i>et al.</i> , 2022)
	<i>OsRR22</i>	2 × 35S pro Pubi-H	OsU6a	Enhance shoot length, salinity tolerance, dry weight, and fresh weight	(Tabassum <i>et al.</i> , 2021)
	<i>OsmiR535</i>	UBI, 35S pro	OsU3, OsU6	Salinity tolerance, osmotic tolerance, shoot length increased by 86.8%, number of lateral roots increased by 514% compared to the line overexpressing MIR535, and main root length increased by 35.8%	(Yue <i>et al.</i> , 2020)
	<i>DST</i>	OsUBQ	OsU3	Salinity and osmotic tolerance	(Lou <i>et al.</i> , 2017)
Drought Tolerance	<i>OsSAPK2</i>	Pubi-H	U3	Reduced tolerance to osmotic stress, drought stress, salt stress, gene role in ABA signaling, stomatal conductance, and ROS scavenging.	(Lou <i>et al.</i> , 2017)
	<i>OsPYL9</i>	Pubi-H	OsU6a, OsU6b	Grain yield, drought tolerance, transpiration rate, stomatal conductance, antioxidant activity, ABA accumulation, chlorophyll content, and leaf cuticle wax	(Usman <i>et al.</i> , 2020)
	<i>OsERA1</i>	Not defined	pCAMBIA1300	More ABA sensitivity, drought tolerance, stomatal conductance	(Ogata <i>et al.</i> , 2020)
	<i>OsSRL1</i> , <i>OsSRL2</i>	Pubi-H	U6a, U6b, U6c, U3m	Enhanced survival rate, number of panicles, ABA content, superoxide dismutase (SOD), catalase (CAT), and drought resistance	(Liao <i>et al.</i> , 2019)
	<i>OsmiR535</i>	UBI 35S pro	OsU3, OsU6	ABA intolerance, drought tolerance, 73% more lateral roots, 30% longer shoots, and 30% longer main roots	(Yue <i>et al.</i> , 2020)
	<i>DST</i>	OsUBQ	OsU3	Leaf design, decreased stomatal density, improved water retention, drought tolerance	(Le <i>et al.</i> , 2022)
Cold Tolerance	<i>OsMYB30</i>	2 × 35S pro Pubi-H	OsU6a	Cold tolerance	(Gao <i>et al.</i> , 2020)
	<i>OsAnn3</i>	UBI 35S pro	U3	Cold tolerance response	(Ahmar <i>et al.</i> , 2023)

Disease resistance

Effectively controlling diseases requires a comprehensive understanding of how resistance develops and how to produce resistant crops. Recent discoveries have identified *Magnaporthe oryzae* chrysovirus as a virus associated with biomolecular studies in rice blast disease (Kondo *et al.*, 2022). Some methods, such as cross-species transformation, host-induced gene silencing, and gene editing, may facilitate the creation of broad-spectrum resistant types.

Rice blast disease, caused by the fungus *Magnaporthe oryzae*, is a very prevalent and globally distributed infectious disease that impacts rice crops. It is responsible for significant production losses, ranging from 10% to 30% (Simkhada and Thapa, 2022). According to findings from recent studies, creating a CC9 SSN (C-ERF922) that targets the *OsERF922* gene in rice can potentially increase the plant's resistance to blast. Twenty-one transgenic plants (T0) were identified as possessing mutant plants induced by C-ERF922 (42.0%). At the seedling and tillering stages, the six mutant lines exhibited significantly fewer blast lesions following pathogen infection than wild-type plants. These plants were analyzed using Sanger sequencing, which identified unique insertion or deletion (InDel) alterations at the specific location (Le *et al.*, 2022). Younas *et al.* (2024) suggest that augmenting blast resistance in rice can be accomplished effectively via CC9 to modify genes. *OsRSY1*, *OsMPK5*, *OsALB1*, *OsPi21*, *MPK2*, *MPK5*, *OsSEC3A*, and *MPK6* are some disease-resistant genes that have been the subject of further studies. The CC9 technology was used to change these genes for functional analysis. This was accomplished via the utilization of gene editing.

