

CRISPR/Cas9 applications for improvement of soybeans, current scenarios, and future perspectives

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Abstract

The soybean is one of the most widely grown legume crops which serves as a source of protein and oil. Soybean production has increased in recent years due to several breeding techniques. The use of conventional breeding approaches does not fulfil the rapidly growing demand of the world population. Newly developed genomic approaches opened the windows of opportunities to bring more genetic variation in soybean germplasm. Clustered regularly interspaced short palindromic repeats (CRISPR) has emerged as a renowned gene-editing tool that has broadened soybean research. CRISPR/Cas9 has been extensively applied to improve several essential traits in soybeans. Soybean yield, quality, and other agronomic traits have been enhanced, and research is being conducted to revolutionize the genomic area of soybeans. The development of specific soybean mutants has shown better yield and quality. In this review, we have enlisted the potential use of clustered regularly interspaced short palindromic repeats (CRISPR) in soybean improvement and highlighted the significant future prospective. Research of applied sciences revealed that CRISPR/Cas9 could improve the traits of the commercially essential soybean crop, including yield, quality, and resistance to certain biotic and abiotic factors. The use of this tool has lifted the scope of genome editing and laid a foundation for the bright future of human beings. This updated review will be helpful for future research studies focusing on the successful use of CRISPR/Cas9 in soybeans.

Keywords: biotic and abiotic stresses; Cas9; CRISPR; soybean; yield; quality

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Introduction

The soybean is one of the most important legume crops grown worldwide (Ke *et al.*, 2022). The soybean yield is one of the main breeding objectives to meet the increasing demand worldwide (Umburanas *et al.*, 2022). Soybean oil has a wide range of uses, including baking, cooking, frying, and as a source of biodiesel and industrial use (Butzen and Schnebly, 2007). Soybeans have lot of nutritional value like, soybean milk, oil, tofu, vitamins, and minerals which have lot of health benefits for humans as well as livestock (Saha and Mandal, 2019). The USA, Argentina, and Brazil produce 80% of total soybeans worldwide. Besides this, India and China are major soybean producers in Asia. China is the largest importer of US soybeans. Soybean production has been increased rapidly because of the increasing demands from China (Hart, 2017). Hence the world soybean market is driven by the USA, Brazil, and China (Gale *et al.*, 2019). Humans and livestock largely consume soybeans and its food products. Soybean oil and bean meal have a lot of nutritious value. Due to the high demand for soybean products and the growing population, it has become necessary to increase the number of soybean products by using several technologies (Hu *et al.*, 2020). Multiple factors are responsible for low-yield soybeans in China, such as, the lack of valuable genetic information, germplasm, less planted area and importation of foreign produced soybeans. Climatic factors have significantly impacted the soybean yield (Zhang *et al.*, 2020a).

Although many advances have been made in classical breeding, developing superior crop cultivars is not quick and leads to increased food demand globally (Gao, 2018; Ahmar *et al.*, 2020). Without the proper investigation on germplasm, it is impossible to get information about natural variation used for the successful breeding programs for the desired traits in any crop (Marathe *et al.*, 2018). Soybeans have a complex genetic architecture, and the use of reliable breeding techniques for its improvement is imminent (Schmutz *et al.*, 2010). The use of traditional approaches to overcome these obstacles is often time-consuming and costly, as well these traits have complex nature of inheritance (Warner and Gupta, 2005). Hence, it is essential to adopt the newly developed molecular techniques for significant improvement of soybeans and to meet the growing demands in the future. Soybean breeders continuously worked to improve the soybean genome and satisfy the hunger of a rapidly growing population. Transgenic techniques have been used extensively in soybeans for improvement of commercially important traits (Natarajan *et al.*, 2013). Transgene techniques focused on transforming potent alleles into the genome of elite cultivars to improve essential features. The use of foreign genes in selected varieties can cause off-target effects and raise public safety concerns. Genetically modified crops are restricted in some areas because of the various biosafety issues (Xu *et al.*, 2020). Soybean germplasm has been improved by using transgenic techniques. Many genes have been mutated using certain mutagens, like X-rays, and biological mutagens, like T-DNA insertion (O'Rourke *et al.*, 2017; Shiming *et al.*, 2017). Random mutation is often heritable, but it needs screening and particular techniques to identify the mutant phenotype (Shiming *et al.*, 2017). Sometimes it is impossible to detect a specific allele for a specific trait because of the random mutation. Multiple tools have been developed for GE in the crop in recent years.

These methodologies have certain drawbacks like low editing efficiency, type of vector used, and generation of off-target effects (Adli, 2018); however, genome editing tools like meganucleases (MNs), zinc finger nuclease (ZFNs), and transcription activators like effector nucleases (TALENs) have broadened the era of genome editing and lead to the development of several novel cultivars. GE is being used as a powerful tool for crop improvement; however, its efficiency can be enhanced for crops with complex genomes (Carrijo *et al.*, 2021). CRISPR/Cas9 has emerged as a reliable tool because of its simplicity of use and target recognition efficiency (Wolter *et al.*, 2019). These tools have been integrated for successful breeding programs (Chen *et al.*, 2019a; Zhang *et al.*, 2019b). It has improved a lot of essential traits in soybeans like yield, quality, and resistance to stresses Figure 1. CRISPR/Cas9 has broken the biological barriers and expanded the use of molecular techniques for a wide range of crop improvement (Chilcoat *et al.*, 2017). Improvement of traits by CRISPR/Cas9 in soybeans does not involve any foreign DNA elements and increases its rate of acceptance in

society without any harmful effects on human health (Xiao *et al.*, 2022). The future of genome editing in soybeans is expected to be bright and will bring targeted results (Rasheed *et al.*, 2021a). As more and more studies on different Cas9 variants are being conducted currently, their future use will shed more light on improvement of key traits in soybeans. The development of high yielding soybeans can lead to a green revolution in agriculture. Being a significant crop of the legumes family, it is receiving more and more attention by the research community (Wang *et al.*, 2020b).

We have reviewed the role of CRISPR/Cas9 in soybean genetic improvement and presented a specific future perspective. It is a tireless task to sum up all progress made by CRISPR in soybeans; however, we have demonstrated the most recent and significant developments regarding several traits. Additionally, the limitation of CRISPR/Cas9 and regulatory ethics are briefly discussed. The overview of the use of CRISPR/Cas9 in driving the food chain in the ecosystem is shown in Figure 1.

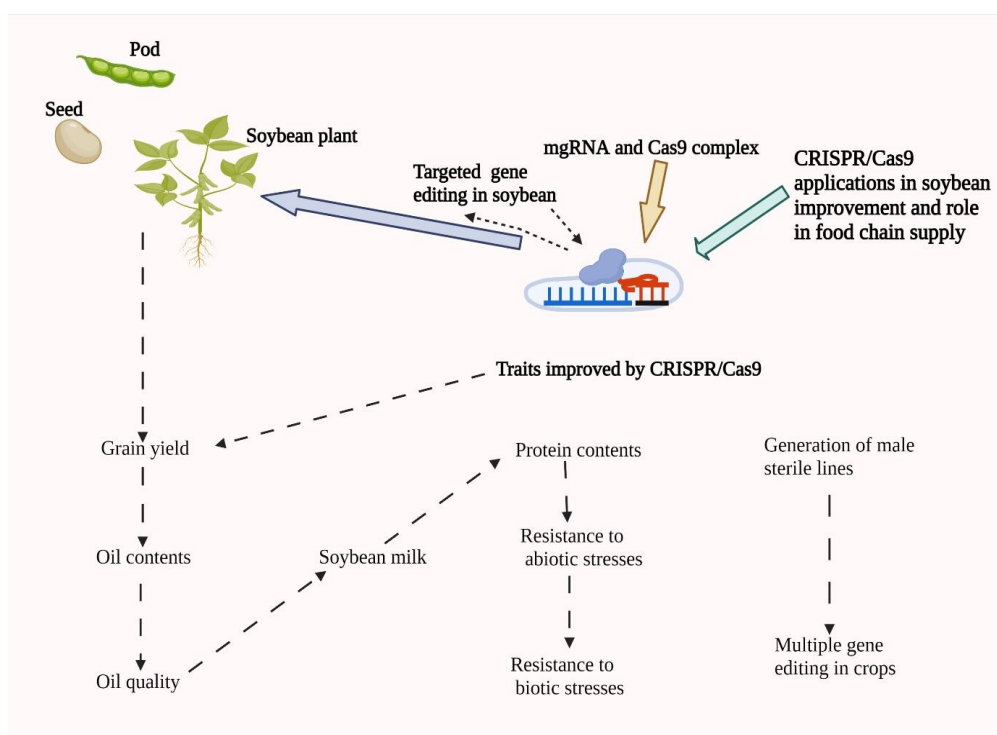


Figure 1 CRISPR/Cas9 mediated genome editing in soybean to improve specific traits and its role in food chain supply

CRISPR/Cas9 helps to increase crop yield and maintain the food supply chain in the ecosystem. The current Figure was made with [BioRender.com](https://www.biorender.com/).

