# Research On Genetic Therapy with CRISPR Genome Directed Editing Technology

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**Abstract.** CRISPR/Cas is an acquired immune response mediated by RNA that is ubiquitous in bacteria and archaea. CRISPR/Cas9 is considered a new generation of gene editing technology due to its simple design, high editing efficiency and strong adaptability. This article summarizes the development and application of CRISPR/Cas technology in gene editing field, and analyzes its main impact factors for the reference of researchers.

**Keywords:** CRISPR/Cas; Gene Editing; Plants; Genomic Site-Specific Modification; Guide RNA.

## 1. Introduction

Genome-specific editing is an important molecular tool that can regulate the expression of target genes at the transcriptional level. It can achieve the target gene knockout and spot knockout, or regulate the expression of the target gene at the transcriptional level. The existing DNA point editing technologies include ZFNs, TALEN, CRISPR, etc., which can realize the accurate localization of DNA. The CRISPR/Cas system is composed of Cas proteins encoded by CRISPR-related sequences. It has many advantages such as simple operation, low cost and good effect.

## 2. CRISPR/Cas architecture

CRISPR, a small fragment located near the K12-base phosphatase (ALP) gene in E. coli, has been reported to be found in about 40 percent of the genome in recent years. Cas is a group of replication-related proteins located in the CRISPR region that interact with CRISPR and have important implications for CRISPR resistance. Next to the Cas gene is a transcription-activated RNA segment that helps CRISPR RNA mature. Using CRISPR/Cas technology, pathogens have developed a unique immune system that can effectively defend against foreign genes. The CRISPR/Cas system can be divided into types I, II and III according to the function of Cas genes. Cas9 technology is CRISPR/Cas9 technology, which is the most commonly used CRISPR/Cas9 technology at present. As shown in Figure 1, the spacer sequence of CRISPR is homologous to the sequence of some phages or plasmids (image cited in Genome-editing technologies and their potential application in horticultural) crop breeding, which provides the host cell with an ability to resist heterologous gene infection, is an acquired immune system evolved from bacteria and archaea. In the CRISPR region, there are a number of highly conserved CAS-binding proteins, which are usually connected to the replication region of the CRISPRs region and constitute the immune response pathway in the CRISP region. Cas is a polymorphic group that encodes proteins containing domains that bind to DNA to make site-specific cuts to DNA that invades the cell, thereby cutting DNA inside the cell. In the first repeat region of the CRISPR locus, the precursor structure can act as a promoter to generate a series of CRISPRRNAs, which work collaboratively with Cas proteins and play an important role in the host immune response. The completion of the project will reveal the role of CRISPR/Cas in host cells.

