Chapter

Role of CRISPR/Cas9 in Soybean (*Glycine max* L.) Quality Improvement

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Abstract

Biotechnology has made significant advances in recent years, and the area of genetic engineering is progressing day by day, generating several advantages. Through the new ability to precisely change and modify the genomes of living organisms, genome editing technology has transformed genetic and biological research. Genome editing technology first appeared in the 1990s, and different approaches for targeted gene editing have subsequently been created. The fields of functional genomics and crop improvement have been transformed by advances in genome editing tools. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat)-Cas9 is a versatile genetic engineering tool based on the complementarity of the guide RNA (gRNA) to a specific sequence and the endonuclease activity of the Cas9 endonuclease. This RNA-guided genome editing tool has produced variations in plant biology fields. CRISPR technology is continually improving, allowing for more genetic manipulations such as creating knockouts, precise changes, and targeted gene activation and repression. Soybean is a leguminous crop, high in protein and oil contents that are used for poultry and livestock feed industry. In this chapter, we focus on the recent advances in CRISPR/Cas9-based gene editing technology and discuss the challenges and opportunities to harnessing this innovative technology for targeted improvement of traits in soybean and other crops.

Keywords: clustered regularly interspaced short palindromic repeats, genome editing (GE), guide RNA (gRNA), nonhomologous end joining (NHEJ), homology-directed repair (HDR), Cas9

1. Introduction

Nowadays, almost one billion people suffer from malnourishment due to increasing population, and our agricultural system is degrading by the loss of biodiversity and climate change [1]. To overcome the malnourishment, there is a need to improve the crop plants. To achieve this goal, conventional breeding approach is labor-intensive, and it takes several years to form the commercial varieties. Genome editing tools are advanced biotechnological techniques to modify an organism's genome efficiently and precisely. Although recently developed genome editing technologies, such as zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs),

have many advantages but also has some drawbacks too. CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology has site-specific genome editing with precision, efficiency, and ease of use.

The type II CRISPR/Cas system is a prokaryotic adaptive immune response system that guides the Cas9 nuclease to induce site-specific DNA cleavage using noncoding RNAs as a template. The CRISPR/Cas type II system is a flexible genome editing tool for crop improvement [2]. It is a simple, effective, and cost-effective approach that can target several genes. Many plants have advantage from the CRISPR/Cas9 system, including rice, maize, wheat, soybean, sorghum, and barley [3].

The CRISPR/Cas9 system has been utilized for genome editing in all mammalian cells, which may be used to make gene knockouts (through insertion/deletion). A single-guided RNA (sgRNA) is used to guide the Cas9 nuclease to a specific genomic region in order to disrupt genes (**Figure 2**). Double-strand breaks caused by Cas9 are repaired by the NHEJ DNA repair mechanism. Because the repair is prone to errors, insertions and deletions (INDELs) might occur, causing gene function to be disrupted. Cellular DNA repair processes, either the nonhomologous end joining DNA repair pathway (NHEJ) or the homology-directed repair (HDR) pathway, fix the DNA damage or DNA repair pathway (i.e., HDR).

Mechanism of CRISPR/Cas9-mediated gene disruption is as follows: (1) A single-guide RNA (sgRNA) binds to a recombinant form of Cas9 protein with DNA endonuclease activity, consisting of a crRNA sequence specific to the DNA target and a tracrRNA sequence that interacts with the Cas9 protein. (2) The resultant complex will cleave double-stranded DNA that is particular to the target. (3) Then, cleavage efficiency of sgRNAs will be tested.

Crop development techniques should enable to increase production, biotic and abiotic stress resistance, as well as quality and nutritional value. Over several decades, innovative agricultural technology has considerably enhanced crop productivity. Consumers are more concerned about crop quality since it provides many nutrients such as proteins, fiber, vitamins, minerals, and bioactive substances, all of which are directly linked to human health [4]. In addition, scientists and breeders have switched their focus from increasing production to enhancing quality. Traditional crossing breeding, chemical and radiation-mediated mutation breeding, molecular marker-assisted breeding (MAB), and genetic engineering breeding have all proven successful in improving various crop qualities [5–8]. Traditional mutagenesis-based breeding techniques are time-consuming and labor-intensive, especially for polyploid crop production [9]. Recently, crop breeding has advantage from genome editing (GE) technology, which alters plant genomes in a precise and predictable manner [10].