Genome Editing Tools

Several genome editing tools have been developed to bring desired variations in the crop genome (Rasheed *et al.*, 2021a). This aims to revolutionize agriculture to ensure the global food security (Gao, 2021). Among the discovered tools, MNs are first-class GE tools (Namo and Belachew, 2021) and have been extensively used in the genome editing of crops. They can identify the targeted genomes of 12-40 base pair (Figure 2) site. They have been termed as the most reliable restriction enzymes because of their nature and target recognition efficiency (Mishra and Zhao, 2018). After double-stranded breaks, the repairing process is initiated by non-homologous end joining (NHEJ), which acts to search and repair the mismatched sequences (Townsend *et al.*, 2009). Alteration of MNs is not possible because of their complex nature (Puchta, 2005).

ZFNs are the most efficient and reliable tools and bring targeted breakdown in double-stranded DNA (Durai *et al.*, 2005). They were used as manipulating tools in crop improvement. The MNs structure has a DNA binding area of 300-600 repeats (Carlson *et al.*, 2012). Individual ZFNs can be seen between 9-18 base pairs. The second component of ZFNs structure is the DNA slicing area (Carroll *et al.*, 2006). Targeted DNA is sliced by enzyme containing the Fok1 protein domain. The zinc finger domain has a particular position order and reads the 24-30 base pairs (Gaj *et al.*, 2012). ZFNs are targeted to slice a particular genome sequence. The cleavage event encouraged by the ZFNs incites cellular repair procedures that in turn facilitates efficient alteration of the targeted locus (Urnov *et al.*, 2010).

The TALENs have been extensively used in crop improvement for many years. They were developed by combining the Fok1 slice region with the DNA binding area of TALENs proteins. They can effectively edit a single base pair with a 34 amino acid duplication complex (Zhang *et al.*, 2019a). TALENs trigger gene-editing by causing double-stranded breaks at specific sites (Gaj *et al.*, 2013). The protein efficiency to alter the DNA was studied in 2007. Few studies have been reported for TALENs in crop gene editing however, and more extensive research needs to be carried out to unfold more breakthroughs in this area (Gaj *et al.*, 2012). Haun *et al.* (2014) successfully used TALENs for targeted editing of soybean gene *FAD2* for shelf life and used NHEJ for the repairing process. A comparison of GE tools is shown in Figure 2.

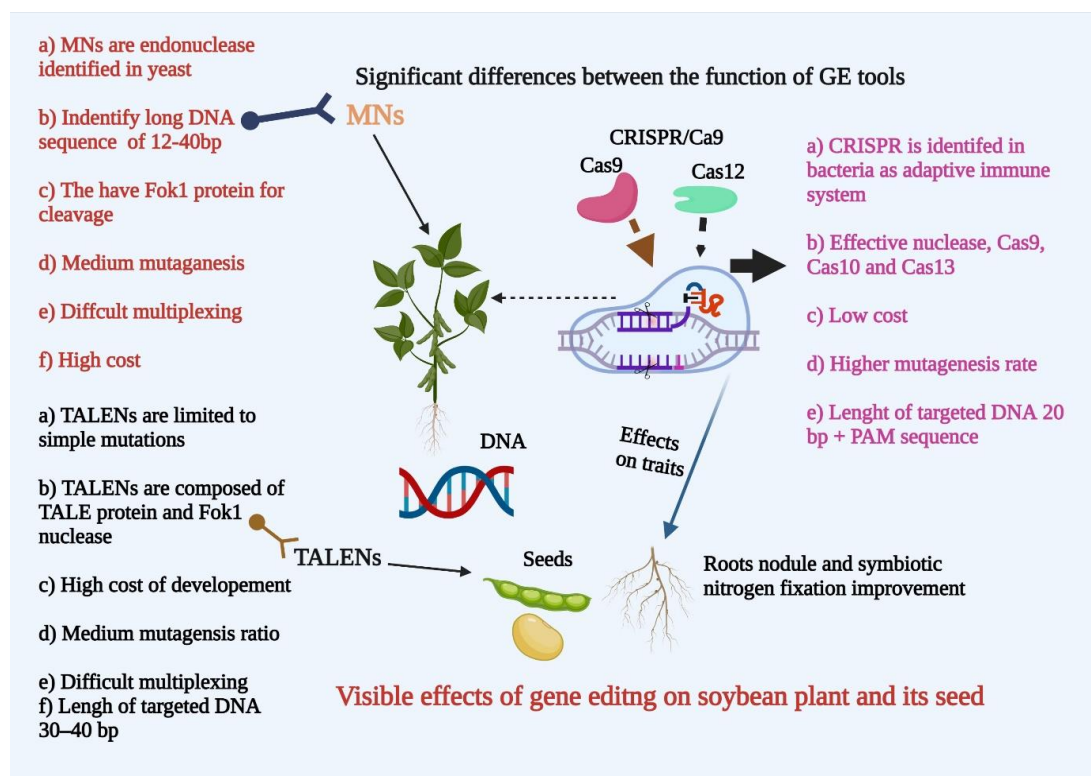


Figure 2 A schematic representation of significant differences among different genome editing tools, TALENs, MNs, and CRISPR/Cas9. These differences highlight the significance of CRISPR/Cas9 over other tools. This figure was created with BioRender.com.

CRISPR/Cas9 Overview

CRISPR/Cas9 technique was first discovered in 2012 (Lander, 2016; Rasheed *et al.*, 2021a) by Jennifer Doudna and Emmanuelle Charpentier. CRISPR/Cas9 consists of an adaptive immune system found in bacteria that provides resistance against phage and other foreign invaders, and it consists of CRISPR locus and

Cas9 protein (Jinek *et al.*, 2012). The role of CRISPR/Cas9 as a biological system was reported in earlier 2007 (Barrangou *et al.*, 2007). CRISPR has specific systems and types; however, the most common and widely applied system is type 11. This system is discovered in bacteria called *Streptococcus pyogenes* (Marraffini, 2016). The single guided (sgRNA) and Cas9 protein group together, and bring the targeted cleavage of any specific site (Jiang and Doudna, 2017). The protospacer adjacent motif (PAM) is necessary for efficient cleavage by Cas9 nuclease (Jiang and Doudna, 2017). PAM sequences are mandatory for recognizing the target, and Cas9 will search for it before unravelling the foreign DNA to cut (Hsu *et al.*, 2014). Cas9 enzyme identifies the NGG nucleotide sequence as PAM. It shows two catalytic domains (HNH, RuvC), which cleave the strand and its opposite strand to bring double-stranded disruptions in the genetic material of any foreign invader (Ishino *et al.*, 2018). The targeted double-stranded breaks are precious in genomic study to create a specific point mutation for particular traits of interest. The Jinek *et al.* (2012) and fellows presented the role of the CRISPR/Cas9 system to improve essential crops. Nowadays, the CRISPR type 11 system is being used for targeted mutagenesis. CRISPR/Cas9 is a two-step process, induction of double-stranded breaks at the targeted site by Cas9 enzyme and repairing the damaged site by specific mechanisms like homology direct repair (HDR) (Chen *et al.*, 2019b). The HDR repairing pathway is vital to the success of CRISPR based targeted mutation (Figure 3). It works by replacement of an exogenous stretch of donor DNA template at the targeted site (Van Vu *et al.*, 2019). There is another type of Cas variant called Cas10 which is attached to an HD family nuclease area that is different from the HD areas of type I CRISPR-Cas systems and, unlike the latter, comprises a circular variation of the conserved motifs (Makarova *et al.*, 2006). There are some other variants of CRISPR which are being used for gene editing in crops. CRISPR/Cas12 based genome editing was first used in *Clostridium difficile* for multiple genomes editing with higher efficiency. The Cas13, uses a guide RNA. It has two different catalytic functions, RNase activity, and gRNA maturation activity (O'Connell, 2019). The small size of Cas13 makes it suitable for molecular genetics (Huynh *et al.*, 2020).

