ISSN 2959-409X

The Potential of Gene Editing Technology in Agriculture: Cre-Loxp and CRISPR-Cas9

Rou Duan

College of Agronomy, Hunan Agricultural University, Hunan, 410124, China
Corresponding author: 20021010170@stu.hebmu.edu.cn

Abstract:

In recent years, gene editing technology has made breakthrough progress. This article uses Cre-loxP and CRISPR-Cas9 as examples to explore its application prospects in the agricultural field. Current research has made substantial progress in using these technologies for genetic modification of crops. However, there is currently a lack of comparative studies on Cre-loxP and CRISPR-Cas9. This article analyzes the Cre-loxP and CRISPR-Cas9 systems that rely on Cre enzyme and Cas9 endonuclease for targeted cleavage respectively, summarizes the mechanism and functional research of the two technologies, and discusses their application in agricultural gene editing. The shortcomings and challenges of both technologies are discussed, and some suggestions for improvements are made. The significance of this study is to provide a comprehensive review of the development trends of gene editing in the agricultural field. The findings contribute to the existing literature by highlighting the potential of Cre-loxP and CRISPR-Cas9 in the genetic modification of crops. However, issues such as technology limitations and off-targeting still require close attention. All these challenges and shortcomings will be addressed in future studies to promote the application of CRISPR-Cas9 and Cre-loxP in agriculture.

Keywords: Cre-loxP; CRISPR-Cas9; agriculture.

1. Introduction

Agriculture is the foundation of human survival and development. In order to meet people's growing requirements for crops, improve crop yields and crop quality, and enhance disease resistance and stress resistance to biotic or abiotic stresses, crop breeding technologies must be innovative and Continuously improve. Gene editing technology is a precise method to edit genes for the study of gene functions and the improvement of crop breeding. It refers to using gene editing tools to accurately insert, replace, and delete target genes, thereby changing the transcription and translation processes. This ultimately leads to changes in protein expression. Commonly used methods for gene editing include CRISPR-Cas9 and Cre-LoxP, both of which have attracted attention due to their accuracy and simplicity. This article will use CRISPR-Cas9 and Cre-LoxP as examples to explore the application scenarios and development prospects of gene editing technology in gene function research and agriculture.

CRISPR-Cas9 and Cre-LoxP use different methods to change genes at target sites, and both methods have their advantages and disadvantages. Cre-LoxP is based on E. coli phage P1. Cre is a DNA recombinase that specifically recognizes and cleaves the Loxp site, which is a 34 bp DNA sequence. When Cre recombinase is expressed, depending on the initial arrangement of the LoxP recombination sites, cells containing LoxP recognition sites in the genome will undergo three recombination events: inversion, translocation, or shearing [1].

Compared with Cre-loxP, CRISPR-Cas9 is a more efficient gene editing tool. CRISPR-Cas9 uses Cas9 protein and single guide RNA (sgRNA) complex to bind to target DNA. This causes the Cas9 protein to localize to a specific site and form a Double-strand break, DSB [2]. After the Cas protein cuts the DNA, the DSB will be repaired using the cell's own DNA repair mechanism. There are two different repair pathways, namely non-homologous end joining (NHEJ) repair and homology-directed repair (HDR). This leads to gene knockout, gene insertion, or gene modification at the target site [3].

The continuous innovation and development of gene editing technology have brought huge opportunities and challenges to the agricultural field. CRISPR-Cas9 and Cre-LoxP are both naturally occurring genome editing tools. Although they have not been discovered for a long time, they are both powerful gene editing tools. This article discusses the latest developments and prospects of CRIS-

PR-Cas9 and Cre-LoxP in agriculture, compares the advantages and limitations of CRISPR-Cas9 and Cre-LoxP, and provides optimization suggestions. It is possible to better understand the importance of gene editing technology to agricultural development and provide strong support for the sustainable development of future agricultural production.

2. Research on Working Mechanisms and Gene Function

2.1 Cre-LoxP

2.1.1 Principle

Cre(Cyclization Recombination Enzyme)is a recombinase derived from bacteriophage P1. Its coding region has a full length of 1029 bp, 38 kDa, and consists of 343 amino acids. The C-terminal domain of the Cre protein contains a catalytically active center, which can perform DNA recombination at different positions on the DNA; at the same time, Cre can also recognize a specific DNA sequence (loxP), thereby achieving gene recombination at two loxP (Locus of X-overP1)sites.