Genome editing can produce predictable and inheritable mutations in specified regions of the genome, with minimal off-target effects and no external gene sequence integration. Deletions, insertions, and single-nucleotide substitutions (SNPs) are all examples of GE-mediated alterations. There are four SDN (site-directed nuclease) families in a nucleotide excision process, i.e., homing endonucleases or mega-nucleases (HEs) [11], zinc-finger nucleases (ZFNs) [12], transcription activator-like effector nucleases (TALENs) [13], and CRISPR-associated protein (Cas) [14]. The majority of SDNs can precisely target double-stranded template DNA and produce a double-strand break (DSB). The DSBs are naturally repaired by a plant's endogenous repair system, which uses one of two major DNA damage repair mechanisms: nonhomologous end joining (NHEJ) or homologous-directed recombination (HDR). A FokI cleavage domain and a particular DNA-binding domain from TALE proteins make up TALENs. TALENs technology has a greater target binding specificity and decreased off-target effects when

compared with ZFNs [15]. In rice [16], wheat [17], maize [16], and tomato [18], it was widely used as a gene-editing method. However, ZFN and TALENs have long construction procedure, which has limited their use in plants on a wide scale. CRISPR (clustered regularly interspaced short palindromic repeat) was first discovered in *Escherichia coli* in 1987 and described as an immunological response to viral and plasmid DNA invasion [19]. CRISPR/Cas systems have become the most popular GE technology in recent years. Because the specificity of editing is governed by nucleotide complementarity of the guided RNA to a specific sequence without protein engineering, the CRISPR/Cas systems are more efficient for genome editing than other SDNs [20].

Soybean is a leguminous crop, has a great economic value, and is high in protein and oil. With the growing demand for soybeans around the world, it is more important to understand gene function and speed up functional gene research and breeding to increase yield and improve quality. Traditional soybean breeding procedures are insufficient to meet the growing demand for soybean products and the problems posed by the agricultural environment. As a result, it is critical to implement quick, precise, and effective breeding procedures in order to develop improved varieties, particularly with improved yield, quality, and stress tolerance or resistance [21, 22]. Genome editing technology is a highly desired technology given the advantages listed above, and it is also an excellent tool for improving soybean genetics. The number of crops engineered by genome editing has increased day by day. Crop quality is one of the most important objectives among the different target traits for crop improvement. Here is a brief description of different quality traits improvement through CRISPR/Cas9-mediated tool.

2. CRISPR/Cas9 gene-editing system in plants

CRISPR/Cas systems are split into two classes and five kinds based on the Cas protein classification. The *Streptococcus pyogenes* type II CRISPR/SpCas9 system has

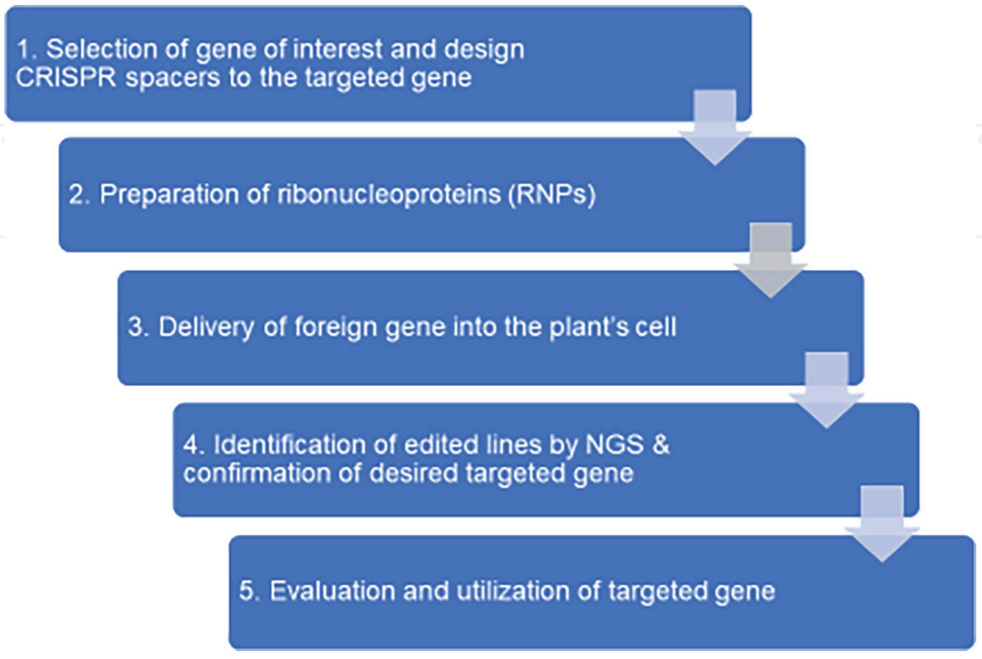

Figure 1.
The workflow of CRISPR/Cas9-based gene editing in plants.