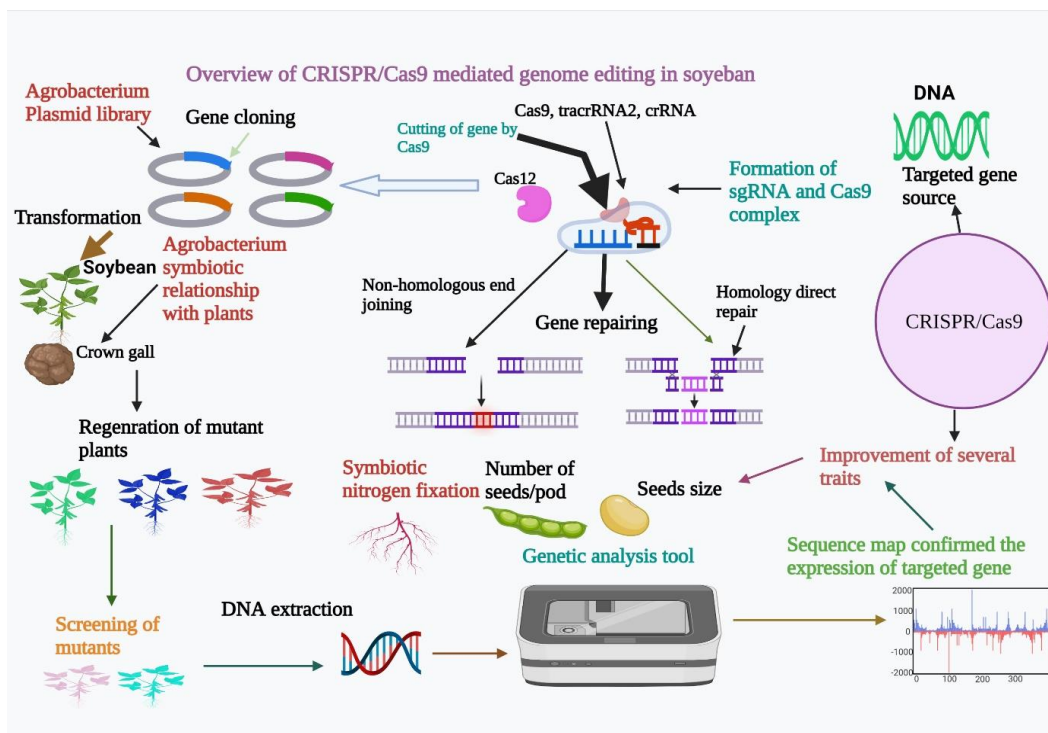


Figure 3 A detailed overview of CRISPR/Cas9 mediated genome editing in soybeans. The GE steps involve, identifying the targeted gene, cutting by Cas9, cloning, transformation, regeneration of mutants, DNA extraction, and sequence analysis for confirmation of the targeted gene. This figure is created with BioRender.com.

CRISPR/Cas9/mediated genome editing in soybeans

CRISPR/Cas9 mediated genome editing has been a powerful and versatile tool for significant improvement in soybeans. There are several successful stories in which CRISPR/Cas9 has been successfully applied for targeted gene editing for a specific trait. CRISPR/Cas9 has ended the biological barriers which have been a major obstacle in crop improvement for decades. Being a valuable legume crop and being a major source of oil, soybean genetics and breeding has gained tremendous attention after the use of CRISPR/Cas9 for development of certain economically important traits (Chilcoat *et al.*, 2017). By use of CRISPR/Cas9 in soybeans certain traits have been improved like, yield, quality, early flowering, diseases resistance, and development of male sterile lines (Lu and Tian, 2022). Genome editing in soybeans is comprised of several steps like, identification of gene, its cloning, transformation, and regeneration (Jaganathan *et al.*, 2018). A schematic overview of CRISPR/Cas9 based genome editing in soybeans is presented in Figure 3. The repairing process in gene editing is initiated by two factors: HDR and NHEJ. HDR is a specific repair mechanism that uses homologous donor DNA to repair DNA damage, while NHEJ is an error-prone mechanism in which cracked ends of DNA are combined together, often resulting in a varied pool of supplements and removals. The tools work in three phases, (1) recognition of double stranded breaks (DSB), (2) processing of nonligatable DNA (3) linking two appropriate DSBs (Ma *et al.*, 2016; Liu *et al.*, 2019).

Role of CRISPR/Cas9 in soybean multiple traits improvement

The use of CRISPR-mediated gene editing is now revolutionizing the field of molecular science by effectively modifying the targeted genes. Yield and quality are one of the main objectives of any crop breeding program (Rasheed *et al.*, 2020a; 2020b; Rasheed *et al.*, 2020; Rasheed *et al.*, 2021b; Rasheed *et al.*, 2021c) (Figure 3).

CRISPR/Cas9 for yield and quality improvement

Plant architecture plays a crucial role in soybean yield and quality improvement. CRISPR/Cas9 has revolutionized the GE area, and now significant progress has been made to improve plant architecture in soybeans. Yield is the ultimate goal of any breeding program. Higher yield leads to the prosperity and sustainability of the farmers and country. Targeting yield and its related components by CRISPR/Cas9 will lead to increased output of crops from a given area (Bao *et al.*, 2019a). Targeted mutagenesis of soybean architecture gene *GmSPL9* was carried out and transferred by *Agrobacterium tumefaciens*. The soybean mutant plants showed a transient phenotype nature. This significant breakthrough demonstrated that short stature soybean genotypes could be developed using CRISPR/Cas9 (Bao *et al.*, 2019b).

The number of pods per plant is one of the significant soybean yield components. CRISPR/Cas9 mutagenesis of *GmJAGGED1* gene improved yield in soybean cultivars. These results showed that targeting pod numbers could enhance yield and contribute to global food security (Cai *et al.*, 2021). Cheng *et al.* (2019) applied CRISPR/Cas9 tool for successful knock out of gene *GmLHY* and used *A. rhizogenes* K599 for gene transformation. The resulting mutants showed a reduced height and short internode nature. These results suggested that the *GmLHY* gene can be transferred to develop short stature genotypes (Cheng *et al.*, 2019). Kanazashi *et al.* (2018) applied sgRNA to mutate *GmPPD1* and *GmPPD2* to get the mutants with altered pod phenotypes. Hence, these reports mentioned above on soybeans showed that CRISPR/Cas9 mediated GE could revolutionize agriculture to address global food security issues. In the future, the use of different Cas variants and efficient transformation methods would increase the application of CRISPR in soybean genetic improvement. The current need is to explore the wild type and increase the quantity of germ plasm to identify targeted genes. Likewise, in another study, the targeted mutagenesis of two genes, *Glyma 08g02290* and *Glyma 12g37050*, was done in a study. Soybean protoplast was used for detection of gene mutation. Mutations of these three genes were detected in soybean protoplasts. *Agrobacterium rhizogenes* was used for transformation. These

findings showed that targeted mutagenesis efficiency of soybeans could be improved further to get satisfactory results (Sun *et al.*, 2015).

Soybean quality is one of the most important breeding objectives. Good quality soybean cultivars are often liked by consumers. Soybean genes, *GmFAD2-1A* and *GmFAD2-1B* for high oleic acid content were edited by CRISPR/Cas9 tool. The working efficiency of gRNA and Cas9 was tested previously. The *Agrobacterium rhizogenes* K599 strain was used for hairy root transformation. The resulting mutants showed a significant increase in oleic acid content in soybeans. The results demonstrated the significance of gene editing for better quality soybean products (Do *et al.*, 2019). Soybean seeds are an important foundation for the agricultural system. Soybean seeds serve as a principal source of protein and oil in the industry. The soybean proteins, glucinins, and conglycinins families account for 70% of total soybean protein (Thanh and Shibasaki, 2002). Soybean protein quality and quantity are primary breeding objectives in a gene-editing program (Poysa *et al.*, 2006). Li *et al.* (2019) set out the testing efficiency of CRISPR/Cas9 for editing soybean seed storage protein gene, *Glyma.20g148400* using *Agrobacterium* hairy root transformation method. The results showed a higher editing ratio in the soybean seed storage protein gene. These results provide resources for future studies to develop high-quality soybeans (Li *et al.*, 2019). Soybean breeding for low saturated fatty acid content is being done to reduce the risk of heart diseases. CRISPR/Cas9 based gene knock out of *GmFATB1a* resulted in mutants with reduced fatty acid content. The mutants showed a 42% decrease in palmitic acid, and this could be of great significance for developing high-quality soybean genotypes (Ma *et al.*, 2021).