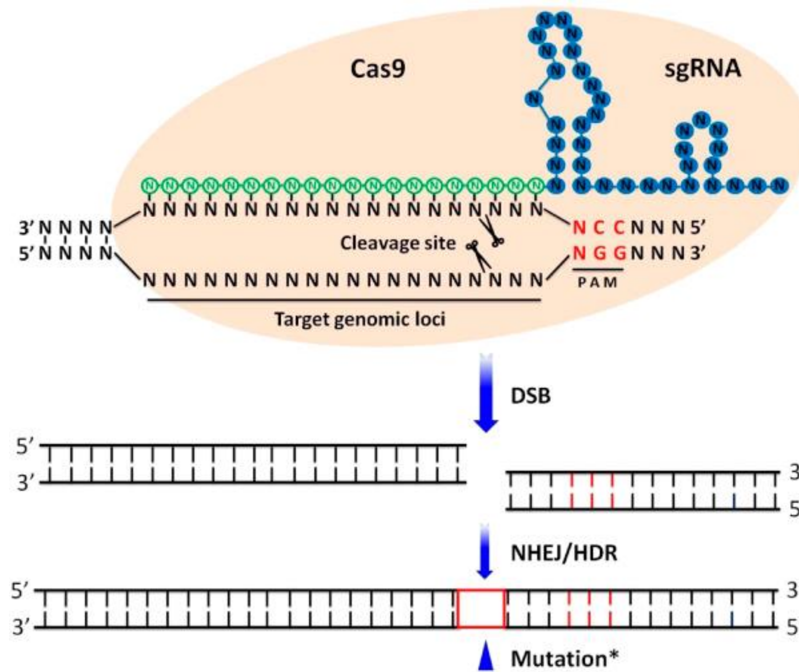


**Fig. 1** CRISPR site structure

Makarova et al. divided CRISPR systems into three types based on the difference of Cas genes in the mechanism of action and the difference of repeated sequences in CRISPR gene clusters: Type I system exists in bacteria and archaea, type II system mainly exists in bacteria, and type III system mainly exists in archaea. In type I and Type III CRISPR/Cas systems, a complex of multiple proteins, such as Cas3, Cas6 and Cas10, plays a role, and its working mechanism is very complex. The Class II CRISPR/Cas system requires only the participation of Cas9 Nuclease, which is the most suitable for gene editing [1]. The Cas9 nuclease complex consists of the Cas9 protein and two non-coding Rnas -pre-crRNA and trans-activating crRNA, both of which are transcripts at CRISPR loci, pre-crRNA forms a heterodimer with trans-activating crRNA, which co-binds to the Cas9 protein, which specifically cuts the pre-crRNA based on the invading DNA, resulting in longer and longer crRNA. The guide sequence gRNA with a length of 20 bp is attached to it, and the guide Cas9 complex cuts the target DNA under the complementary action of the guide sequence and the target DNA sequence. At the 3' terminal of crRNA, Cas9 is required to form a PAM region at the 3' terminal of crRNA, and three bases upstream of this region are cut by Cas9 enzymes. Due to the selectivity of the Cas9 binding site, the Cas9 nuclease, pre-crRNA, and tracrRNA3 genes must be directed at a 20 bp sequence. Some scholars proposed to use cas9 and sgRNA2 components to build CRISPR/Cas system to achieve specific and efficient cutting of target genes. How the CRISPR system works in bacteria is shown in Figure 2.