Application	Crop	Gene target
Physical appearance and quality	Rice	GS3, Gn1a, GW2, GW5, TGW6, GL2/OsGRF4, OsGRF3, GS9, GW5, OsGS3, OsGW2, and OsGn1a, ANT1, SlMYB12, SlMYB12, Psy1, CrtR-b2
	Tomato	OVATE, Fas, Fw2.2, fas, lc, ENO, CLV3,
	Wheat	TaGW7, TaGW2
	Maize	Psy1
Texture, palatability, quality	Tomato	ALC, PL, PG2a, TBG4
	Banana	MaACO1, OsGBSSI, OsGBSSI
	Rice	OsAAP6, OsAAP10, OsBADH2, SH2, GBSS
	Maize	Wx1
Nutritional quality	Rice	OsGAD3, OsNramp5, OsFAD2–1, OsPLD*a*1
	Tomato	SlGAD2, SlGAD3, slyPDS, BnFAD2
	Wheat	*a*-gliadin genes

Table 1.
List of research on crop quality improvement by using CRISPR/Cas gene-editing technology.

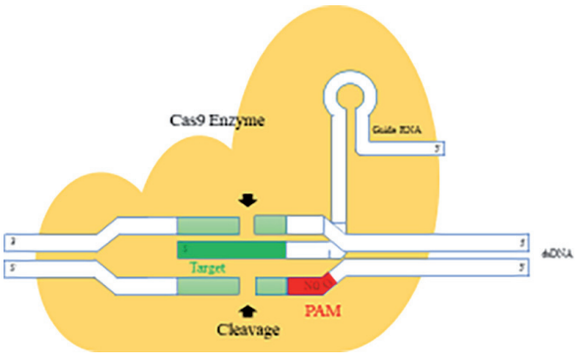

Figure 2.
Mechanism of CRISPR/Cas9 system.

been adapted and evolved as a powerful GE tool for several purposes [2]. The Cas9 protein and the guide RNA (gRNA or sgRNA) are the two main components. CRISPR RNA (crRNA) and trans-activating crRNA are both made up of gRNA (tracrRNA). A 20-nt fragment (also known as spacer, complementary to a certain region of target genes) is followed by a protospacer adjacent motif (PAM) in the target genes of interest in the gRNA. Cas9 nuclease generates DSBs to three base pairs upstream of the PAM motif under the supervision of gRNA [23]. Gene deletion or loss of protein function is common outcome of NHEJ cleavage repair [24]. **Figure 1** indicates the general steps of CRISPR/Cas9 gene editing technique.

Using CRISPR/Cas9, several scientists have been working to improve crop yield, quality, and stress resistance. CRISPR/Cas9-mediated genome editing has been reported in 41 food crop species, 15 industrial crops, 6 oil crops, 8 ornamental crops, 1 fiber crop, and 1 feed crop (**Table 1**) [25]. Mechanism of CRISPR/Cas9 system breakage is presenting in **Figure 2**.

3. CRISPR/Cas9-mediated molecular breeding enhances the crop quality

Crop quality has been an important factor in determining market value of crop. External and internal traits, in general, determine crop quality. Physical and visual aspects such as size, color, texture, and aroma are examples of external quality attributes. Internal quality factors, on the other hand, consist of nutrients (such as protein, carbohydrate, and fats etc.) as well as bioactive chemicals (carotenoids, lycopene, –aminobutyric acid, flavonoid, etc.).

3.1 Improvement in the physical appearance of crop

3.1.1 Modification of shape and size

CRISPR/Cas9 technology was utilized to alter the shape and size of the crops to the consumer's demand. Several genes/quantitative trait loci (QTLs) have been proposed to be responsible for crop appearance quality. Rice and tomato supplied the most information about fruit shape and fruit size. The first QTL found to increase grain length, GS3 (GRAIN SIZE 3), has been successfully knocked out in five japonica rice varieties. The grain length of T1 lines has been increased in all genetic backgrounds compared with wild type [26, 27]. Grain shape affects grain weight (GW) as well as quality [28]. For example, disruption of many grain weight negative regulators, GW2, GW5, and GW6, enhanced the rice grain weight. The disruption of TaGW7 in wheat using CRISPR/Cas9 technique helps in increasing grain width and weight [29]. Researchers can manipulate tomato fruit form and size in horticulture species by changing the expression of OVATE, CLV [30], fas and lc [31], and ENO [32]. OVATE and SUN, for example, are involved in asymmetric and symmetric fruit elongation [33, 34], whereas SlWUS and SlCLV3 are genes that modulate tomato locule quantity. CLV3 mutations that cause gain-of-function and WUS mutations that cause partial loss of function are referred to as the fas and lc locus, respectively. Both mutations increase the size of the fruit [35–37]. This was further validated by disrupting the CLV-WUS cis-regulatory genes [31].