Soybean seed flavour has a crucial significance in soybean quality. Soybean quality traits are improved and liked by the consumers. CRISPR/Cas9 mutagenesis of beany flavor gene *GmLox1*, *GmLox2*, and *GmLox3* (Table 1) were mutated, and 60 T₀ plants were developed. Sequencing of T₁ plants revealed that mutation in *GmLoxi* inherited to subsequent progeny. The development of lipoxygenase-free mutants would be helpful in the reduction of beany flavour without regulatory restrictions (Wang *et al.*, 2020a). Reduced fatty acid content can attract soybean consumers because it prevents heart diseases. Wu *et al.* (2020) used the CRISPR/Cas9 genome editing technique for mutagenesis of gene *GmFAD2-1A*, which showed 95% target efficiency. The T₂ generation showed a significant decline of 62.91% in linolic acid content, and protein content increased to 37.69%. In the T₃ generation, linolic acid content decreased to 56.58% (Wu *et al.*, 2020). Likewise, Al Amin *et al.* (2019) studied targeted disruption of *FAD2-2* to improve fatty acid profile in soybeans. Results showed that soybean mutant plants had lower fatty acid content. Oleic acid content were higher, and this showed a huge diversity in the biochemical contents of soybeans (Al Amin *et al.*, 2019).

In soybeans a high content of phytic acid is always a main concern for people's health. Breeding of soybeans to reduce phytic acid content is being done to improve soybean nutritional qualities. Now Cas9 made it possible to successfully edit the genes *GmIPK1* and *GmIPK2* responsible for phytic acid synthesis. Carrijo *et al.* (2021) studied targeted mutagenesis of these genes using Cas9, and mutant plants showed reduced phytic acid content. *A. rhizogenes* strain K599 was chosen for gene transformation. The development of highly nutritious soybeans is not a challenge anymore because of the emergence of the latest innovative and precise genome editing tools (Carrijo *et al.*, 2021). Soybean isoflavone is a critical secondary metabolite with many health benefits and protects plants from environmental stresses. In a recent study, CRISPR/Cas9 was successfully used to edit the *GmF3H1*, *GmF3H2*, and *GmFNSII-1* to increase isoflavone content in soybeans. The resulting mutants showed a large frequency of variation in T₀ transgenic plants (Zhang *et al.*, 2020b). These reports revealed that soybean quality could be enhanced with Cas9 and consumer preference regarding soybeans would boost the soya industry in future.

CRISPR/Cas9 for development of early flowering soybean genotypes

Flowering time is a crucial determinant of the regional adaptability of soybeans. Improvement of flowering time is a key trait that helps in the early maturity behaviour of genotypes. Early maturing crops can

escape from harmful effects of different abiotic and biotic stresses (Kong *et al.*, 2014). Cai *et al.* (2018) applied CRISPR/Cas9 in targeted mutagenesis of *GmFT2a* and *gmFT5a* to study the flowering time in soybeans (Table 1). The mutants showed improved flowering time, and hence it is proven that large fragment deletion can be inherited in soybeans. Likewise, CRISPR/Cas9 mediated mutation of *Gmpr37-ZGDD* in soybeans improved early flowering and overexpression of *GmPRR37* caused a delay in flowering time under long photoperiod conditions. The study showed that the soybean gene *GmPRR37* regulates flowering time in soybeans and highlights the opportunities to develop varieties with characteristics of adaptability to a wide range of ecological conditions (Wang *et al.*, 2020b). The flowering gene *LNK2* was successfully mutated by CRISPR/Cas9 in soybeans. *Agrobacterium tumefaciens* strain EHA101 was used for stable soybean transformation. The mutants showed an earlier flowering time than later wild types. These findings supported the potential use of CRISPR/Cas9 in developing early flowering soybean genotypes (Li *et al.*, 2021). Cai *et al.* (2020) applied CRISPR/Cas9 based targeted mutagenesis of gene *GmFT2a* and *GmFT4* for flowering time in soybeans and used *Agrobacterium tumefaciens* for transformation (Table 1). The resulting mutants showed a significant difference in flowering time and a high frequency of mutation was observed in T₁ generations. Early flowering soybean genotypes could provide higher yields and escape from one set of biotic and abiotic stresses (Cai *et al.*, 2020).

Use of CRISPR/Cas9 for development of male-sterile soybean lines

Development of soybean male sterile lines is one of the main breeding objectives. Many attempts have been made for introgression and integration of valuable genetic material from diverse soybean genotypes. Due to development and labour costs, the level of genetic breeding level in soybeans is relatively low. The lack of diverse soybean germ plasm is one of the key hindrances in generating effective male sterile lines. Hence, it is essential to develop male sterile lines to overcome this deficiency (Jiang *et al.*, 2021). For the first time in soybean history, AMS homologs' targeted mutagenesis was done to develop male sterile lines. The mutagenesis of *GmAMS1* resulted in male-sterile soybeans. This gene increased pollen formation in soybeans (Chen *et al.*, 2021b). Likewise, Jiang *et al.* (2021) successfully applied the CRISPR/Cas9 tool to edit the *MSI* gene to develop soybean male sterile lines. These male sterile mutants can be used to develop out-crossing soybean populations (Jiang *et al.*, 2021). More than twenty male-sterile mutants have been made to date and these can be valuable for basic research in the molecular field (Zhao *et al.*, 2019b). The further use of CRISPR/Cas9 in development of male sterile lines will be useful to harness hybrids for hybrid seed production. This will lead to the development of the hybrid seed industry to enhance soybean yield. Hence, we can say that CRISPR/Cas9 is well suited for targeting genomes for development of male sterile lines. Application of CRISPR/Cas9 for improvement of different traits of soybeans are given in Table 1.

Table 1. CRISPR/Cas9 mediated gene knockout for yield and quality traits

Crop	Gene	Trait	Tool	Transformation	References
	<i>GmFAD2-1A, GmFAD2-1B</i>	Higher oleic acid	CRISPR/Cas9	<i>Agrobacterium rhizogenes</i> (K599 strain)	(Do <i>et al.</i> , 2019)
	<i>Glyma.20g148400</i>	Improved the seed storage protein content	CRISPR/Cas9	<i>Agrobacterium rhizogenes</i> (K599 strain)	(Li <i>et al.</i> , 2019)
	<i>GmFEI2, GmSHR</i>	Hairy roots	CRISPR/Cas9	<i>A. rhizogenes</i> K599	(Cai <i>et al.</i> , 2015)

