

# Progress of Construction of Gene-Modified Soybean Based on the CRISPR/Cas9 System

Yuyang Tao

College of Life Science, Sichuan Agricultural University, Sichuan, China

202104426@stu.sicau.edu.cn

**Abstract.** From the first discovery by Japanese scholar Ishino in 1987 to being named and classified in 2002, CRISPR technology was proven by the Nobel Prize for Chemistry in 2020. In 2010, the type II CRISPR/Cas system (CRISPR/Cas9) was developed as an RNA-targeted genome editing system, which, as a novel and emerging genome editing technology in recent years, has a promising application prospect because of its high efficiency, simplicity and low cost. There is a serious mismatch between the plant area and yield of soybeans in China with market demand. Soybean is an important source of oil and biofuel. It is closely related to national food security. The large demand has led to a high dependence on imports of soybeans in China. Therefore, there is an urgent need to breed Chinese soybean varietal strains with outstanding forms in soybean selection and breed in order to breeding good strains and realize the goal of basically satisfying independent production. This review summarizes the research of the CRISPR/Cas9 system in constructing biological models of specific genes or genomic deletions in soybeans, introduces the latest development of this technology, and discusses for the advantages of CRISPR/Cas9 system in breeding target soybeans with good resistance and high economic production value.

**Keywords:** CRISPR/Cas9, soybean, gene editing.

## 1. Introduction

In 1987, Ishino, a Japanese scholar, specific repetitive sequences in a study of the alkaline phosphatase gene encoded by *E. coli*. Later in 2002, Mojica and Jansen formally defined this repeat structure as CRISPR, identified the Cas protein, and made the first classification of CRISPR systems. And in 2010, researchers realized that the Type II CRISPR/Cas system was simpler and developed it as an RNA-targeted genome editing system. The technology has evolved at a rapid pace over time. In 2016, Feng Zhang's team discovered that the Cas13 protein has the property of non-specific cutting of RNA and developed a genomic diagnostic technology based on the CRISPR/Cas13 system. The course of history shows that the technology has a broad application prospect. Eventually, in 2020, CRISPR technology became known when it won the Nobel Prize in Chemistry.

In 1987, Ishino, a Japanese scholar, first discovered that when an exogenous nucleic acid invades for the first time, the CRISPR/Cas system processes it into spacer sequences, which form clustered regularly interspaced short palindromic repeats (CRISPRs). This sequence is transcribed and processed into a mature crRNA. When the exogenous nucleic acid invades again, the crRNA can then recognize it and guide the Cas protein to cleave it. Nowadays, CRISPR/Cas systems are mainly classified into three types: type I, II, and III, as type II CRISPR systems have evolved to develop a mechanism containing the Cas9 protein that is different from that of type I and type III CRISPR systems.

The Type I system is the most complex and has the most Cas proteins. Among them, Cas3 proteins function as deconjugating enzymes and nucleases. Multiple Cas proteins bind to mature crRNA to form CRISPR associated complex for antiviral defense (CASCAD), CASCAD binds to invading exogenous DNA, and the crRNA within the crRNA pairs with the complementary strand of the exogenous DNA to form an R-loop structure, which recognized by the nuclease of Cas3. The Cas3 nuclease recognizes the R-loop structure and opens the complementary and non-complementary strands successively. The Cas10 protein of the Type III system has RNAase activity and a CASCAD

function similar to that of the Type I system. Cas10 is mainly involved in crRNA maturation and shear invasion of exogenous DNA.

The main components of the CRISPR-Cas9 system are the Cas9 protein and the small guide RNA (sgRNA), while the Cas9 protein has the function of processing and producing crRNA as well as cutting specific exogenous nucleic acids. The principle of CRISPR/Cas9 genome editing technology is that tracrRNA: crRNA is designed as a sgRNA, which utilizes the complementary sequences of the target DNA to locate the site to be edited and binds to Cas9. The Cas9 protein has an HNH nucleic acid structural domain in its intermediate position, which cuts the template strand complementarily paired with the crRNA. Cas9 protein has a HNH nucleic acid structural domain in the middle position, which can cleave template strands that are complementary to crRNA, and the outside of the third base of the 5' end of the Protospacer adjacent motif (PAM) is the cutting site. Between 3 and 8 bases upstream of PAM is its cutting site [1]. At this point, the target gene is cleaved, and the double-stranded DNA is broken and introduced into the specific target gene [2]. Once a double-stranded DNA break occurs in a gene, it causes two automatic repair mechanisms. These are homology-directed repair (HDR) and non-homologous end joining (NHEJ) auto-repair mechanisms.