Within the regions of Southeast Asia and West Africa, the *Xanthomonas oryzae* pv. *oryzae* (Xoo) strain is the major agent responsible for the bacterial blight of rice, which is a significant rice disease (Tran *et al.*, 2018). The CC9 was used in a separate experiment to change the corresponding coding sequence of *OsSWEET14* in rice Zhonghua 11. This was done to impair the functioning of *OsSWEET14* (Ma *et al.*, 2017). During the disruption of *OsSWEET14*, it was found that the height of the plants increased without a consistent loss in yield. Gene editing was facilitated by identifying numerous TALE (transcription-activator-like effectors) variants for *SWEET13* alleles in 63 Xoo strains via sequence analysis of TALE genes (Blanvillain-Baufumé *et al.*, 2017). Rice lines with genome-edited *SWEET* promoters have been shown to display potent and broad-spectrum resistance, as evidenced by the findings of paddy experiments conducted by Oliva *et al.* (2019). Furthermore, the rice gene *Os8N3* was deleted utilizing CC9 technology to increase Xoo resistance. These mutations were genotyped and passed on to succeeding generations.

Rice tungro disease (RTD) develops through the interaction of the rice tungro spherical virus (RTSV) and the rice tungro bacilliform virus (RTBV). RTSV enables vector transmission by leafhoppers, while RTBV induces disease symptoms. Both viruses are essential for RTD development (Azzam and Chancellor, 2002). To produce novel sources of resistance to RTDs, mutations in eIF4G were introduced into the cultivar IR64, which was sensitive to RTSV, using the CC9 technology. The rates of mutation ranged from 36.0% to 86.6%. These mutations were successfully transmitted to succeeding generations. Under glasshouse conditions, the Cas9 sequence was absent from the final products that exhibited resistance to RTSV, increasing yield. Consequently, RTSV-resistant plants possessing innovative eIF4G alleles are invaluable in searching for cultivar variation for RTSV resistance (Macovei *et al.*, 2018).

Insects/Pests Resistance

Biological and environmental factors account for the loss of approximately 52% of rice production worldwide; parasites contribute to this loss by about 21%. *Bacillus thuringiensis* (Bt) is a Gram-positive soil bacterium found in soil and can generate an extensive range of insecticidal protein toxins that exhibit restricted specificity towards distinct species of insects (David *et al.*, 2019). In the crystalline inclusions found inside the bacterium, these toxins, called crystal (Cry) and cytolytic (Cyt) proteins, can assemble. This bacterium also produces toxins known as vegetative insecticidal proteins (VIPs). During bacterial expansion, they are expressed.

To produce transgenic rice plants without marker genes, researchers used Agrobacterium-mediated transformation with the *rbcs* promoter, which is specific to green tissue. The *cry2AX1* gene, responsible for gene expression, was expressed in these plants. The transgenic plants exhibited a modest level of resistance against the rice leaf folder (*Cnaphalocrocis medinalis*) and the rice yellow stem borer (*Scirpophaga incertulas*) (Le *et al.*, 2022). Rice plant susceptibility to *Chilo suppressalis* larvae was reduced following RNA interference (APN1) and APN2 knockdown of two aminopeptidase N genes. Two *HaABCA2* knockout mutants were created using the CC9 genome editing approach. These mutants showed sensitivity to the SCD strain of

Helicoverpa armigera, a susceptible strain maintained without insecticide exposure. The purpose of this study was to investigate the causal relationship between *ABCA2* genes (*HaBCA2*) and *Cry2Ab* resistance in *H. armigera*. Both knockout mutants were found to have a loss in the exon location that was either 2 base pairs or 5 base pairs, respectively. The participants exhibited a sizeable resistance to Cry2Aa (more than 120 fold) and Cry2Ab (more than 100 fold), whereas they showed either no resistance or a meager resistance to Cry1Ac (4 fold) (J Wang *et al.*, 2017).