Soybean	<i>Glyma08g02290</i> , <i>Glyma12g37050</i>	Yield	CRISPR/Cas9	<i>A. rhizogenes</i> K599	(Sun <i>et al.</i> , 2015)
	<i>GmSPL9</i>	Short plant architecture	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i> EHA105	(Bao <i>et al.</i> , 2019b)
	<i>GmAMS1</i>	Male sterile lines	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i> EHA105	(Chen <i>et al.</i> , 2021b)
	<i>GmFT2a</i>	Flowering time	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i> EHA105	(Cai <i>et al.</i> , 2018)
	<i>GmPRR37</i>	Flowering time	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i> EHA105	(Wang <i>et al.</i> , 2020b)
	<i>GmFT2a/5a</i>	Early flowering	CRISPR/Cas9	<i>Agrobacterium</i> strain EHA105	(Han <i>et al.</i> , 2019)
	<i>GmJAGGED1</i>	Yield	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i> EHA105	(Cai <i>et al.</i> , 2021)
	<i>MS1</i>	Male sterility	CRISPR/Cas9	<i>Agrobacterium</i>	(Jiang <i>et al.</i> , 2021)
	<i>GmFATB1a</i>	Reduced content of saturated fatty acid	CRISPR/Cas9	<i>Agrobacterium</i> strain LBA4404	(Ma <i>et al.</i> , 2021)
	<i>LNK2</i>	Early flowering	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i> strain EHA101	(Li <i>et al.</i> , 2021)
	<i>GmLHY</i>	Alter plant height and internode length	CRISPR/Cas9	<i>A. rhizogenes</i> K599	(Cheng <i>et al.</i> , 2019)
	<i>GmLox1</i> , <i>GmLox2</i> , <i>GmLox3</i>	Beany flavor	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i> GV3101	(Wang <i>et al.</i> , 2020a)
	<i>GmFAD2-1A</i>	Low fatty acid content	CRISPR/Cas9	<i>Agrobacterium</i>	(Wu <i>et al.</i> , 2020)
	<i>FAD2-2</i>	Fatty acid profile	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i> strain EHA105	(Al Amin <i>et al.</i> , 2019)
	<i>GmDr2ba</i> , <i>GmDrb2b</i>	Yield and quality	CRISPR/Cas9	<i>A. rhizogenes</i> K599	(Curtin <i>et al.</i> , 2018)
	<i>GmPPD1</i> , and <i>GmPPD2</i>	Pod number	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i>	(Kanazashi <i>et al.</i> , 2018)
	<i>GmFT2a</i> and <i>GmFT4</i>	Flowering time	CRISPR/Cas9	<i>Agrobacterium tumefaciens</i>	(Cai <i>et al.</i> , 2020)
	<i>GmIPK1</i> and <i>GmIPK2</i>	Low phytic acid	CRISPR/Cas9	<i>A. rhizogenes</i> strain K599	(Carrizo <i>et al.</i> , 2021)
	<i>GmF3H1</i> , <i>GmF3H2</i> , <i>GmFNSII-1</i>	Increased isoflavone content	CRISPR/Cas9	<i>Agrobacterium rhizogenes</i> strain K599	(Zhang <i>et al.</i> , 2020b)

Application of CRISPR/Cas9 in development of resilient soybeans and other genomic modifications

Development of resilient soybeans by CRISPR/Ca9 is one of the key breeding objectives. Resilient soybeans can adapt to different environmental conditions.

Development of soybean resistance to abiotic stresses

Biotic stresses are causing a significant loss in soybean yields annually (Almeida-Silva and Venancio, 2022). The application of CRISPR/Cas9 for the development of resilient soybeans has been studied, but there is a need to apply newly developed methods for improving the efficiency of soybeans. Until now, few case studies have been presented that showed the successful use of Cas9 for improving biotic stress tolerance in soybeans. In recent research, CRISPR/cas9 was successfully used to edit the *GmF3H1*, *GmF3H2*, and *GmFNSII-1* to increase isoflavone content in soybeans. The resulting mutants showed a large frequency of variation in T0 transgenic plants. The large isoflavone content showed increased resistance to the soybean mosaic virus (SMV). Results suggested the potential use of Cas9 to improve soybean resistance to viruses (Zhang *et al.*, 2020b). Cyst nematode is one of the potent threats for soybean production (Dong and Hudson, 2022) in the world, which alone causes than 1.2 billion loss/year in yield in the United States alone. For this reason, planting resistant soybeans is the only solution. The QTL, *Rbg1*, and *Rbg4* are resistant to CN (Table 2). However, recently emerged GE tools like CRISPR/Cas9 can be applied to bring targeted mutations in the genes for CN resistance (Kang, 2016).

Development of soybean resistance to herbicides

Abiotic stresses significantly reduce crop yield worldwide. The use of herbicides in soybeans has been a primary concern because of their toxic effects on the crop (Sharkey *et al.*, 2021). Herbicide-tolerant crops have been grown to reduce the use of herbicides to avoid environmental contamination and reduce health hazards. In recent years, herbicide resistance has been incorporated in crops, mainly soybeans, using GE tools like CRISPR/Cas9 (Cheng *et al.*, 2021). Some examples of CRISPR/Cas9 mediated gene editing for herbicide-tolerant soybeans are given below. Li *et al.* (2015) applied CRISPR/Cas9 to edit the *DD20*, *DD43* gene for chlorsulfuron-resistant soybeans using particle bombardment. The resulting mutants showed a significant improvement in the desired trait. The targeted editing efficiency of soybeans was tested in an experiment. Cas9 successfully edited the two genes *GmPDS11* and *GmPDS18*. The resulting mutants showed a dwarf bud phenotype, which indicated the higher targeted editing efficiency of Cas9. The successful transformation was done using *Agrobacterium rhizogenes* strain K599 (Du *et al.*, 2016) (Table 2). Likewise, Cheng *et al.* (2021) edited the two soybean genes *GUS* and *GFP*, for herbicide tolerance by using Cas9, which showed the development of eco-friendly soybean genotypes. Due to the modernization of agriculture, different kinds of herbicides, fungicides, and insecticides are contaminating our food and causing severe health hazards for human beings. Hence, CRISPR/Cas9 is a potent tool for developing resistant soybeans, which can yield higher without any toxic effect in its products (Cheng *et al.*, 2021). A list of different genes for herbicide tolerance and other critical traits is given in Table 2. The application of CRISPR/Cas9 to edit the targeted gene of desired traits has not been broadly studied in soybeans. Time is needed to explore the soybean genome for identification of different genes for their transformation into commercial cultivars.

Table 2. List of CRISPR/Cas9 based edited genes for improvement of numerous traits in soybeans

Crop	Gene	Trait	Tool	Transformation method	References
Soybean	<i>GmPDS11</i> , <i>GmPDS18</i>	Dwarf buds	CRISPR/Cas9	<i>Agrobacterium rhizogenes</i> strain K599	(Du <i>et al.</i> , 2016)
	<i>DD20</i> , <i>DD43</i>	Chlorsulfuron-resistant	CRISPR/Cas9	Particle bombardment	(Li <i>et al.</i> , 2015)
	Large chromosomal segment	Multiple gene editing	CRISPR/LbCp f1	<i>Agrobacterium rhizogenes</i> strain K599	(Duan <i>et al.</i> , 2021)
	<i>GlymaFAD2-1A</i> and <i>GlymaFAD2-1B</i>	Editing efficiency	CRISPR/LbCp f1	Polyethyl glycol (PEG)	(Kim and Choi, 2021)
	<i>GmAGO7a</i> , <i>GmAGO7b</i>	High somatic mutation in soybean hairy root	CRISPR/Cas9	<i>Agrobacterium rhizogenes</i> strain K599	(Zheng <i>et al.</i> , 2020)
	<i>GUS</i> , <i>GFP</i>	Herbicide tolerance	CRISPR/Cas9	<i>A. rhizogenes</i> strain K599	(Cheng <i>et al.</i> , 2021)
	<i>GmF3H1</i> , <i>GmF3H2</i> , <i>GmFNSII-1</i>	Resistance to soybean mosaic virus	CRISPR/Cas9	<i>Agrobacterium rhizogenes</i> strain K599	(Zhang <i>et al.</i> , 2020b)
	<i>Rhg1</i> and <i>Rhg4</i>	Resistance to soybean cyst nematode	CRISPR/Cas9	<i>Agrobacterium</i>	(Kang, 2016)

CRISPR/Cas9 to drive soybean domestication events

Domestication of wild crops species is an attractive way of meeting the increasing demand for food; therefore, domesticating wild species or using semi-domesticated crops is an attractive way to meet the ever-increasing demand for food. Traditional domestication is a lengthy process, and only a few loci have been identified which are involved in crop improvement. CRISPR/Cas9 can undoubtedly speed crop domestication (Yang *et al.*, 2019; Zhu *et al.*, 2020). Soybean domestication is one of the most critical factors to introduce the desired gene pool into existing elite cultivars. Many genetic events happened during the soybean domestication in Asia (Hyten *et al.*, 2006). Unfortunately, more than 50% of genetic diversity (Zhou *et al.*, 2015) existed in soybean landrace, *Glycine soja* was lost during the domestication events. Currently, only 19 soybean landraces in the North American breeding pool have contributed to genetic variation for most of the desired traits (Gizlice *et al.*, 1996). The present-day soybean originated from a wild-type species from East Asia about 6000-9000 years ago in China (Kim *et al.*, 2012).