LoxP is a 34 bp coding sequence located in phage P1, which contains two 13 bp palindromic sequences and an 8 bp spacer sequence. The reverse palindromic region is the recognition and binding region of Cre recombinase, while the spacer sequence controls the direction of LoxP [4].

2.1.2 Induced Gene Recombination Methods

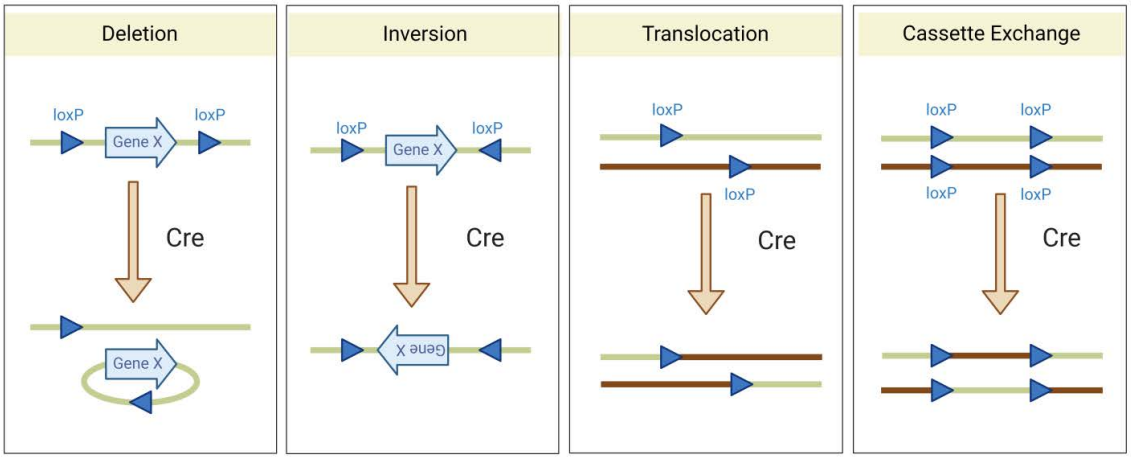

Fig. 1 The location of the Loxp site

As shown in figure1, according to the location of the Loxp site, there are the following four situations:

① If two LoxP sites are located on one DNA strand and in the same direction, Cre recombinase can effectively delete the sequence between the two LoxP sites (Deletion);

② If two LoxP sites are located on a DNA strand in opposite directions, Cre recombinase can induce sequence inversion between the two LoxP sites(Inversion);

③ If the two LoxP sites are located on two different DNA strands or chromosomes, Cre recombinase can induce the exchange of the two DNA strands or chromosomal translocation, that is, gene transposition (Translocation) [4].

④ If the four LoxP sites are located on two different DNA strands or chromosomes, Cre recombinase can induce sequence exchange between LoxP (cassette exchange).

2.1.3 Epigenome Editing

DNA methylation is a chemical modification of DNA that changes the genetic status without changing the sequence of the DNA. DNA methylation plays an important role in the developmental regulation of plant gene expression and is also an important means for plants to defend against foreign intrusion of DNA. However, this is a direct cause of transgene position-dependent silencing. Transgene inactivation caused by other reasons is often related to DNA methylation or directly manifested through methylation. Expression of Cre enzyme in plants causes hypermethylation of DNA, and methylation of the lox site inhibits gene deletion. Ruochen Liu et al. improved the efficiency of Cre-mediated DNA recombination in tobacco by reducing DNA methylation (including CG and non-CG methylation) by inhibiting the expression of de novo methyltransferases [5].

2.2 CRISPR-Cas9

2.2.1 Principle

CRISPR-Cas systems have significant diversity and are divided into type 1 and type 2. CRISPR-Cas9 belongs to the type II CRISPR-Cas system.