Improve herbicide tolerance

An additional critical desirable characteristic scientists attempted to incorporate into rice varieties is herbicide resistance. Numerous efforts have been undertaken to utilize transgenic lines; nevertheless, their effectiveness could be moderate because genetically modified organisms are subject to legal and economic constraints. Genome editing techniques enable the production of modified organisms devoid of transgenes. CRISPR/Cas technology has been shown in several experiments to produce herbicide-resistant plant types that are not genetically modified (Marone *et al.*, 2023; Yadav *et al.*, 2023). In the work conducted by Zuo *et al.* (2020), a total of 1,760 base pairs (bp) were removed from the α -6 nicotinic acetylcholine receptor (nAChR). The deletion was achieved by using *S. exigua* and the CC9 system, leading to the generation of a homozygous mutant called *Sea6-KO*. The *Sea6-KO* mutant exhibited a 373-fold resistance to spinosad and an 850-fold resistance to spinetoram, in comparison to the WH-S mutant. This resistance was seen without any changes to the genetic background. Genetic research discovered that this mutant is passed down through successive generations as an incomplete recessive characteristic.

Sun *et al.* (2016) used the CRISPR/Cas method together with homologous recombination (HR) repair to substitute the normal *ALS* gene with a modified form containing two particular mutations (W548 L and S627 I) that provide resistance to herbicides. Two single guide RNAs (sgRNAs) were designed to specifically target a genomic region ranging from 1,625 to 1,888 base pairs of the gene. The plasmid contains sgRNAs, a template for homologous recombination with precise point mutations, all contained within the Cas9 expression cassette. Since the target region for both single guide RNAs (sgRNAs) is present on the template, Cas9 has the ability to cut both the genomic DNA and the plasmid using the homologous recombination template. This allows *Cas9* to accomplish its objective. To facilitate recombination, the template is surrounded by two arms that are homologous to the target region: a left arm that is 100 base pairs long and a right arm that is 46 base pairs long. Particle bombardment of rice (Nipponbare) calli introduces the free DNA donor fragment and plasmid to produce adequate donor fragments for HR. This technique involves HR in all plants but in varying efficiencies. Sequencing every plant verifies the target area edition. Maize has employed a similar method (Singh *et al.*, 2023) still, it works significantly less well in rice, illustrating the need for adapting a successful technique from one plant species to accommodate other species. Furthermore, Cas9 combined with a base editor, such as cytidine deaminase (PmCDA1), which is found in the bacterium *Petromyzon marinus*, is an additional method that may be used to generate point mutations via the utilization of CRISPR/Cas. Target-activation-induced cytidine deaminase is also referred to as Target-AID. Two mutated versions of Cas9, dCas9 (without nuclease function) and nCas9 (with nickase function), are paired with the primary editor (Wu *et al.*, 2019). This allows the base editor to be guided to a particular location using small guide RNAs (sgRNAs). Rice plants resist the herbicide imazamox due to a specific point mutation in the ALS gene called *C287T* (Mishra *et al.*, 2020).

Improve nutrient use efficiency

The CRISPR/Cas technology can potentially speed up breeding processes that typically occur over years and involve conventional crossing and backcrossing methodologies. For instance, introducing an improved allele into commercial cultivars is an example of this occurrence. Specifically, the increased nitrogen utilization efficiency in indica rice may be attributed to a single nucleotide polymorphism in the gene that codes for the nitrate transporter *NRT1.1B* (Yang *et al.*, 2023). Substituting this gene may result in commercial cultivars that

have a higher efficiency in utilizing nitrogen. To replace the indica allele with the japonica allele throughout the process, Li *et al.* (2018) used the technique with some alterations. At first, the two tiny guide RNAs, also known as sgRNAs, are directed toward distinct DNA strands to lessen the amount of non-homologous sequences created when the vector is released. Following the completion of the HR implementation process, the next step is to make modifications to the PAM site inside the template sequence in order to avoid additional cuts. As a last point of consideration, a longer right arm has been proposed to increase the frequency of the homologous recombination (HR). Using this exceptional approach, the correct replacement of the allele, regardless of whether it is homozygous or heterozygous, is accomplished within 6.72% of the plants that have undergone this transformation. In addition, it was observed that partial HR and reverse complementation took place; nevertheless, no off-target changes were found in the investigated plants. The outcomes of this research indicated that the CRISPR/Cas technology has the ability to be utilized to accomplish adequate allelic replacement by HR in rice within a single generation, hence accelerating the process of agricultural progress. This was proven by the fact that the technology displayed the potential to be applied.