3.1.2 Color modification

Plant pigments made up of carotenoids, anthocyanins, and polyphenols determine plant color. The color of the fruit, leaves, and flower buds, especially in plant edible parts, affects the consumer's taste. Europeans and Americans like red tomatoes, whereas Asian customers prefer pink tomatoes [38]. It was reported that the absence of flavonoid pigments in the peel caused the pink phenotype. CRISPR/Cas9 can thus be used to manipulate the color of fruits by interrupting genes involved in the pigment production pathway. MYB12 regulates the accumulation of flavonoids and controls the pink skin phenotype as a flavonoid biosynthesis pathway transcription factor. SlMYB12 has been successfully knocked out, resulting in pink-fruited tomatoes [39]. In addition, by targeting PSY1 and ANT1, researchers were able to develop yellow and purple tomatoes. The PSY1 gene controls the early stages of carotenogenesis by encoding phytoene synthase. PSY1 mutations lowered total lycopene levels, resulting in yellow flesh tomato fruit [40, 41], whereas ANT1-modified tomatoes increased anthocyanin accumulation, resulting in purple plant tissue [42]. The anthocyanin biosynthetic structural genes are predominantly controlled by R2R3-MYB,

bHLH, and WD-repeat proteins in all crop species investigated. Yellow roots occurred from CRISPR/Cas9 knockout of DcMYB7, an R2R3-MYB, in the solid purple carrot [43]. Flower color influences the market value of ornamental crops, and plant breeders are continually looking for new colors. Several groundbreaking flower color alteration research studies have previously been completed. Flavanone 3-hydroxylase (F3H), a major enzyme involved in flavonoid production, is required for anthocyanin accumulation. Disrupting F3H with CRISPR/Cas9 has resulted in pale blue flower torenia variations and pale purplish-pink flowered petunia varieties [44, 45].

3.2 Improving crop texture

3.2.1 Prolonging shelf life

Fruit texture is another important factor in commercial crop production. CRISPR/Cas9 technology has a pivotal role for extending the shelf life of tomatoes and bananas. Several naturally occurring mutant genes, such as Nr, alc, rin, nor, and Cnr [46], have the potential to extend shelf life. These modifications, however, are accompanied by a lack of color, an unpleasant flavor, and a low nutritional value [47]. According to one study, alc mutation not only extended the shelf life of fruits but also preserved their color and smell [48]. To induce tomato ALC gene mutations, HDR-mediated gene replacement was used, and the intended alc homozygous mutants in T1 generation displayed good storage performance [49]. Cell wall degrading enzymes can alter the texture of fruits [50]. During fruit softening, the pectate lyase enzyme can degrade the cell wall [51]. Pectate lyase enzyme interferes with RNA for prolonging life in tomato to exhibit a good fruit phenotype [52]. Similarly, SlPL gene knockout mutations based on CRISPR/Cas9 showed a harder phenotype and longer shelf life without compromising organoleptic or nutritional quality [53, 54]. Downregulating endogenous ethylene production, in addition to silencing genes involved in cell wall disintegration, can be another effective technique for delaying the fruit softening process [55]. Ethylene is the most important element affecting banana post-harvest preservation and shelf life. MA-ACO1 is involved in the ethylene production process and has an impact on the after-ripening process [55]. After ethephon treatment, the after-ripening process in MA-ACO1-mutant lines was slowed by around 2 days. More intriguingly, the amount of vitamin C and sugar in the fruit was increased without any negative effects on the fruit's quality [56].

3.3 Improving palatability

3.3.1 Improving eating and cooking quality

Consumer acceptance and market value are both determined by the eating and cooking quality (ECQ). Amylose production requires the Waxy (Wx) gene, which codes for granule-bound starch synthase I (GBSSI). After cooking, rice varieties with a somewhat low amylose concentration (7–10%) have a soft and sticky texture, making them more popular among Asian buyers. Several genetic improvement studies have effectively used the CRISPR/Cas9 system to alter the Wx gene in japonica background rice accessions, resulting in accessions with grain amylose content of 5–12% without sacrificing other desirable features [57, 58]. A number of rice mutants with fine-tuned amylose contents have been created by precise alteration of particular bases of Wx genes to fulfill the different demands on ECQ [59]. Meanwhile,

by disrupting the Wx gene with CRISPR/Cas9 [60], waxy maize mutants have been generated in 12 elite inbred lines. Furthermore, rice with low palatability has a high grain protein content (GPC), which is inversely connected to ECQ. As a result, many elite rice cultivars with good ECQ have a low GPC content (often less than 7%) [61]. The GPC-related QTL qPC1 was discovered in rice for the first time. In rice, a positive regulator of GPC was found in an amino acid transporter (OsAAP6) found in qPC1 loci [62]. Rice cultivars with high ECQ can be quickly reduced in GPC and improved in ECQ using targeted mutagenesis of OsAAP6 and OsAAP10 [63].