ZFN is a nucleic acid endonuclease formed by fusing zinc finger proteins with the nucleic acid endonuclease FokI, which creates DNA double-stranded incisions at specific locations in the genome. Transcription activator-like effector nuclease (TALEN), which is also formed by fusing a DNA-binding protein with FokI, can also modify complex genomes. However, due to the complexity and high cost of ZFN preparation, TALEN is not very specific and efficient. In contrast, the CRISPR/Cas9 system requires only the successful design of gRNA sequences to enable Cas9 to localize to new DNA sequences for modifications such as knockouts, insertions, and targeted mutations. It has the advantages of simple design operation, short time-consuming, and a small workload. Therefore, most current studies use the CRISPR/Cas9 system.

The unsaturated fatty acid content of about 20% of the fat content of soybeans is close to 80%, which is one of the very important raw materials for vegetable oils and biofuels. Soybeans are also very rich in protein, making them an important source of high-quality protein for human intake. Soybean meal is one of the most important feed ingredients in the farming industry. The growing demand for soybean oil and soybean meal in China's oilseed market and farming industry is an important reason for the year-on-year increase in China's soybean imports in recent years. External dependence is extremely high and occupies an important position in producer employment, national food security, and international strategic approaches. Therefore, there is an important goal in recent years. CRISPR/Cas9 system, with the benefits of easy design, short time-consuming and low cost, has been widely applied to cultivate crops with outstanding forms. Therefore, application of CRISPR-Cas9 system genome editing technology to construct a biological model of soybean-specific gene deletion, to explore the resistance, agronomic traits, metabolism, and secondary metabolites of soybean is a promising approach to meet the emerging increased market need.

## 2. The use of CRISPR/Cas9 technology in soybean breeding

Jacobs et al. first constructed editing vectors for soybean endogenous genes using CRISPR/Cas9 technology in 2015 and demonstrated that CRISPR/Cas9 technology could play an editing role in the hairy root transformation system induced by *Agrobacterium* transformation [3]. In the same year, Cai et al. designed six sgRNAs and detected the presence of mutations targeting DNA in the hairy roots of soybeans, which was the first time to verify that the CRISPR/Cas9 system could edit two endogenous soybean genes at the same time with one sgRNA [4].

Currently, the use of CRISPR-Cas9 genome editing technology for soybean gene editing has become mature. However, the efficiency of soybean transgenesis is too low compared with that of model plants such as *Arabidopsis thaliana*, and the stable transgenesis of soybean requires the use of *Agrobacterium tumefaciens* K599, which produces adventitious roots, to conduct PCR tests on new roots in healing tissues to determine the editing efficiency of the target site before transgenesis [5].

Current research using CRISPR/Cas9 is mainly in enhancing soybean stress tolerance, changing the ratio of soybean secondary metabolites, improving soybean metabolism, and improving soybean agronomic traits. The following are all relevant studies using CRISPR/Cas9 gene-editing technology.