Challenges and ethical considerations

Even though the CRISPR/Cas9 technology offers a wide range of possible uses in crop breeding, it does have limitations (Le *et al.*, 2022) (Figure 5). The restricted gene pool that regulates crucial agronomic qualities presents a significant issue, given that these genes are necessary for this technology. In this context, there is a critical need to explore high-quality genetic resources for crop growth and to comprehend information about genomic sequences. It is crucial to highlight that further problems include inefficient transformation systems and the difficulties linked to plant regeneration from tissue cultures. Both challenges demand methods that are complex, technically demanding, and time-consuming. In addition, concerns about the biosafety of CRISPR/Cas9 may prevent its continued development.

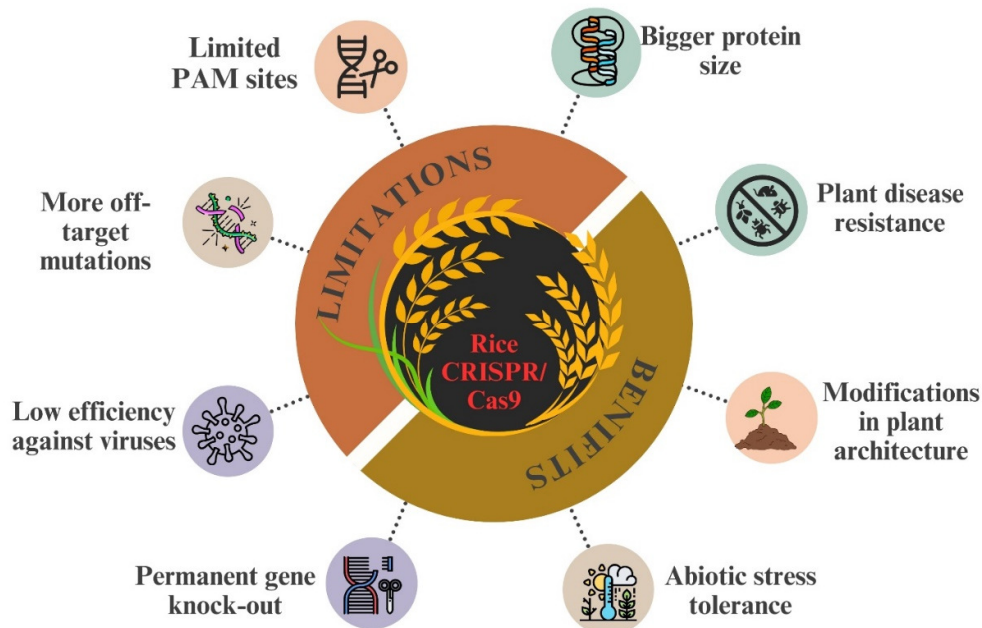


Figure 5. Limitations and beneficial aspects of CRIPSER/Cas9 genome editing in rice

One of the primary concerns about CRISPR technology is that it might change genes that are not supposed to be changed, which could have biological effects on the organism's surroundings that were not meant to happen. Random mutations may activate disease susceptibility genes. Moving chromosomal regions around and making the genome less stable can also occur when you change genes. By improving the design of the sgRNA, many ways have been thought of to lower the risks of Cas9 going off-target. Studies have shown that using sgRNA that has been cut short can reduce the number of unwanted mutations that happen at some off-target sites without affecting the efficiency of genome editing that happens on-target. Müller *et al.* (2016) and other researchers reported on a *Streptococcus thermophilus* Cas9 variant with an extended PAM sequence and reduced activity, which was unexpected. The NmeCas9 technology provides a safer alternative for precise genome editing; however, it is not as successful as SpCas9. Using paired Cas9 nickases was shown to create two single-strand breaks (SSBs) or nicks on distinct DNA strands. It is specific and works well without doing any damage to other DNA.

By picking the suitable sgRNA genome and experiment settings, the Cas9 off-target activity can be controlled. Minimal off-target activity can also be achieved by adjusting the length of gRNA and the amount of Cas9 enzyme present in living cells (Naeem *et al.*, 2020). Whole genome sequencing can be used to find the off-target mutants. According to GUIDE-Seq technology, DSBs can be found across the whole genome without bias using Cas9 RGEN. Digenome-seq and LAM-HTGTs can also be used to watch for changes not meant to happen (Manghwar *et al.*, 2020). To get close to isogenic lines in plants, mutants that do not have the CRISPR cassette can be crossed back with their wild-type parents with little risk. Like many other technologies, CRISPR is quickly changing and will be perfect.