3.3.2 Improving flavor

Next to ECQ, aroma is a preferred quality feature. Rice-eating communities in Asia and Europe both prefer rice cultivars with aroma [64]. According to research, most fragrant rice cultivars are particularly high in the 2-acetyl-1-pyrroline (2AP) chemical [65], which is also found in fresh bread and popcorn and gives food products a popcorn or cracker-like aroma [66]. BADH2 (encoding a betaine aldehyde dehydrogenase) has been linked to fragrance generation in genetic research [67, 68].

Functional BADH2 was found to engage in the conversion of -aminobutyraldehyde (GABald) to GABA, whereas nonfunctional BADH2 mutants convert GABald to 2AP [69]. As a result, RNAi technology has been employed to disrupt OsBADH2 and boost 2AP production [70]. In 2015, the first fragrant rice was generated by employing TALENs to target the OsBADH2 gene [71]. Researchers have recently made a breakthrough in the creation of novel OsBADH2 alleles using CRISPR/Cas9, successfully converting an unscented rice variety, ASD16, into a unique aromatic rice [72].

3.4 Biofortification of nutrient elements

Consumer preferences are changing toward healthier, more nutritionally rich foods. As a result, researchers have been struggling to develop new goods to meet the needs of this expanding industry. Many nutrients found in fruits and vegetables have anti-inflammatory, anticancer, and antioxidant properties. Biofortification of several nutrients, such as carotene, γ-aminobutyric acid (GABA), iron, and zinc, has been implemented in many crops through breeding programs. Through gene editing for biofortification, it has been attempted to satisfy the "hidden hunger" with high-quality nutrients.

3.4.1 Increasing carotenoid content

Carotenoids have been linked to antioxidant mechanisms and the prevention of eye diseases. Humans, on the other hand, are unable to produce carotenoids and must obtain them from their food. Lycopene and phytoene also aid in the prevention of cancer and cardiovascular disease. Previously, researchers used traditional genetic engineering to add CrtI and PSY genes into rice, as well as manufacture β-carotene. Many anti-GMO researchers believe that golden rice may not offer enough β carotene to eliminate vitamin A deficiency; and allergies and antibiotic resistance are potential dangers of planting and eating golden rice. GMO crops may also have an adverse effect on the ecosystem and biodiversity [73]. Carotenoid biofortification in rice, tomato, and banana has been achieved using CRISPR/Cas9 genome editing. Due to the lack of external gene integration in host genomes, those created by this technique have a good chance of avoiding GM regulation. Carotenoid biofortification

was generally accomplished using one of two methods. First, carbon input into the carotenoid biosynthesis pathway is imposed by overexpression of phytoene synthase genes using CRISPR/Cas9-mediated knock-in. A carotenogenesis cassette including CrtI and PSY genes was successfully integrated into the target site in rice, yielding marker-free gene-edited mutants with 7.9 g/g β carotene in dry weight [74]. Another technique is to prevent their precursors from being converted or to silence associated genes, such as LCYe, BCH, ZEP, and CCD4. The loss of the LCYe gene, for example, resulted in a golden fruit banana mutant with a sixfold increase in β-carotene content [75]. Similarly, a fivefold increase in lycopene content was achieved by disrupting five carotenoid metabolic-related genes (SGR1, LCYe, BLC, LCY-B1, and LCY-B2), resulting in a lycopene-enriched tomato [76].

3.4.2 Increasing γ-aminobutyric acid content

GABA is a nonprotein amino acid inhibitory neurotransmitter that regulates blood pressure and acts as an antianxiety agent [77]. As a result, the food sector has turned its attention to generating new GABA-rich foods. Glutamate decarboxylase (GAD) is a crucial enzyme that catalyzes glutamate decarboxylation to GABA. GAD has a C-terminal autoinhibitory region that inhibits GAD action. The C-terminal has been fully removed using CRISPR/Cas9 in order to boost GABA content. GABA accumulation increased sevenfold in mutant tomatoes [78]. Furthermore, researchers generated GABA-rich rice by truncating the C-terminal of the OsGAD3 gene using the CRISPR/Cas9 system, resulting in a sevenfold increase in GABA content [79]. GABA-rich vegetables clearly have a positive impact on human health. However, pursuing a high GABA content without regard for the phenotype of the fruit could result in not only a reduction in glutamate but also a faulty phenotype [80]. Li et al. [81] employed a multiplex CRISPR/Cas9 approach to remove SlGABA-Ts and SlSSADH, resulting in a 20-fold increase in GABA levels but significant reductions in tomato fruit size and yield [82].