### 2.1. Enhancing Soybean Resilience

*Mycosphaerella* spp. can cause root and stem rot in soybean, which is one of the major causes of loss in the global soybean industry. Tankknocked out, replaced, and backfilled the genes encoding the RxLR effector of *M. spp.* in soybean to validate the screening role of the Nat1 screening marker gene against *M. spp.* for use in genetic transformation experiments of *M. spp.* [6] Ma et al. reported that GmLMM2 encoded a co-protoporphyrinogen III oxidase, which played an important role in the synthesis and defense system governed by light conditions of soybean BADH tetrapyrrole [7]. Knockdown of soybean GmLMM2 by the CRISPR system disrupts tetrapyrrole biosynthesis thereby decreasing chlorophyll content and enhancing resistance to soybean blight. The BADH gene regulates plant response to adversity stress, and Shi created three targeting mutations occurring in the coding region and regulating salt tolerance traits in soybean, including the production of truncated BADH1 proteins with a shifted code mutation [8] The GmBADH1 gene was shown to positively regulate salt tolerance traits at seedling emergence, and GmNF-YA16, GmNF-YB2 and GmNF-YC14 formed a heterotrimer that activated the GmPYR1-mediated abscisic acid (ABA) signaling pathway to regulate soybean stress tolerance. Yu et al. found that mutant plants knocked out of the GmNF-YC14 gene were more sensitive to drought stress compared to wild-type soybean [9]. Some of the proteins in the HD-Zip family are involved in plant response to environmental conditions. Zhong constructed the overexpression vector pTF102-GmHdz4 and the gene editing vector pBGK041-GmHdz4 and found that GmHdz4 has the function of regulating drought stress [10]. Sucrose non-fermentation-related protein kinases (SnRKs) have important regulatory roles in plants. Among the four SnRK1 homologous genes in soybean, GmSnRK1.1 and GmSnRK1.2 are the major genes, and Li et al. targeted knockdown of the above two genes reduced the sensitivity of plants to ABA and the tolerance to alkali stress, which demonstrated that SnRK1 kinases play important roles in the plant response to abiotic stresses [11].

### 2.2. Changing the proportion of soybean secondary metabolites

Soybean secondary metabolites are a class of nonessential small-molecule organic compounds, including terpenoids, alkaloids, flavonoids, and saponins, that are produced from primary metabolites by further transformation under specific conditions. Isoflavone synthase (IFS) is the key enzyme in the pathway of isoflavone synthesis. Flavanone-3-hydroxylase (F3H) and flavone synthase II (FNSII) interact with each other because they share the same substrate with IFS [12]. Zhang et al. compared Glycine max L. Merr. flavanone-3-hydroxylase1, Glycine max L. Merr. flavanone-3-hydroxylase2 and Glycine max L. Merr. flavanone synthase II-1 was simultaneously targeted for knockdown, resulting in unrestricted isoflavone synthase substrates and a significant increase in isoflavone content in the mutant strain [13] A total of 54 soybean saponins have been detected, which are classified into seven types of saponins: A, DDMP, B, E, H, I, and J [14]. The GmSg-5 gene is a key enzyme gene for the synthesis of A saponins, and the development of soybean varieties free of A saponins is conducive to the improvement of the taste and quality of soybeans. Shen targeted and edited the GmSg-5 gene and obtained nine lines with 57 pure GmSg-5 soybean mutants. Two molecular markers were developed to identify the GmSg-5 mutants [15]. The seeds of the mutants contained no A saponins, and the contents of B, DDMP, and E saponins were significantly higher than those of the control soybean cultivar Williams 82.

### 2.3. Improving soybean metabolism

Diacylglycerol acyltransferase (DGAT) is key speed-limiting enzyme that catalyzes the transform of diacylglycerol (DAG) to triglyceride (TAG), which is the main storage form of plant seed lipids, and DAG is more beneficial to human health than TAG. Yang et al. obtained seven effective editing