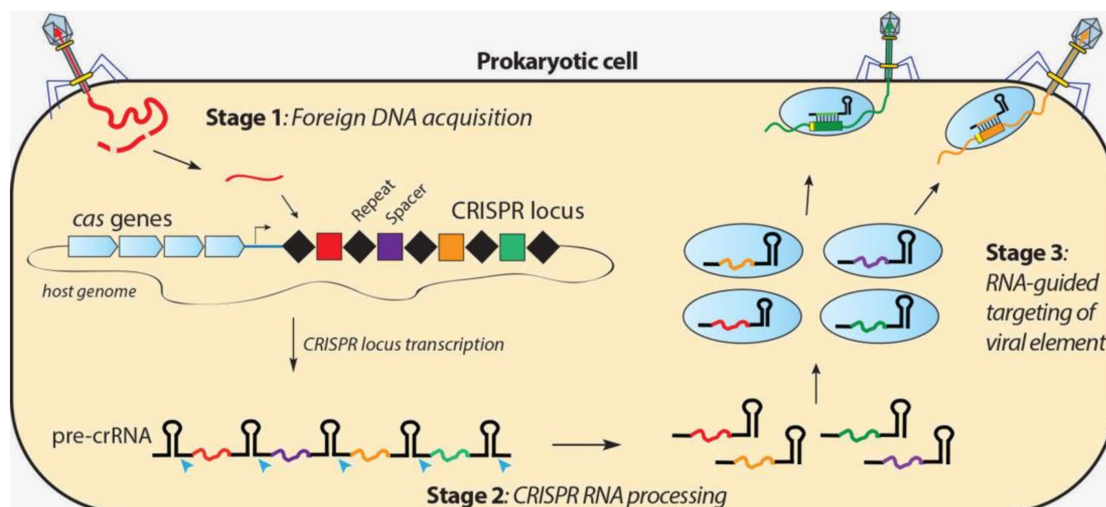


**Fig. 2** Mechanism of action of the CRISPR system in bacteria

## 3. Important factors affecting gene editing

### 3.1. Oriented gRNA screening

The existing crRNA and tracrRNA combine to form a single loop RNA (sgRNA). In the previous work, crRNA and tracrRNA were expressed independently to construct a double-directed RNA. Previous studies have found that compared with sgRNA, display RNA is more likely to produce Indel gene mutations in the error-prone repair system NHEJ. In addition, the assembly of the two components of sgRNA-tracrRNA is more efficient than that of CrNA-TracrRNA, which is one of the reasons why sgRNA-tracrRNA is faster than double-strand RNA. The length of tracrRNA is also an important factor [2]. In human gene editing, although sgRNA (+48) is highly efficient at cutting target DNA, its mechanism of action remains unclear. However, in organisms, sgRNA (+72) and sgRNA (+84) have longer tracrRNA3' terminus (+72) and sgRNA (+84), respectively. The increase of stem ring structure can enhance the stability of sgRNA and promote its spatial conformation in Cas9: SGRna-DNA trimer. Existing Sgrnas are generally in the 100 nucleotide range, including the 5' terminal 20 nucleotide sequence and the 3' terminal 70-80 nucleotide sequence.

### 3.2. Design of target DNA

In the design of the target DNA, it should first determine whether the 3' end of the target DNA contains PAM, and then select the target DNA according to the characteristics of the promoter. Both the U6 promoter and T7 promoter require G and GG to be the transcriptional starting points for RNA polymerase III. Therefore, the target sequence of sgRNA is restricted to GN16 to 19 or GGN15 to 18. In order to solve this problem, the academic community proposed two solutions: one is to take the first or first two bases of the 5' terminal of sgRNAs as the wrong sequence, ignoring the influence of promoters on gene expression. The second is to add G or GG to the end of sgRNA5'. Both existing methods can produce efficient sgRNAs, but both can reduce the effect of gene editing [3]. The academic community intends to verify the effect of sgRNAs deletion and prolonged sgRNAs on gene editing through large samples.

### 3.3. Expression and distribution of Cas9 and gRNA in genetic engineering

After obtaining the corresponding sgRNA, the next step is to study the application of Cas9 and sgRNA in target animals and cell lines. Currently, the technology to transfer the Cas9 gene into sgRNA has been developed in a variety of animals and cells. The rapid and efficient expression of Cas9 and sgRNA in mammalian mammary glands was achieved by electroporation, nuclear transfection and liposome transfection. At present, Cas9 genetic engineering technology has been successfully applied to Cas9 gene editing in humans and mice [4]. In addition, the RNA can be

injected into zebrafish, fruit flies and mice. The complex formed by Cas9 and sgRNAs was cultured in vitro and then injected into the parasite. In addition to model animals and cell lines, Cas9 is also widely used in a variety of crops, such as wheat, rice, sorghum, tobacco, Arabidopsis, and others. A large number of experiments have proved that Cas9 and sgRNAs can achieve efficient gene editing [5]. Although combining Cas9 with sgRNAs can enhance gene editing, there is also an "off-target" phenomenon. In addition, Cas9 is Cas9 from a variety of pathogens, and its expression in eukaryotes needs to enter the nucleus to achieve gene editing. It is necessary to add eukaryotic nuclear localization signals (NLS) to the N and/or C terminus of the Cas9 protein.

### 3.4. Strategies to improve targeting specificity.

At present, the molecular mechanism of Cas9 nuclease specificity has not been fully elucidated, but some new methods have been developed to enhance Cas9 nuclease specificity and reduce "off-target" phenomenon. One is to reduce the way sgRNA binds to Cas9, but the effectiveness of this approach is unclear. Reports have shown that this new technique can effectively reduce the variation of off-target/localised genes, but it has also been suggested that this new technique may reduce both variants. The second approach is to use Cas9 gene editing technology (D10A and H840A) to achieve DNA damage repair [6]. This is because damage to a single strand of DNA results in more fidelity than damage to two strands of DNA. In order to increase the number of bases that are specifically complementary to the target gene, some scholars plan to use Cas9 lyase to double-split sgRNA. Two widerspaced gaps were created on the same target gene, thus realizing the regulation of the double-stranded structure. Because the technology can accurately repair gaps at off-target sites, the specificity of the technology is more than 1,500 times that of wild Cas9. Since NHEJ is clipped by N-acetylase at the N-methylase flat and sticky ends of Cas9, N-methylase clipping and non-methylase splicing result, thus initiating damage repair of NHEJs. Some scholars plan to introduce a sgRNA, a Cas9 shear enzyme and a genetically engineered gene of a homologous recombinant gene into cells to achieve efficient and accurate repair of homologous recombinant genes. In addition, due to the long homologous arm in the homologous gene sequence, the production of ectopic genes can be effectively reduced [7]. The third class is to truncate and modify the sgRNA, by truncating the 3' end of the sgRNA and the sequence corresponding to tracrRNA, adding two GG at the 5' end, or adding 2-3 bases at the 5' end, to enhance the selectivity of Cas9 to sgRNA. The former will lose part of the gene editing effect, and the latter will obtain the same effect as the full length of SgRNA, and can reduce the off-target variation of SgRNA, and improve the sensitivity of SgRNA to SgRNA: DNA single base mismatch [8]. The above approaches and strategies can be combined to reduce the occurrence of off-target sites. Through in-depth analysis of the structure and function of Cas9 protein, as well as reasonable modification and directed evolution of Cas9 protein, the specificity of Cas9 protein can be further enhanced.