3.4.3 Biofortification of micronutrients

Micronutrient deficiencies, such as selenium, zinc, iron, and iodine, affect around two billion people worldwide. For those who have an imbalanced diet, biofortification of crop plants with micronutrients would be a long-term solution. Knocking down vacuolar iron transporter (VIT) genes, such as OsVIT2, to increase Fe content in rice grain is a potential use of the CRISPR/Cas9 technology. In a recent study, OsVIT2 mutation resulted in increased Fe distribution to the grains' embryo and endosperm, as well as higher Fe content in the polished grain without affecting yield [83]. Furthermore, the rice gain-of-function arsenite tolerant 1 (astol1) mutant enhanced the grain content of selenium (Se), an essential mineral with antioxidant properties for humans. Gene editing can also help in the formation of micronutrient-rich rice and wheat grains by regulating the expression of genes involved in ion homeostasis [84].

3.4.4 Improving fatty acid composition

Olive oil is high in monounsaturated fatty acids (MUFA), such as oleic acid (18:1). Diets high in oleic acid have been shown to improve cardiovascular health. Saturated fatty acids and trans-fatty acids, on the other hand, are frequently referred to as "unhealthy" fats and have been associated to cardiovascular disease [85, 86]. Soybean oil, the most extensively produced and used edible oil, contains just 20% oleic acid,

compared to 65–85% in olive oil [87]. For controlling the fatty acid composition in soybean, several fatty acid desaturase genes, such as FAD2 and FAD3, were targeted and altered. By altering two homologous genes of GmFAD2, researchers have already boosted oleic acid levels from 20% to 80% in 2019, while lowering linoleic acid levels from 50% to 4.7% [88]. Similar breeding tactics have been used in rapeseed and camelina, with increases in oleic acid content of 7% and 34%, respectively [89, 90]. The first gene-edited high oleic soybean line, with 80% oleic acid and up to 20% less saturated fatty acid, was recently marketed for sale in the US market [91].

3.4.5 Eliminating antinutrients

Phytic acid, gluten protein, and cadmium (Cd) are only a few of the chemicals that have a negative impact on crop nutritional quality. Genome editing can also be used to reduce the amount of unwanted compounds in the body. Due to a lack of comparable degrading enzymes, humans are unable to metabolize phytic acid. Because phytic acid can interact with minerals and proteins to form complexes, absorption of minerals and protein is inhibited when large amounts of it are consumed by people [92]. CRISPR/Cas9 was used to knock out an ITPK gene encoding an enzyme that catalyzes the penultimate phase of phytate production in rapeseeds in order to lower phytic acid concentration [93]. The ITPK mutants had a 35% decrease in phytic acid without affecting plant performance [94]. Furthermore, gluten proteins in wheat can cause celiac disease in people who are gluten-intolerant [95]. Due to the more than 100 loci in the wheat genome that code for gluten protein, traditional breeding strategies are unlikely to reduce gluten concentration. Low-gluten, transgene-free wheat lines have been generated using CRISPR/Cas9 to target a conserved area of the α-gliadin genes [96]. Furthermore, the CRISPR/Cas9 technology has aided in the development of heavy metal pollution-resistant rice varieties. Cd is a human carcinogen, and long-term use of Cd-contaminated rice can result in chronic diseases such renal failure and cancer [97]. As a result, scientists face a problem in developing low-heavy-metal rice in Cd-contaminated areas [98]. Researchers created new Indica rice lines with reduced Cd accumulation in grain by altering OsNramp5, which mediates Cd root uptake. Furthermore, when cultivated in high Cd circumstances, osnramp5 mutants' agronomic characteristics and grain yield were unaffected [99].

4. Challenges and future perspectives

Gene editing in crops is now progressing at a considerably faster rate than in other disciplines. The CRISPR/Cas9 technology has successfully transformed and improved several quality-related features in various crops, as shown in **Table 1**. Although several gene-edited crops, such as the TALEN-fad2 soybean, TALEN-ppo potato, and CRISPR-wx1 maize, have been commercialized, we are still at the beginning of the gene-editing revolution.