targets of GmDGAT1/2. GmDGAT1-Cas9 contains DGAT1a and DGAT1c, and GmDGAT2-Cas9 contains DGAT2a, DGAT2b, DGAT2c, DGAT2d, and DGAT2e [16]. GmFAD2 is a key gene controlling the conversion of oleic acid to linoleic acid, which determines the content and proportion of polyunsaturated fatty acids in soybean seeds. Zhou et al. constructed five single-gene editing vectors with transformation efficiencies of more than 80%. Exon sequences with high specificity and high GC content (35-65%) were screened to design gRNA target sequences [17]. Ma et al. knocked out one or two mutants of the GmFATB1 gene and found that the contents of palmitic acid and stearic acid were reduced, and the content of beneficial linoleic acid was increased, which could effectively improve the quality of soybean oil [18]. He et al. tested soybean GmFAD2-1A, GmFAD2-1B, GmLOX1, GmLOX2, and GmLOX3 genes in the GmLOX3 and GmLOX4 genes, respectively, to determine the content and proportion of polyunsaturated fatty acids in the soybean seeds, GmLOX2 and GmLOX3 genes were edited [19]. Seven excellent new germplasm with different allelic variants were obtained. Lipoxygenase activity was significantly reduced, and oleic acid content was increased to 81.7%-83.5% in the mutant seeds. Stability and edible quality of soybean edible oil affected by High oleic acid. Zhang knocked out two genes, GmFAD2-1A and GmFAD2-1B, which negatively regulate the oleic acid content of soybeans, and obtained a soybean mutant line with high oleic acid content. The oleic acid content of the pure mutant lines was much higher than that of the wild type, with the highest oleic acid content exceeding 80% [20]. The main role of the GmLOX genes (including GmLOX1, GmLOX2, and GmLOX3) in soybean is to encode lipoxygenase [21]. Lipoxygenase produces soybean odor under conditions of enzymatic oxidation, and lipoxygenase catalyzes the oxidation of linoleic acid to generate lipid hydroperoxides that also form soybean odor, and they limit the application of soybean products. Wang constructed the gmlox1gmlox2gmlox3 triple mutant and the gmlox1gmlox2 double mutant and obtained transgene-free mutants from the offspring, which can be used to reduce soybean odor [22]. Thiamine is an important cofactor for a variety of enzymatic reactions in all organisms. Feng et al. constructed the Gmpgl1 mutant and found that the deficiency of thiamine in it resulted in reduced activities of pyruvate dehydrogenase (PDH) and pyruvate decarboxylase (PDC), and six amino acids were lower than that of the wild type [23]. It was demonstrated that GmPGL1 plays an important role in thiaminothiazole biosynthesis.

## 2.4. Improving agronomic traits in soybean

The HOS1 gene is not only involved in the regulation of low-temperature responses, but Wang also found the HOS1 gene in the regulatory pathway of plant flowering time [24]. In addition, HOS1 is also involved in hypocotyl elongation and maintenance of circadian rhythms in plants. The four AP1 genes in soybean, which are mainly expressed at the stem tip, were simultaneously knocked out by Chen et al. to obtain a pure mutant, which proved that the AP1 genes could affect flowering time, floral organ development, and plant height in soybean [25]. The SEP4 (SEPALLATA4) gene belongs to the MADS-box gene family, and, as one of the five classes of genes for floral development - ABCDE in the category of E, it has been shown to be involved in the plant's differentiation organization, floral organ differentiation, and floral height. Liao successfully constructed two knockout vectors, GmSEP4-1-Cas9 and GmSEP4-2-Cas9 [26]. And found that the GmSEP4a gene is involved in the regulation of the soybean flowering stage, and the GmSEP4c and GmSEP4d genes are involved in the regulation of soybean floral organs. In addition to the regulation of floral organ development, SEP4 also has a determined role in producing meristem of flowers. There are three soybean homologous genes associated with the regulatory pathway of flowering, GmSVP01, GmSVP02, and GmSVP06. Li constructed the knockout vector Cas9-Gmsvp01/02 and obtained the pure mutant and overexpression plants to further clarify the function of their function in the regulation of flowering [27]. The photoperiod-related FT family genes are the integration nodes of multiple flowering pathways, and Cai et al. found that the mutant GmFT2a null allele pure plants showed late flowering and stable inheritance by targeted mutagenesis of GmFT2a [28]. Litargeted knockout of ZH39GmFT1a and GmFT4, and successfully created the GmFT1a pure mutant material and pure GmFT4 mutant plants that flowered early in LD conditions [29]. GmFT4 mutant material under LD