In the endogenous system, the complex composed of mature CRISPR RNA (crRNA) and trans-acting CRISPR

RNA (tracrRNA) is called single guide RNA (sgRNA). Directing Cas9 to the target site CRISPR/Cas9 uses the base-pairing interaction between crRNA and the target site to achieve the cleavage of specific sequences [6]. This sequence is adjacent to a short sequence called a protospacer adjacent motif (PAM). PAM is an important recognition sequence that determines the cleavage of the target strand. After the target sequence is combined with Cas9, the nuclease domain of Cas9 is used to cleave HNH and RuvC. During this process, the RuvC and HNH regions cause DNA breaks, leading to DNA double-strand breaks (DSB). DNA double-strand breaks are completed by the cell's own DNA repair mechanism. Achieved through two methods: non-homologous end joining (NHEJ) and homology-directed repair (HDR) [3].

2.2.2 Functional Study

As shown in Figure 2, CRISPR/Cas-mediated genome editing has numerous functions.

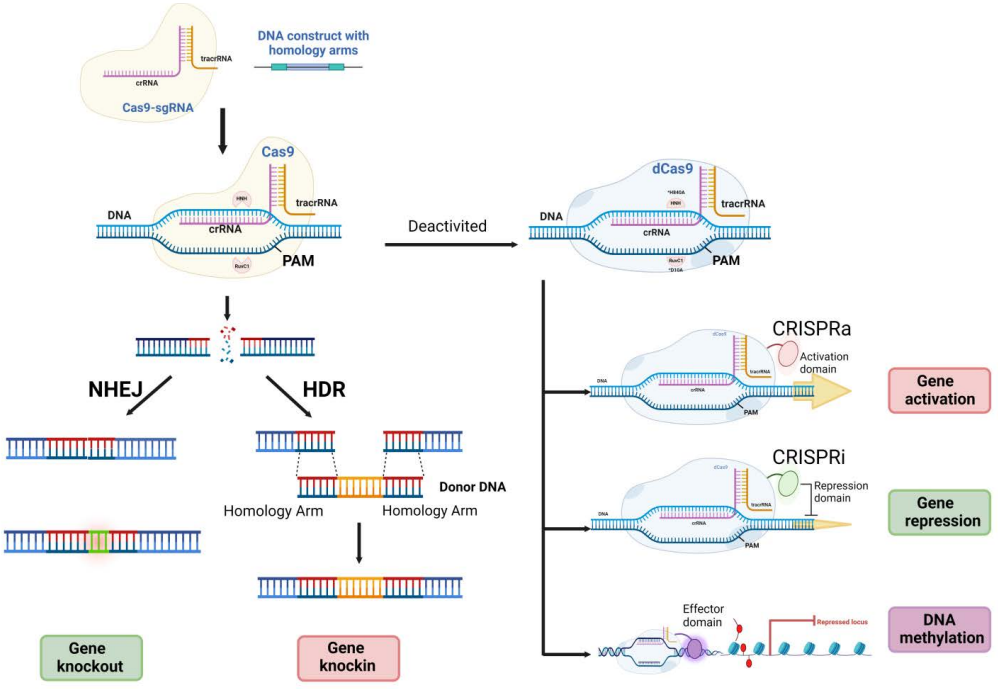

Fig. 2 Functions of CRISPR-mediated genome editing

1) Gene knockout/silencing:

NHEJ is a common way to repair DNA damage. Still, the damaged DNA ends usually undergo small insertion or deletion mutations in the genome, interfering with or deleting certain functional genes or genome target elements. Based on this, using CRISPR-Cas9 technology, NHEJ can achieve site-specific knockout of target genes without relying on donors. Tianyuan Su et al. proposed CA-NHEJ, a DNA damage repair technology based on NHEJ, which is suitable for large-scale DNA deletions [7].

Gene silencing caused by CRISPR-Cas9 has many advantages. CRISPR-Cas9 can target specific genes and avoid interference from other genes to achieve precise and efficient gene silencing. Silencing of different genes can also be achieved through simple design with high flexibility.

2) Gene knock-in:

After the DNA strand breaks, the DNA damage repair system prefers NHEJ to HDR, which is a major challenge for effective gene editing. The HDR pathway is only active in the S/G2 phase and is greatly affected by donor concentration. Recent studies have shown that by changing the structure of the hairpin and introducing it into the mutation site, HDR-mediated gene knock-in can be effectively improved, and PPRH-mediated damage repair can be achieved [8].