To begin with, gene-edited crop rules and regulations different countries have distinct regulatory frameworks. Most countries' regulatory frameworks for genetically modified organisms (GMOs) govern the development and commercialization of novel gene-edited crops. The United States and some South American countries, such as Argentina, Brazil, Chile, and Colombia, have used product-based regulations that exempt gene-edited products from GMO supervision if the final products do not contain exogenous DNA [100, 101], whereas the European Union (EU) and New Zealand have

strict process-based regulations for genome-edited crops, resulting in costly and time-consuming GM safety tests. China also has a process-based GMO regulation framework, as any gene-edited crops are scrutinized closely and no gene-edited crop has yet to be sold. The benefits of genome editing have been negated as a result of such rigorous restriction. As a result, establishing a worldwide unified and specialized regulatory system for genome-edited crops is vital. Thirteen WTO countries recently released a declaration supporting the use of gene editing in agricultural innovation, marking the first step toward building a global regulatory framework [102].

Furthermore, the delivery of CRISPR/Cas9 would be the most difficult challenge in using plant gene-editing technology. The recipient genotype has a big impact on biolistic bombardment and Agrobacterium-mediated transformation efficiency, especially in monocots. Some elite rice cultivars, for example, are notoriously difficult to change due to a lack of culture and regeneration-friendly traits [103]. Furthermore, T-DNA incorporation is unavoidable, and following plant regeneration methods are frequently technical and time-consuming. As a result, creating delivery systems that do not require tissue culture and can be used to a variety of plant species is important. Exogenous genes were delivered into pollen grains of many model crops using "pollen magnetofection," a unique approach that uses magnetic NPs as DNA transporters. About 1% of transgenic plants were produced after pollination with magnetofected pollen [104]. Some scientists, however, questioned pollen magnetofection's reproducibility [105]. It will be a shortcut to establish heritable gene alteration in transgenic seeds without tissue culturing if CRISPR/Cas9 can be delivered to reproductive cells and stably expressed via the pollen magnetofection approach [106]. Furthermore, because nano delivery technologies are nonintegrating and nonpathogenic, nanomaterial-mediated gene-edited crops may be exempt from GMO [107].

The specificity of plant CRISPR/Cas9 systems for targeted gene editing is another issue. According to several research studies, CRISPR/Cas systems have a high potential for off-target activity, and sgRNA/Cas9 complexes could create mismatched DNA sequences in mammals [108, 109]. Despite this, whole-genome sequencing demonstrated that the incidence of off-target mutations caused by CRISPR/Cas9 in plants is extremely low [110]. Off-targeting can be a problem in gene functional investigations since it might influence the phenotype of interest and lead to incorrect results interpretation [111]. Off-target effects can, however, be avoided when utilizing CRISPR technologies in crop breeding. Beneficial off-target mutations can be retained in progeny because off-target mutations with detrimental phenotype consequences are rejected throughout the breeding process [112]. As a result, in the breeding of gene-edited crops, screening advantageous mutations is more crucial than discovering off-target variants. To reduce off-targeting, several solutions have been proposed [113]. First, by developing highly precise sgRNAs with the fewest projected off-targets, the majority of off-targeting can be avoided. Second, high-fidelity Cas9 enzymes such as eSpCas9 [114] and SpCas-HF [115] can improve the specificity of CRISPR systems. Finally, the ribonucleoprotein (RNP) delivery approach can be employed to shorten the time that genomic DNA is exposed to CRISPR reagents, reducing off-targeting rates [116].

5. General procedure of genome editing in soybean and factors for success

The first successful genome editing in soybean was done in hairy roots, where ZFNs were used to target the GmDcl4a and GmDcl4b genes. ZFNs were also used

to develop the first viable GE soybean plants with a GmDcl4 gene mutation (either GmDcl4a or GmDcl4b). The first TALENs-mediated GE events with two target sites were reported by Haun et al. [117]. Jacobs et al. [118] reported the first successful CRISPR GE in soybean. The majority of CRISPR/Cas9 research first concentrated on developing a GE system and analyzing its targeting effectiveness in hairy roots, and the multiplex property of CRISPR to target pairs of genes at the same time was also confirmed. Meanwhile, target gene knockout [119] and homology-directed recombination (HDR) in whole plants have both been successful [120].