conditions. The plants effectively shortened the flowering period and had great breeding potential at high latitudes. The GmJAGGED1 mutant plants constructed by Cai et al. improved soybean yield at low latitudes [30]. Campbell B W et al. mutated the CPR5 direct homolog with CRISPR/Cas9 and obtained mutants that showed that the CPR5 direct homolog was essential for the normal growth and development of soybean trichomes [31]. GmPRR3b has been well known as a major flowering time regulator, and Li et al. knocked out the main form of the gene, GmPRR3b H6, and found that it delayed the growth and floral transition [32]. Guan et al. demonstrated that simultaneous mutation of GmAS1 homologs and GmAS2 homologs resulted in the bending of soybean leaves toward the distal axial plane, crumpling, and petiole shortening [33]. Mutations in both GmSHP1 homologs and GmSTK homologs resulted in the arrest of pod development. Mutations in the GmSHP1 homolog and GmSTK homolog resulted in the cessation of pod development. SPL3 is an important flowering regulator gene, and Wu et al. constructed a quadruple mutant of the soybean GmSPL3 gene [34]. The spl3abcd mutant was found to exhibit smaller leaves, reduced number of nodes, shorter node spacing, and lower plant height under short sunlight conditions, indicating that GmSPL3 has the function of regulating the morphology of soybean plants. Legume crops have an autoregulation of nodulation (AON) mechanism, Bo et al. constructed nodulation Gmnrk mutants and found that five mutants among eight different mutation types had the phenotype of hyper nodulation [35]. The phenotypes were analyzed for hyper tuberculate, dwarf plants, and plants with dark green leaves.

### 3. Editing efficiency of the CRISPR/Cas9 system in soybean breeding

Before transgenicizing the whole soybean plant, the editing efficiency of the target can be tested using a hairy root transformation system [36]. Different promoters were used in different studies resulting in different editing efficiencies. In response to the inconsistency of the methods for judging the editing efficiency, Niu proposed for the first time a new method to determine the editing effect of the CRISPR/Cas9 system by measuring the morphology of the peak maps (high peaks/low peaks) of the target sites in the hairy roots [37]. Niu used seven promoters (four known Cas9 promoters p35S, pZmUbiquitin, pGmYAO, and pAtRPS5A from the Arabidopsis ecotype Col4, as well as three soybean endogenous promoters pGmRPS5Ab, pGmRPS5Ac, and pGmHE). The respective editing efficiencies of these promoters were recorded against the soybean genes GmEID1 and GmSPA1a, respectively. The pGmRPS5Ab and pAtRPS5A promoters were shown to be the most suitable Cas9 promoters in the soybean CRISPR/Cas9 system with high editing efficiency and good editing effect. The occurrence of off-target effect is also an important reason affecting the editing efficiency. Computer prediction and experimental prediction (including a cell-free method that reconstructs the nuclease reaction to directly recognize gene cleavage, cell culture methods such as WGS, Cas9 ChIP-seq, IDLV, GUIDE-seq, BLISS and in vivo assays such as Discover-seq, GUIDE-tag that found the original Cas off-target and sequenced it) were applied to perform off-target prediction [38]. The nuclease activity of the Cas9 protein in experiments may also induce off-target mutations that researchers do not want. Benjamin P Kleinstiver et al. Studies using SpCas9-HF1 have made off-targeting essentially undetectable by whole-genome break capture and targeted sequencing methods [39]. Provides an alternative to wild-type SpCas9 for research and therapeutic applications. The editing efficiency of CRISPR is largely affected by the inefficiency of homology-directed repair (HDR). Studies in recent years have shown attempts to improve the efficiency of HDR using chemical modulation, synchronized expression, and overlapping homology arm.

### 4. Conclusion

This paper summarizes the application of CRISPR/Cas9 gene editing technology on soybean genes edition in recent years. The corresponding plants meeting the expectations were obtained for soybean resistance, soybean secondary metabolites, soybean metabolism, and agronomic traits of soybean. sgRNA and Cas9 protein expression levels, expression times, and variants of both need to be

regulated in a complex manner [40]. The principle of action has been clarified and many studies have been conducted, and efforts are underway to address issues such as avoiding off-targeting, homologous genome redundancy, and other issues related to the use of the technology, but relatively little attention has been paid to safety issues. The U.S. Food and Drug Administration (FDA) conducted a safety review of the technology in October 2023 and granted it priority review status, while the world's first CRISPR gene editing therapy was approved for marketing in November 2023 by the U.K.'s Medicines and Healthcare products Regulatory Agency (MHRA). Recent clinical studies illustrate that the safety of CRISPR technology is still under review before finalization. Although gene editing of soybeans using this technology has been accomplished in many ways, there is still a lack of research related to disease resistance in soybean caecilians. Knockout of relevant disease-causing genes using CRISPR/Cas9 gene technology could be considered to explore resistance to *Caulobacter*.

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