3) Gene enhancement and knockdown:

The two endonuclease domains of RuvC and HNH were mutated so that aspartic acid at position 10 of RuvC was mutated to alanine (D10A), and histidine at position 840 of HNH was mutated to alanine (H840A), the Cas9 protein will lose its activity, called dead Cas9 (dCas9) [9]. Although dCas9 loses the ability to cut DNA double strands, it can still bind to specific DNA sequences.

dCas9 can perturb target genes during the transcription process by binding to various activators (such as VP64, p300, etc.) or inhibitory factors (such as KRAB, LSD1, DNMT3A/3L) [10].

CRISPR-mediated gene transcription activation (CRIS-

PRa) technology is a new gene editing technology that can achieve site-specific expression of genes. It has been widely used in high-throughput functional acquisition genome testing and cell model design [11]. CRISPR-dCas interference technology (CRISPRi) can effectively inhibit gene transcription and expression and knock down genes. Nicolas Marx et al. demonstrated that promoter methylation can stably downregulate gene expression by targeting the dCas9-DNMT3A3L complex [12]. In mammalian cells, it has been demonstrated that the CRISPR-dCas system can directly act on the TSS region or downstream of the gene transcription start site. However, there is still little research on CRISPRi in plants. Currently, there is only one case of partial suppression of a maize gene through CRISPRi.

2.2.3 Epigenome Editing

Using CRISPR/Cas technology to modify DNA methylation is an important means to regulate gene expression and traits. Although dCas9 has lost its catalytic function, it still has a strong binding ability to target proteins. Therefore, dCas9 can achieve methylation and demethylation of DNA through fusion with methyltransferase or demethylase. Papikian et al. achieved efficient localization of DNA methylation, including the FWA promoter, through the dCas9-SunTag system [13].

3. Application Progress

3.1 Cre-LoxP

3.1.1 Insect-resistant and Herbicide-Resistant

Pests and weeds have a serious impact on crop yield and quality. The long-term use of chemical pesticides and herbicides has led to increased resistance to pests and weeds, hindering the growth of crops. Therefore, Cre-LoxP can be used to develop insect-resistant and herbicide-resistant transgenic varieties to reduce the harm caused by chemical agents. In recent years, Zhang used the Cre/loxP system to control the expression of the Cre gene in the endosperm using the tissue-specific promoter gluC. By feeding stem borer and spraying glyphosate, the gene was tested for effective insect resistance and herbicide resistance. At the same time, the specific knockout efficiency of this recombinant system in rice endosperm was measured, and insect-resistant and herbicide-resistant transgenic rice without exogenous genes in the endosperm was cultivated [14].

3.1.2 Delete Screening Marker Genes

Genetically modified technology has the advantages of high yield and high efficiency, but most of the currently used selectable marker genes come from non-food-borne microorganisms, which poses food safety risks. Therefore, it is very important to establish an expression vector that does not require a selectable marker gene in transgenic plants or to delete its marker in transgenic plants.

Wang used the Cre gene as the research object and compared and analyzed the effects of different promoters on the Cre gene. He found that only the promoter on the pollen/seed promoter can achieve complete recombination of the Cre gene, proving that the PAB5 promoter is used to regulate the genes of the Cre/LoxP system. Knockout efficiency [15]. Zhang et al. used an Agrobacterium tumefaciens-mediated method to transform the Cre-loxP GmPTF1 gene into Yudou 22. Under β-estradiol induction conditions, the selectable marker gene was eliminated through the Cre-loxP system, and GmPTF1 transgenic soybeans without selection markers were obtained [16].

The Cre-LoxP system is not only highly efficient, accurate, and fast in eliminating screening marker genes, but the system can also be expressed in the T0 generation, thus simplifying the operation and saving time.

3.1.3 Creation of Artificial Male Aterile Lines and Restorer Lines

Based on the characteristics of Cre-LoxP and the characteristics of the F1 generation of plant hybrids, a sterility gene was inserted between two homologous loxp loci to transform the plant into a sterile line. Using the Agrobacterium-mediated method, the Cre gene was introduced into the sterile line to separate the expression between loxp sites in the same direction, thereby restoring its fertility. Cao, Bihao, et al. used the Cre-loxP system to control the expression of Barnase gene in plants to achieve plant fertility regulation and then produce eggplant hybrid seeds [17].