## 4. Targeted editing application of Cas gene

CrRNAs and tracrRNAs can form a Mosaic RNAs called a guide RNAs. Under the regulation of sgRNAs, Cas9 cuts the target gene into DSB, thus achieving CRISPR/Cas gene editing [9]. Using the Type II CRISPR/Cas system, researchers have developed a set of convenient and fast gene editing techniques and conducted extensive studies in E. coli, Diplococcus pneumoniae, yeast cerevisiae, zebrafish, mice, mice, multiple human cell lines, fruit flies, nemworms, rats, rice, wheat, Arabidopsis and tobacco, among other tissues and organs. Some studies have used CRISPR technology for site-specific mutation of target DNA for the first time, and obtained the double strand break (DSB) on target DNA, which laid a good foundation for the follow-up research of RNAi. The results showed that the HNH and RuvC regions of Cas9 were double-stranded with the first 3-8 bp of the target DNA PAM. In addition, when crRNA binds to tracrRNA to form a single RNA, Cas9 also induces the cas9 protein to bind to the target gene, thus cutting the DNA. Subsequently, Church et al. used Type II CRISPR/Cas technology to successfully find a series of gene variants caused by NHEJ damage repair

patterns in multiple tumor cell lines, and the variation rate varied with tumor cell types and RNA carriers, with a variation rate of 2%-38%. In addition, Cong et al. established a type II CRISPR/Cas system and confirmed that Cas9 nuclease can specifically cut human and animal genes at the lower site mediated by lncRNA. The error matching test showed that the base error at the 5' terminal of crRNA had no significant effect on its shear ability [10]. The effect of CRISPR/Cas9 technology for site-directed mutation of EMX1 site is comparable to that of TALENs technology for site-directed mutation of EMX1 site, and it is better than TALENs technology. Some scholars have conducted in-depth studies on sgRNA and found that the lengthening of the 3' end of RNA can increase the mutation rate of the target DNA. In this process, the combination of sgRNAs and Cas9 is the key link [11]. Previous studies have found that CRISPR/Cas9 technology with sgRNA as the core can cut specific DNA fragments of zebrafish, and its mutagenicity and pathogenicity of fh1, apoea and other related genes are comparable to that of TALENs. Some studies used CRISPR/Cas9 technology to construct the mutant strain drd3,gsk3b. Previous studies have found that GRNA-targeted CRISPR/Cas9 technology can carry out synchronous site-specific mutations on multiple genes such as Tet1,Tet2,Tet3, Sry, and Uty. Figure 3 shows a model of the CRISPR/Cas9 system (image cited in CRISPR/Cas9 Mediated Targeted Mutagenesis in the Liverwort Marchantia polymorpha L).