Soybean origin is still a mystery because of the lack of detailed genetic information. Newly emerged molecular techniques like CRISPR/Cas9 have shed light on the origin of this valuable crop (Lu and Tian, 2022). The published literature showed that GE could instantly cause domestication; however, there are numerous factors involved in the long-term domestication process. The long-term interaction of genetic and cultural practices drives the domestication process (DeHaan *et al.*, 2016). GE editing has been applied to edit multiple genes like *SP5G* to improve traits like, fruit number in wild tomato *Solanum pimpinellifolium* to make it a valuable and attractive cultivar (Li *et al.*, 2018; Zsögön *et al.*, 2018), but there is currently no proper study reported on the use of CRISPR/Cas9 in soybean domestication. The studies mentioned above strongly recommended the use of GE in soybeans. The wild soybean relatives could be used to successfully apply CRISPR/Cas9 to edit the candidate genes for multiple traits to speed up or drive the domestication events in the future (Li *et al.*, 2022). As is evidenced from the above results, the domestication of soybeans is one of the

main strategies to improve the wild soybean for yield and quality traits. Until now, there is no report of use of CRISPR/Cas9 for soybean domestication. However, the studies are being conducted to expand the use of CRISPR/Cas9 to drive the soybean domestication events (Li *et al.*, 2020a). Earlier, Li *et al.* (2020a) reported the CRISPR/Cas9 mediated knockout of gene, *GmPRR3b* which result in delayed growth as well as floral transition. This evidence indicates the possible use of CRISPR/Cas9 to drive the domestication process in soybeans. A comparison of conventional and CRISPR/Cas9 mediated soybean domestication process is presented in Figure 4.

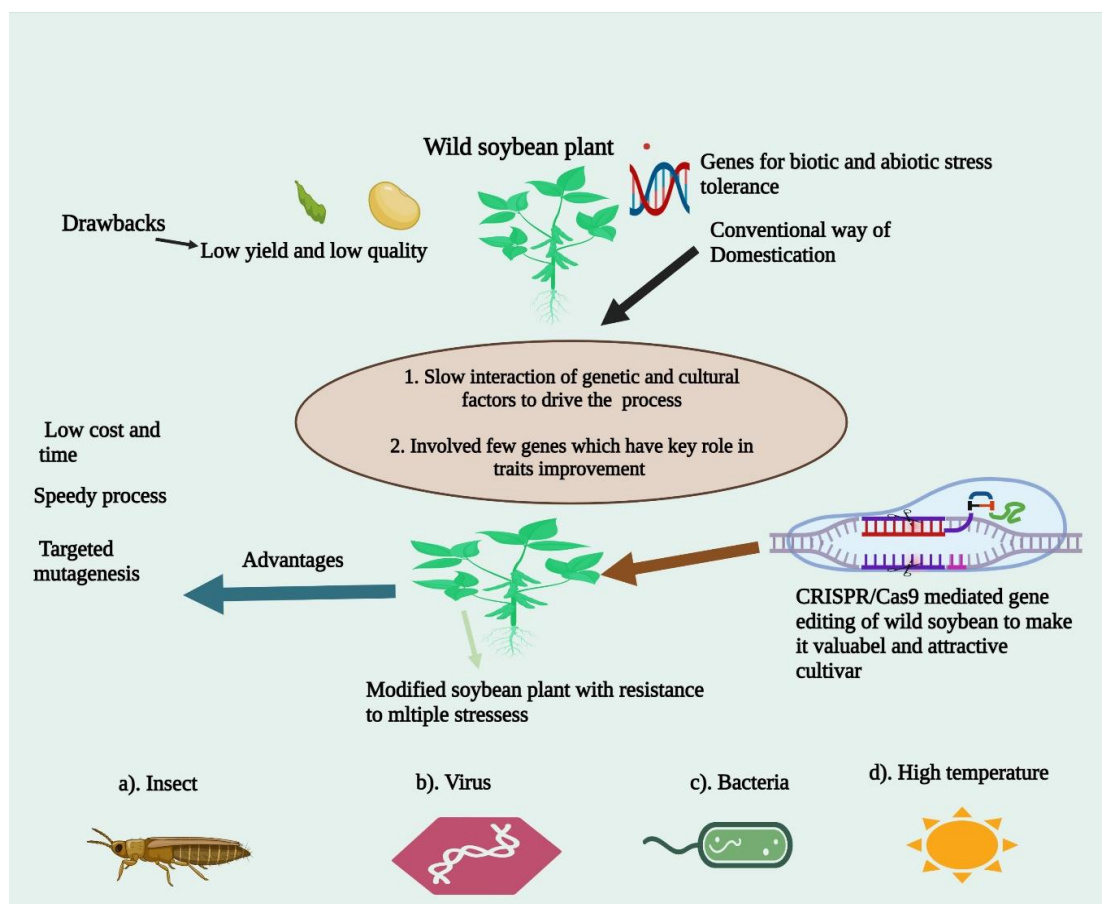


Figure 4 Advantages of CRISPR/Cas9 mediated the domestication process's driving over the traditional breeding plans

It involves the identification of wild genotypes, identification of genes, knockout of genes and improvement of traits to bring the crop under domestication. This is a speedy process with targeted mutagenesis, low cost and time. The modified plants are high yielding and have resistance to certain biotic and biotic factors. This Figure was created with BioRender.com.

Efficiency and development of new gene-editing systems (CRISPR/LbCpf1) in soybeans

The development of new soybean genotypes is mainly limited because of the duplication and redundancy of the genome. Hence, deletion of large chromosomal segments and multiplex editing using CRISPR/Cas9 proteins is one of the most promising strategies to address these challenges. Cpf1 is a versatile GE tool for large chromosomal segment deletion; however, its successful applications have been reported in only a few species. The first successful CRISPR/LbCp1 application report for segment deletion was written by (Duan *et al.*, 2021). They have optimized the CRISPR/LbCpf1 for direct repeats and gRNA lengths in crRNA display. The editing efficiency was checked by using LbCp1 driven by CaMV35S and ubiquitin promoter of soybeans. Results showed that targeted genes could be mutated in a single step when a single eight gRNA target

crRNA array was applied with an editing efficiency of 17.1. This system was successfully used for large fragment and small fragment deletion involving four gene clusters in soybeans. In the conclusion section, these results revealed that CRISPR/LbCpf1 is precise and reliable for editing multiple genes simultaneously, which is not possible with Cas9 alone. This tool demonstrated a remarkable breakthrough in multiple gene editing. Future studies would be needed to improve various traits and develop a cultivar with numerous desired characteristics (Duan *et al.*, 2021). Kim and Choi (2021) studied the targeted mutagenesis of two soybean genes, *GlymaFAD2-1A* and *GlymaFAD2-1B*, by CRISPR/LpCpf1 in soybean protoplast. Among the other systems, LbCpf1 was highly efficient in multiplex editing of genes in soybean cotyledon. These findings demonstrated that soybean protoplast-based CRISPR selection is a novel tool for sorting out the ccRNAs efficiency and heritability for the next generation (Kim and Choi, 2021). Zheng *et al.* (2020) studied the editing efficiency and validation of editing systems in soybeans. The successful mutation of *GmAGO7a*, *GmAGO7b* was done using Cas9 and *Agrobacterium rhizogenes* strain K599 for transformation. These findings suggested a new platform to create inheritable soybean mutants by avoiding the complications of somatic mutations. This can be used to characterize the targeted gene in soybeans (Zheng *et al.*, 2020). Besides all of this, base editing and prime editing systems have been used in crops. BE has been emerged as a novel technique which enables accurate substitution of nucleotide in a defined manner, without gene disruption (Komor *et al.*, 2016). BE is used to convert one base into another (Eid *et al.*, 2018). A new type of technique is PE which can overcome challenges in crop improvement. This new ground-breaking genome-editing tool can bring targeted changes with less off-target events (Anzalone *et al.*, 2019).

Hence, the editing efficiency of CRISPR/Cas9 can be improved and used to bring heritable mutations to the genome. Besides all of these aspects, CRISPR/Cas9 can also be used for development of a mutant library in soybeans which has a complex genome. Bai *et al.* (2020) successfully applied CRISPR/Cas9 to develop mutants' library in soybeans to overcome gene redundancy issues. These mutants have different characteristics, like increased nodule number which can be a key factor in improving nitrogen fixation in soybeans.