However, there are still some problems with how gene editing is used. These problems can be lessened in rice and other crops so that this extraordinary technology can be used more effectively to improve crops. The first task for CRISPR-based genome editing devices is to make the PAM requirement less strict. A critical factor in CRISPR/Cas specialization is the unique PAM spot that SpCas9 needs. The exact PAM requirement is stringent, and it might slow down genome editing by reducing the regions that can be changed (Roy and Soni, 2021). On top of that, Khan *et al.* (2022) found that using 5'-NAG-3' by itself or with 5'-NGG-3' edits the rice genome effectively with few unwanted side effects. New versions of the Cas9 system, called VQR and VRER, have allowed even more gene editing in plants. According to a recent study, scientists have been trying to make changing VQR types in rice more effective. The newly revised CRISPR-Cas9-VQR method works exceptionally well for editing the genome quickly with 5'-NGA-3' PAMs (Uranga and Daròs, 2023). Recently, a new type of SpCas9 called xCas9 was discovered to recognize many PAM sequences, such as NG, GAA, and GAT (Qi *et al.*, 2023). Utilizing many Cas9 variants with distinct PAM specificities would increase the possibilities of genome editing. There is significant potential for enhancing these Cas9 variants to expand their recognition of a broader range of PAM sites and facilitate modifications in many genes of grain crops, particularly rice.

Improving the efficiency of gene replacement editing is the second obstacle that must be overcome. More substantial implications for crop improvement can be derived from precision editing strategies such as sequence substitution and fragment knock-in via human gene therapy. This is because gain-of-function mutations is necessary to develop more favorable characteristics for crop breeding. When it comes to plants, however, the efficacy of HR is relatively low, which makes gene replacement editing a challenging task. In addition to ensuring that the NHEJ and HR pathways are in a state of balance, there is a need for the development of more efficient genome editing tools that rely on homologous recombination (HR). It may be able to improve the delivery methods of the donor template DNA in order to make gene replacement editing easier. Making the modified rice and other crops successful early in the farmer's field is the third difficulty that must be overcome. Most of the research published on genome editing up to this point is only proof of concept under limited conditions. There is a possibility that there are uncertainties about the performance of the altered plant under natural environmental conditions. Having further field trials of the plants that have been modified will make this technical problem more clear.

Future prospects and research directions

Plants have been utilized to evaluate the efficacy of the CRISPR/Cas genome editing technology in inducing site-directed mutations. This technique is frequently applied to functional or agronomic investigations in plant species, most notably rice, which serves as a model for monocot molecular and physiological studies and is significant for agronomic investigations. Due to increased interest and ongoing research for new applications, new information, and advancements have been developed to apply this technique to rice and other plants. According to the studies, this method may produce massive gene deletions, minor InDels that induce frameshift mutations, precise base-editing systems that work with CRISPR/Cas to create point mutations, and homologous recombination at certain genomic regions to replace a gene. This technology makes it possible to make a group of mutants in more than 12,000 genes (Saber Sichani *et al.*, 2023). This method and huge sequencing improvements may create new rice lines with site-directed mutations without off-target mutations.

Furthermore, this method makes the creation of mutant plants without transgenes possible because transgenic organisms are tightly regulated. For the most part, this method makes it easier to study how different genes work and improve the genes of this critical species. Researchers worldwide working to improve rice genes choose this technology over others because it has many benefits.

The two best things about CRISPR/Cas9 compared to other genome editing methods are that it is easy to use and works well. The main benefit of the CC9 method is that it can change a lot of target genes at once. To carefully target six genes, Zsögön *et al.* (2018) did an experiment involving two stages, leading to four gene modifications. It is also possible for CC9 to introduce multiple off-target changes into the genome. On the other hand, modern versions of CRISPR/Cas that recognize different PAMs have made target base editing more effective in the ideal direction.