5.1 Selection of a target trait

The function and properties of the genes influencing the target trait, including sequencing data, transcription data, copy number in target materials, and variances compared to the reference genome, should all be completely known. Genome sequencing and gene discovery in soybeans pave the door for GE. More than 46,000 genes in the soybean genome have been predicted using a soybean reference genome assembly and Williams82 DNA sequences [121]. Hundreds of accessions of *Glycine max* and related species have recently been sequenced for new reference genomes, including a high-quality reference genome of a wild soybean W05 and a popular Chinese farmed soybean Zhonghuang 13 (ZH13) that was recently assembled [122–124]. Moreover, the soybean reference genome assemblies have been used to characterize hundreds of regulatory noncoding RNA loci, such as microRNA (miRNA) and phased small interfering RNA (phasiRNA) loci [125]. Comparative genomics can be used to examine all of the sequencing information in order to find potentially beneficial genes. More than 70% of these genes have been duplicated and survive as numerous copies as soybean is a paleopolyploid and the two duplication events occurred 59 and 13 million years ago, respectively. It is difficult to find genes that are linked to crucial agronomical properties including yield, protein, oil, and biotic and abiotic stress tolerance, which makes soybean breeding projects challenging [126–129]. As a result, finding the genes that govern significant agronomic qualities is crucial for trait selection in soybean genome editing. The fundamental problem for soybean improvement researchers has been a lack of understanding of gene activities and contributions to agronomically important target phenotypes. GE in soybean has concentrated on features with a clear genetic background, such as GmFAD2 for oleic oil content, based on existing knowledge.

5.2 Challenges and prospective for GE and related product development in soybean

In the last four decades, popular transgenic technology has been used to introduce foreign genes into crops, such as soybean, for desired qualities, and it has proven to be a viable option to expanding genetic resources. The random incorporation of transgenes in the genome, on the other hand, has triggered public outrage and rigorous government restriction, drastically increasing the cost and time required to generate a new variety. Instead of going through repeated back crosses to transfer a natural mutation in a traditional breeding method, GE technology allows crop breeders to integrate a desired feature into an elite background in a precise and predictable manner. Traditional mutagenesis breeding introduces mutations that are indistinguishable from those induced by GE. The largest constraint for GE application in soybean, such as other crops [130], is a lack of GE candidate target genes due to poor foundational

research, as described above. Technical issues such as the inability to precisely mutate any target site, the lack of ways to deliver genome-editing reagents into soybean cells, the low efficiency of selecting desired events and regenerating intact plants with targeted mutation, and off-site targeting are among the remaining bottlenecks. Through the use of newly developed GE technologies and a soybean regeneration system, several attempts have been made to reduce the restrictions and enhance the efficiency of recovering GE events. Additional challenges for GE product development include transgenic GE events, intellectual property restrictions, and government control of GE. Before GE can play a significant role in soybean improvement, these challenges must be addressed.

6. Success stories of CRISPR/Cas9 mediated in soybean (*G. max* L.)

The CRISPR-Cas9 method has been used to successfully mutate the genes GmFT2a, FAD2-2, and GmSPL9 in soybean modifying flowering time, seed oil profile, and plant architecture, respectively. This success implies that employing the CRISPR-Cas9 technology to improve soybean agronomical qualities is possible.

Targeted mutants of E1 gene controlling soybean flowering were generated. Two new types of mutations were discovered: 11 bp and 40 bp deletions in the E1 coding area, respectively, and frameshift mutations that resulted in premature translation termination codons and shortened E1 proteins, causing early blooming under long day conditions. Furthermore, by predicting and analyzing the probable off-target areas of E1 targets, no off-target effects were found. The shortened E1 protein disinhibited GmFT2a/5a, and boosting GmFT2a/5a gene expression led in evident early flowering in two new mutants with significantly decreased E1 gene expression [131].

7. Conclusions

Thanks to the CRISPR/Cas9-based gene-editing method, researchers may now change crop-specific traits more accurately and effectively. The CRISPR/Cas9 system has become the most frequently used and versatile technology in crop breeding and functional genomics. Its unrivaled ability to manipulate genes contributed in the development of a number of crop varieties with beneficial agronomic traits. Most crop-improvement gene-editing research, on the other hand, is still in the early stages of uncovering genomic function and regulatory pathways. Gene-edited crops are still a long way from becoming commercially available. In addition, gene-editing approaches have yet to meet all of the requirements for changing plant genomes. Because several quality-related variables are governed by many QTLs and altering individual genes may not generate significant phenotypic change, further development will be critical for the use of CRISPR/Cas in plants. It might be possible to create a CRISPR/Cas-mediated chromosomal rearrangement technology that works well. Furthermore, delivering CRISPR cargoes remains a significant challenge. As a result, it would be advantageous to design new carrier materials. Aside from that, public concerns and the government's rigorous regulatory policies on gene-editing technologies are another roadblock to plant breeding progress. Despite the remaining hurdles, gene-editing technology is expected to become more frequently used in the future and will undoubtedly play a significant part in crop quality enhancement like in soybean.

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