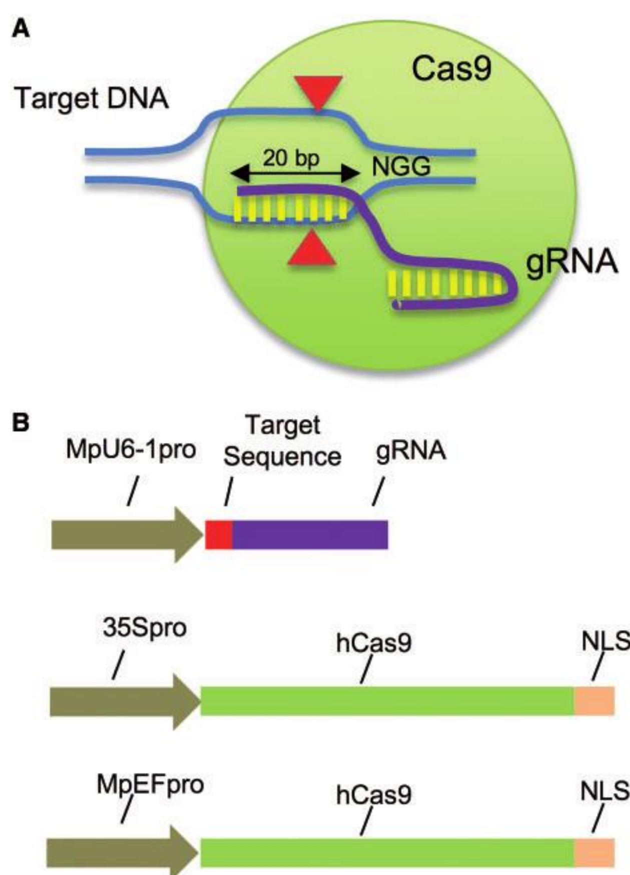


**Fig. 3** Model diagram of CRISPR/Cas9 system

In addition, the research of CRISPR/Cas technology in plants has also made important progress. On August 8, 2013, Nature Biotechnology published a report on the work of three research groups on the site-specific knockout and insertion of multiple genomes of important crops such as rice, wheat, model plants Arabidopsis thaliana, and white-backed planthopper, and conducted four site-specific mutations in rice and one site-specific mutation in wheat [12]. The mutation rate of CRISPR/Cas system can reach 14.5%-38.0%, while the mutation rate of transgenic plants can reach 4.0%-9.4%. In the earlier stage of the study, transgenic plants with three candidate genes, OsPDS, OsMPK2 and OsBADH2, were successfully constructed by RNA-seq technology, and a transgenic plant with white and short traits was constructed [13]. At the same time, the system can also use single strand nucleic acid (ssDNA) as a template to insert 12 bp double-strand DNA fragments into the key site of DNA

damage. Some scholars are the first in the world to prove that CRISPR/Cas can achieve targeted gene editing of crops, which will greatly promote the improvement of traits and molecular target cultivation of important crops such as rice and wheat, and it is also the most played article of NatureBiotechnology in August 2013. Newsfocus, a journal of Science, published an article that highly affirmed the research results of CRISPR/Cas for fixed-point editing and genetic engineering on the genomes of a variety of organisms, and studied the OsPDS gene. Using CRISPR/Cas technology, JenSheen's research group successfully carried out CRISPR/Cas whole gene point mutations in two types of tobacco, and the mutation rate could reach 1.1% to 38.5%. CRISPR/Cas technology allows simultaneous editing of two genes or two different locations in the same gene at the same time. At the same time, using the double-stranded DNA as a template, a restriction enzyme degradation site with the size of 6 bp was accurately embedded into the PDS encoded protein of the plant by using DNA homologous recombination technology. Sophienamoun et al. used CRISPR/Cas technology to successfully obtain a locus of this gene in tobacco with a mutation rate of 1.8%-2.4%. Subsequently, Zhu Jianjiang's research group successfully obtained point mutations of this gene in two different types of rice and Arabidopsis thaliana using CRISPR/Cas technology, and the mutation rate reached 5%-84%. Previous studies have found that after GAI knockout, Arabidopsis T1 showed obvious dwarfization phenotype, while after YSA knockout, Arabidopsis T0 showed obvious bleaching.

## 5. Conclusion

CRISPR/Cas is an emerging gene point editing technology developed in recent years, which has achieved good results in plants, animals and microorganisms. Due to the wide application of this technology, a new revolution will occur in animal and animal gene function, which will bring new opportunities and challenges to the development of life science and life science. At the same time, CRISPR/Cas technology also brings many new problems, such as "off-target effects", the ethical issues involved in genetic modification in embryonic cells, and so on. However, with the further development of CRISPR/Cas technology, CRISPR/Cas technology will become more and more mature and more widely used in all aspects of life science.

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