CRISPR/Cas9 is an extremely effective tool. However, it has substantial disadvantages that inhibit crop advancement. In order to develop and validate gene-edited plants, it is essential that breeders design a CC9-target gene vector transformation technique that is both adequate in quantity and efficient. Each type of plant that you want to target should have the application implemented correctly. This step could be important for making the gene-edited plants work. False gRNA, gRNA-independent methods, or non-specific spots cause off-target mutations. Off-target activity or changes in places other than the planned target spot cause a lot of concerns. The DNA could become fragile, and normal genes could stop working properly. Synchronized expression, genetic manipulation, and overlapping homology arms may precisely modify the gene evolved by preventing NHEJ or promoting HDR. Plant and mutant off-target mutations are frequently tolerated and may be eliminated via segregation or backcrossing. Bioinformatics tools like Cas-OFFinder and CCTop and methods like SELEX, IDLV capture, Guide-seq, and Digenome-seq have been made to find off-targets (Le *et al.*, 2022). The HDR pathway is not as efficient as the NHEJ pathway. One more problem is that not all countries are ready to promote their crops modified through gene editing. Aqib *et al.* (2022) give an overview of how CC9 can be used to alter the genomes of rice plants. It covers CC9 activity, transformation methodologies, agricultural trait improvement utilizing CC9, biosafety, and genome editing regulations. Multiple countries formally assert that crops will remain protected from biosafety regulations in the absence of unusual DNA sequences in the results of genome editing. The CC9 has been used in various genomic architectures to produce non-GMO mutant plants that satisfy the current biosafety standards for GMO plants. These plants may be used to investigate their function, tolerance to biotic and abiotic stresses, and other vital agronomic traits. In general, this method makes it easier to figure out how different genes work and improve the genetics of important species. CC9 is preferred by rice genetic improvement specialists worldwide because of these advantages. To use CC9 technology to create new types with genes that produce essential agronomical

traits, breeders must first find genes that increase output, improve quality, and make plants more resistant to biotic and abiotic stress. They can use these genes to make specific changes to make new types. Breeders should eventually use homology-directed repair (HDR) methods to make it easier to use breeding strategies to develop particular genes that are important for making novel varieties.

Conclusion

In comparison to transcription activators such as Zinc Finger Nucleases (ZFNs) and Effector Nucleases (TALENs), CRISPR/Cas9 technology accelerates, simplifies, and enhances the efficiency of genome editing for researchers. The platform strongly supports primary research and speeds up the process of turning study results into valuable products. Utilizing newly synthesized Cas9 variant proteins and associated proteins, such as SaCas9, Cas9-VRER, Cpf1-RVR, Cpf1-RR, and Cas9-VQR, the genome of rice has been edited. This has significantly expanded the number of genes susceptible to modification. Researchers have made the rice CC9 system much more effective at editing genes by using a natural promoter, improving sgRNA translation elements, and other optimization techniques. This has given them more powerful tools for editing genomes.

Additionally, novel genetic operating systems have been developed using CC9. Single-BE, gene insertion and replacement, and transcriptional control are a few examples. These systems have given scientists and crop breeders new ways to do primary research and improve crops. Transgene-free technology for rice is also a big step forward in ensuring genetic safety for food security. A quick editing or screening method can produce mutants without transgenic elements in their offspring, such as suicide genes or resistance. Although this impact is often controllable, the CRISPR/Cas system may potentially have off-target effects. Cpf1 has a reduced off-target effect on the rice genome compared to Cas9. Nevertheless, there are still significant remaining issues that must be addressed prior to the editing of the rice genome. For example, the CC9 system cannot be delivered efficiently without becoming part of the rice genome. Few accurate ways exist to knock in and swap native genes through HDR repair. Previous research has shown that SpCas9's ability to recognize NAG PAM is very different in human cells compared to rice cells. Therefore, the CC9 system seems to be an effective method for enhancing plant breeding. Nevertheless, the challenges and concerns associated with the widespread use of the CC9 system may impede its advancement. Because plant and animal cells are different, much work still needs to be done on studying the CRISPR/Cas system in rice.

Authors' Contributions

Conceptualization: AM, TAYA and FMA; Writing - original draft: MT, MA; Validation and Visualization: HFNA, AW, BAK, MIA and TAK; Software: AM, ZE, CCT, HA, TAYA and FMA; Funding acquisition: FMA; Writing - review and editing: AM and TAYA and FMA; All authors agree to be accountable for all aspects of this study

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

The authors extend their appreciation to the Deanship of Research and Graduate Studies at King Khalid University for funding this work through Small Research Project under grant number RGP 1/43/46.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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