Challenges and limitations of GE in soybeans

The transgenic techniques developed in soybeans during the last four decades have pooled various foreign genes into the soybean genome to improve multiple traits (Zheng *et al.*, 2021). Due to the random integration of genes in the crop, public concerns have been increased. Due to strict government regulations and measurements, the development cost of a new variety has increased (Turnbull *et al.*, 2021). Hence, CRISPR/Cas9 based genome editing provides a very efficient way of gene editing in a defined manner rather than introducing a gene into a cultivar by several backcrossing events. The CRISPR-based GE can be easily distinguished from the mutations created by traditional breeding methods. Cas9 mediated gene editing can overcome the issues related to transgenic techniques. It is obvious that a large number of GE tools have been developed for soybeans and various outcomes have been achieved as mentioned above (Scheben and Edwards, 2018). Other challenges include the lack of guarantee of defined mutation, limited ways of successfully delivering genes into soybean cells (Figure 5), and low efficiency of mutant screening and regeneration of mutants. Many steps have been taken to reduce these limitations and ensure successful gene editing, transformation, and regeneration of mutants in soybeans; however, various concerns still need additional studies to find reliable solutions (Xu *et al.*, 2020). Despite many applications in crop improvement, CRISPR/Cas9 still has a lot of challenges. The lack of abundance of genes for critical agronomic traits which has limited the gene pool is one of the main challenges of CRISPR/Cas9 use in crop improvement. For this issue, we need to explore the large number of genetic resources for valuable genes to be used in CRISPR/Cas9 system. Lack of efficient transformation systems and regeneration systems is also a main issue to be addressed. In some countries, increased biosafety concerns will hinder the large-scale use of CRISPR/Cas9 in future, however it's a topic of debate and many countries are relaxing their stance on it. With the passage of time, later

and newer methods can be identified which will help to identify and remove the mutants with off-target effects. To overcome the off-target effects in mutants, it is mandatory to select high affinity sgRNA with Cas9 enzyme in the experiments (Bao *et al.*, 2019a). Presence of public concerns in the market as consumer is also pushing the CRISPR tool to a narrow side.

High-efficiency base replacement

Most GE tools mainly focus on targeted mutagenesis of the desired gene. On the other hand, the uses of BE remain very limited, mainly in soybeans, because of the low efficiency of transformation methods and lack of proper knowledge. In crops, traits are often regulated by genetic variation in single nucleotide polymorphisms (SNP); the main challenge is to recognize them and mix them into superior cultivars. This will help to insert a new allele onto the genome by using the base replacement technique. The use of repairing techniques like HRD and NHEJ will hold promise in this way. The prime editing technique used to mutate a particular DNA segment will be helpful in base replacement if used and validated in crops (Anzalone *et al.*, 2019). PE is more advanced than BE, which converts A to G and C to T and it offers the replacement of small DNA regions (Rees and Liu, 2018). Extensive research activities are required to adapt and advance these techniques for various plant classes (Yang, 2020). Recently, Cai *et al.* (2020) successfully applied CRISPR/Cas9 for base editing in soybeans, enabling a base substitution into others by using RNA programmatic way without double-stranded breaks of DNA (Komor *et al.*, 2016).

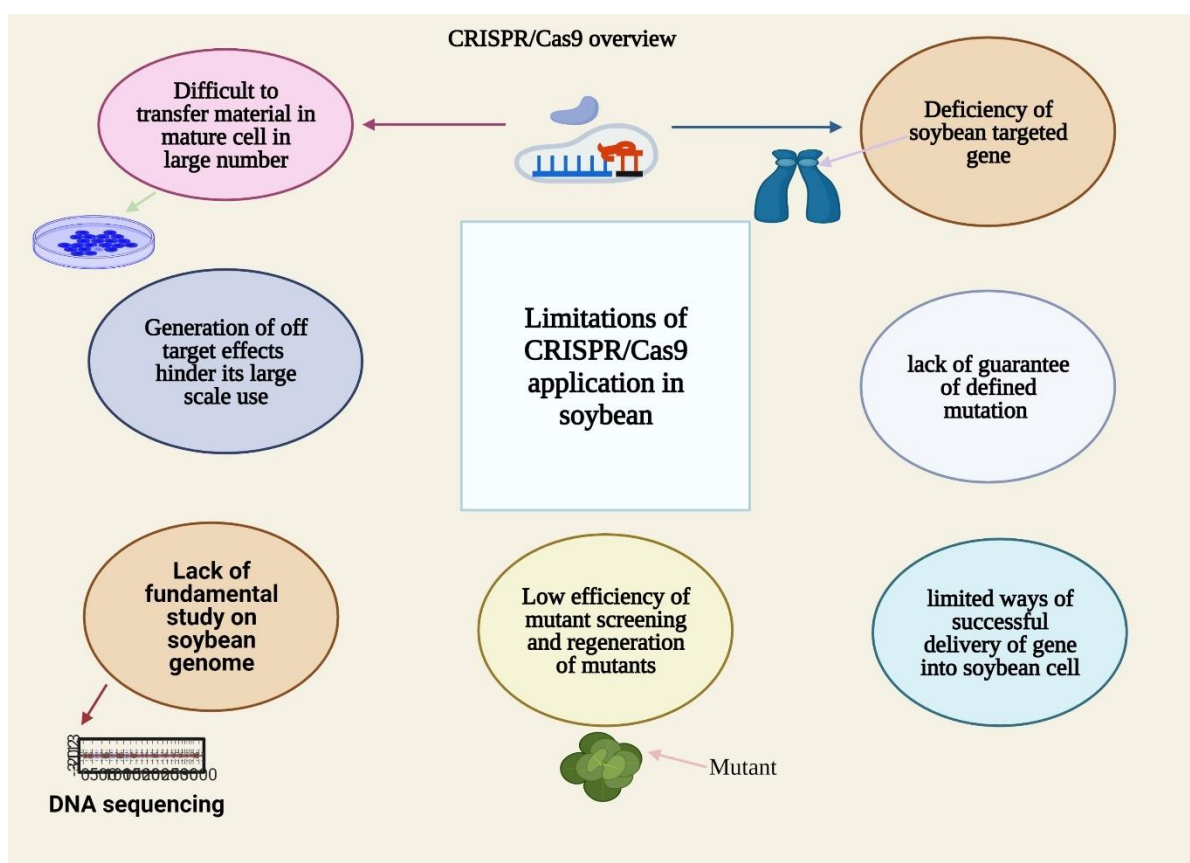


Figure 5. Limitations of use of CRISPR/Cas9 in soybeans

The limitations involve the, deficiency of soybean targeted genes, difficult to transfer the material into mature cell, off target effects, lack of fundamental study on soybeans, and low rate of successful regeneration of mutants. This Figure is created with [BioRender.com](https://www.biorender.com).

Reducing off-target effects and improving the efficiency of CRISPR/Cas9 GE in soybeans

CRISPR/Cas9 mediated GE in soybeans resulted in the generation of off-target effects (Figure 6) compared to GE by ZFNs and TALENs (Sun *et al.*, 2015). For instance, the off-target sites were identified in the soybean genome using the CRISPR-P5 web tool for targeted editing of the *FAD2* gene (Do *et al.*, 2019). The off-target sites in the soybean genome can be minimized by using several web tools like Cas-Offfinder (Bae *et al.*, 2014), and CROP-IT (Singh *et al.*, 2015). The effects can be reduced by enhancing the specificity of CRISPR/Cas9 systems by using highly effective variants of Cas9 nuclease (Zhong *et al.*, 2019) (Figure 6) and transforming cleaved Cas9 ribonucleoproteins (RNPs) into the cell (Kim *et al.*, 2017). There has been a system made for the soybean database to identify and analyse the off-target effects (Zou *et al.*, 2020). This system allows the calculation of off-target number and specificity values for every single CRISPR/Cas9 target site. This would be helpful to reduce off-target effects generated in soybean GE in the future (Xu *et al.*, 2020). The selection of a stable transformation vector (Figure 6) should be a primary objective to successfully transform the desired gene into the soybean genome for a particular trait (Chen *et al.*, 2021a). The efficiency of soybean genetic transformation is always low, which has been a significant hindrance in the use of CRISPR/Cas9 tools to screen the mutant's population (Bai *et al.*, 2020). Bai *et al.* (2020) successfully developed a soybean mutants library using pooled CRISPR/Cas9 system. They have made about 70 CRISPR/Cas9 vectors to edit 102 candidate genes and their homologs exposed to mutual transformation in 16 phases. A progeny containing about 407 T0 lines was found comprising all sgRNAs at a usual editing frequency of 59.2%, counting 35.6% lines carrying multiplex alterations. This study gives a modified strategy to generate multiplex mutant populations to avoid the issue of unnecessary gene copies in soybeans (Bai *et al.*, 2020). The key strategies to improve the efficiency of CRISPR/Cas9 gene editing in soybeans is given in Figure 6.