3.2 CRISPR-Cas9

3.2.1 Resistant to Diseases

Plant diseases are plant diseases caused by fungi, bacteria, viruses, and other pathogens. Diseases will have a certain impact on the normal growth of plants, leading to reduced yield and quality, ecological imbalance, environmental pollution, and other hazards.

Rice blast and bacterial blight are important agricultural diseases in my country. Knocking out OsERF922 in japonica rice Kuiku131 using CRISPR-Cas9 technology can significantly enhance rice resistance to rice blast. Editing the coding region of the OsSWEET14 gene can effectively improve bacterial blight resistance. Editing a disease resistance gene through CRISPR-Cas9 can not only enhance resistance to a certain disease but also achieve the purpose of collaboratively improving multiple resistances. Studies have shown that a single knockout of Pi21 and

ERF922 not only has significant resistance to rice blast but also significantly improves resistance to bacterial blight. Knockout of OsVQ25 in rice confers synergistic resistance to blast and bacterial blight [18].

3.2.2 Resistance to Abiotic Stress

Abiotic stresses include drought, salinity, low temperature, high temperature, heavy metals, etc. These stress factors will not only have a serious impact on the growth, yield, and quality of plants but will also cause serious harm to agricultural production.

By using CRISPR-cas9 technology, the genes of organisms can be precisely edited to make them more resistant to stress. For example, CRISPR-Cas9 technology was used to knock out the Arabidopsis transcription factors AtWRKY3 and AtWRKY4, and found that the AtWRKY3 and AtWRKY4 genes were highly expressed by salt and methyl jasmonate. Using CRISPR-Cas9 technology, the tomato high temperature stress response gene SIAGL6 was gene edited to improve the heat tolerance of tomatoes under high temperature stress. After the three genomes of OsPIN5b, GS3, and OsMYB30 were knocked out using CRISPR-Cas9, phenotypes such as high yield and low-temperature tolerance were obtained [19].

The CRISPR-cas9 gene also has certain limitations in enhancing its ability to enhance stress. For example, its resistance to stress can be reduced through the design of sgRNA and the use of more specific nucleases (such as Cas12a, Cpf1, etc.). At present, transgenic technology represented by Agrobacterium tumefaciens has problems such as low efficiency and easy to cause random integration. However, the use of new gene editing technologies, such as electroporation technology, is expected to achieve efficient and precise gene editing [19].

3.2.3 Improve Quality

CRISPR-Cas9 is used to improve the quality of crops. Currently, there are several rice varieties (GS3, GW2, GW5, GW6, TaGW7, etc.) that are involved in important genes that regulate fruit shape and size. By knocking it out or modifying it, the grain length, thousand-grain weight, grain width, and other characteristics of rice can be improved, thereby improving the appearance and quality of crops. CRISPR-Cas9 can increase the amylose content of rice by regulating Wx, thereby improving the eating quality of rice. Using CRISPR-Cas9, the nutritional quality of carotenoids, GABA, iron, zinc, and other nutrients can also be increased [20].

Plant-specific carriers constructed using CRISPR-Cas9 technology have a certain off-target effect, but their off-target mutation rate in crops is low. During the breeding process, in order to maintain favorable off-target mutations, highly specific sgRNA can be used, and high-fidelity Cas9 digestion and RNP delivery can be used to reduce off-target phenomena [20].

4. Conclusion

Research on two gene editing technologies has found that they have great potential to improve crop quality, increase yields, and resist pests and diseases. The application of Cre-loxP and CRISPR-Cas9 technologies in agricultural production has provided new ideas and methods for agricultural production. The application of this gene editing technology is of great significance. It can not only help farmers increase crop yields and reduce the damage of pests and diseases to crops but also promote the sustainable development of agricultural production. Due to space limitations, this review only focuses on the main mechanisms of action and agricultural applications of Cre-loxP and CRISPR-Cas9. For example, CRISPR-Cas9 can be used for chromatin imaging and gene mapping in addition to genes and epigenetics. Research has discovered that some mechanisms of action are rarely studied in the plant field. For example, there is only one case of inhibiting some genes in corn through CRISPRi. Cre-loxP technology has far fewer applications in agriculture than CRISPR-Cas9. Future research can further explore the application of gene editing technology on different crops and its combination with traditional breeding methods to better promote innovative development in the agricultural field. In addition, social and ethical impacts need to be fully considered in order to contribute to agricultural development better.

References

[1]Yang Xiaoyu, et al. Targeted genome engineering and its application in trait improvement of crop plants. Agricultural Sciences, 2019.

[2]Schulze, Sabrina, Michael Lammers. The development of genome editing tools as powerful techniques with versatile applications in biotechnology and medicine: CRISPR/Cas9, ZnF and TALE nucleases, RNA interference, and Cre/loxP. ChemTexts, 2020, 7(1): 3.

[3]Zhang Dangquan, et al. CRISPR/Cas: A powerful tool for gene function study and crop improvement. Journal of Advanced Research, 2021, 29: 207-221.

[4]Chen Ming-Yang, et al. A review of tamoxifen administration regimen optimization for Cre/loxp system in mouse bone study. Biomedicine & Pharmacotherapy, 2023, 165: 115045.

[5]Liu Ruochen, et al. DNA methylation occurring in Cre-expressing cells inhibits loxP recombination and silences loxP-sandwiched genes. New Phytologist, 2021, 231(1): 210-224.

[6]Rodriguez-Rodriguez, Diana Rucker, et al. Genome editing: A

perspective on human disease using CRISPR/Cas9. International Journal of Molecular Medicine, 2019, 43(4): 1559-1574.
[7]Su Tianyuan, et al. A CRISPR-Cas9 assisted non-homologous end-joining strategy for one-step engineering of bacterial genome. Scientific reports, 2016, 6(1): 1-11.
[8]Ebrahimi, Zahra, et al. Successful CRISPR/Cas9-mediated HDR at individual DNA breakpoints using TFO-based targeted template design. Electronic Journal of Biotechnology, 2024.
[9]Qi, Lei S., et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell, 2021, 184(3): 844.
[10]Li Kailong, et al. Interrogation of enhancer function by enhancer-targeting CRISPR epigenetic editing. Nature communications, 2020, 11(1): 485.
[11]Heidersbach, Amy J., et al. A versatile, high-efficiency platform for CRISPR-based gene activation. Nature communications, 2023, 14(1): 902.
[12]Marx, Nicholas et al. dCas9-DNMT3A3L enhances targeted DNA methylation of CMV and endogenous promoters, leading to subsequent significant histone modification changes in CHO cells. Metabolic Engineering, 2021, 66: 268-282.
[13]Papikian, Ashot et al. Site-specific manipulation of Arabidopsis loci using the CRISPR-Cas9 SunTag system. Nature Communications, 2019, 10(1): 729.
[14]Zhang Mingli. Using the Cre/loxP system to cultivate insect-resistant and herbicide-resistant transgenic rice without foreign genes in the endosperm. Huazhong Agricultural University, 2023.
[15]Wang Guilan. Establishment of automatic marker gene deletion system based on Cre/LoxP recombination system. Southwest University, 2022.
[16]Zhang Xiaofang, et al. Creation and analysis of soybean germplasm transduced tolerant to low phosphorus transcription factor GmPTF1 without selection marker based on Cre/loxP system. Acta Crop Sinica, 2019, 45(5): 683-692.
[17]Cao Bihao, et al. Restoring pollen fertility in transgenic male-sterile eggplant by Cre/loxp-mediated site-specific recombination system. Genetics and molecular biology, 2010, 33: 298-307.
[18]Li Wenlong, et al. Research progress in targeted rice improvement based on CRISPR/Cas9 gene editing technology. Guangdong Agricultural Sciences, 2010, 49(9): 114-124.
[19]Kumar, Manoj, et al. Application of CRISPR/Cas9-mediated gene editing for abiotic stress management in crop plants. Frontiers in plant science, 2023, 14: 1157678.
[20]Liu Qier, et al. Application of CRISPR/Cas9 in crop quality improvement. International Journal of Molecular Sciences, 2021, 22(8): 4206.