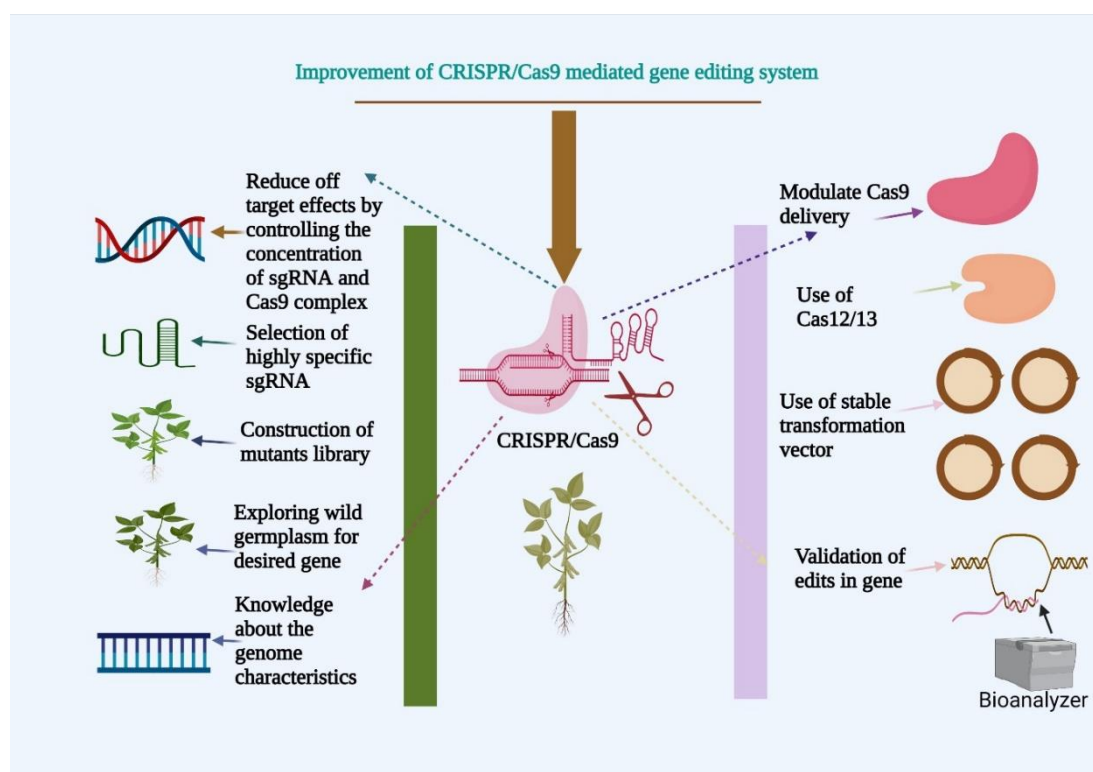


Figure 6. Key strategies to enhance the efficiency of CRISPR/Cas9 genome editing in soybeans

Regulatory measurements of CRISPR/Cas9 gene-editing tool

Top GM crop producers like the USA, Argentina, and Brazil announced that if the newly developed crops have no foreign DNA element, then the crop varieties would be regulated as a traditionally improved variety; however, the opinion of the Europe Union (EU) was in contrast with other countries (Bhowmik *et al.*, 2021). The Secretary of Agriculture of the United States of America (USA) in 2018 revealed that the crop genotypes which are developed without the involvement of any foreign genetic material do not need any type of additional regulatory measurements (<https://www.usda.gov/media/press-releases/2018/03/28/secretary-perdue-issues-usda-statement-plant-breeding-innovation>). Recently, the European Court of Justice stated that the crop produced by the genome-edited method must be subjected to regulation across the union (Callaway, 2018). In Brazil, principles used to elaborate the Biosafety Law of Brazil (N0. 11.105 of 24 March 2005) help scientists to speed up the developments in agricultural areas. According to provisions RN16 of Brazilian Law, the crop genotypes and their products were analysed and considered by CNTBio (Entine *et al.*, 2021) to possess the traits developed in the Normative and were not considered to fall in the scope of law 11.105/2005, which monitors GM crops in Brazil. By following this now, small companies adopt new techniques by considering the unique measurements for GE (Hua *et al.*, 2019; Zhao *et al.*, 2019a; Entine *et al.*, 2021). The Chinese Ministry of Agriculture provides directions for GM crops, mainly focusing on the safety of these products (Ahmad *et al.*, 2021). In 2011, China implemented the laws for GM crops, including soybeans and other crops (Arujanan and Teng, 2018). Still now, China has approved 64 submissions for commercial cultivation of GE crops, including soybeans, rice, and cotton (Li *et al.*, 2020b).

Conclusions

The objective of developing low-cost and safe soybean products to meet the growing population's needs may pose problems. Therefore, using novel techniques for soybean improvement will be necessary. The application of novel breeding techniques allows breeders to quickly alter and transfer the gene into the soybean genome more quickly compared to traditional breeding methods. CRISPR/Cas9 has been applied in many crops for successful trait improvement during the past few years. Though, numerous alterations to this tool will lead to an increase in target efficiency. CRISPR/Cas9 mediated gene editing has emerged as the most powerful and reliable tool for efficient crop engineering for important agronomic traits. Dozens of reports on the successful use of CRISPR/Cas9 in soybean improvement have been presented. CRISPR/Cas9 mediated genome editing is used to develop non-transgenic crops. It has been applied mainly to develop critical agronomic traits in soybeans, including yield, quality, and resistance to biotic and abiotic stresses. This method helps to analyse the crucial role of different genes and increase the genetic makeup of soybeans. Because of these reasons, breeders working on soybeans worldwide favour this tool. The soybean crop relies on targeted gene editing, which breaks double-stranded DNA. To increase the editing efficiency of GE for multiple traits, it is mandatory to develop an efficient system that can recognize targeted regions. CRISPR/Cas9 mediated GE for drought, cold, salinity, and heavy metals needs to be studied to develop resilient cultivars.

Breeders first need to identify the genes that express specific agronomic traits and edit them by Cas9 to develop new varieties. In future studies, soybean breeders can apply the HDR repairing method to aid the breeding techniques for the desired gene to develop new genotypes. The development of the CRISPR/Cas9 library possesses many benefits like multiplexing and targeting the gene of interest. To decrease the off-target effects, it is essential to conduct a quality check of CRISPR mutant's library of soybean crops at every point in the screening test. Investigation of gene role by the above technique is essential to identify the role of genes. Newly uncovered CRISPR/Cas9 approaches and the development of new tools are being successively defined, suggesting that the use of CRISPR/Cas9 toolbox for GE will rise further in the future. Exploring wild germ

plasm will help to identify potent genes used in CRISPR/Cas9 GE in soybeans. The new and efficient system has been developed like CRISPR/LbCpf1, which causes multiple GE in a single step. Further use of this method would revolutionize the soybean industry in the near future. This set of tools will bring new approaches to attain well-defined genome editing without involvement of any foreign DNA in genome-edited soybean plants. New studies in fundamental genetic areas should unlock new gene groups to improve vital agronomic traits. Additionally, to integrate into the soybean crop genome, CRISPR/Cas9 can be reshaped, like GE of mitochondrial and chloroplast gene. Finally, CRISPR/Cas9 has indisputably reshaped soybean genetics and will continue to expand its areas in the future.

Authors' Contributions

GJ, XZ contributed equally to work in preparation of MS. AR prepared Figures. WT, ZQ, ZZ, ZZ, provided technical assistance. JJG and IA reviewed the MS. WX, WJ and GY supervised the study. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

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

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