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CRISPR-Cas9 mediated genome editing in soybean for improving quality traits

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Legumes are the major source of energy for people throughout the world and play a significant role in a balanced diet to satisfy the body's need for protein. Soybean (*Glycine max* L.) is also a poor man's meat which is a highly enriched amount of protein present in it. Day-by-day increase in the worldwide population is also a great challenge to improve the yield and nutritional values. Here are some exciting ways to improve the yield and nutrition values through basic and advanced techniques that are particularly important and worldwide use. A unique idea called "biofortification" involves the enrichment of micronutrients using traditional plant breeding and contemporary technologies. Research on grain bio-fortification has considerably reduced hunger globally over the past few decades. The current bio-fortification programs are now more competitive due to a better understanding of the food matrix. Recent advancements in biotechnology have a variety of positive effects, and genetic engineering is developing quickly. Since genome editing technology has made it possible to precisely alter and change the genomes of living beings, it has transformed genetic and biological research, the simplest example is CRISPR/CAS9. We concentrate on the most recent developments in CRISPR/Cas9-based technology and talk about the

prospects and difficulties of using this ground-breaking technology to improve specific characteristics in soybeans and other crops.

Keywords: *CRISPR/CAS9, soybean, genome editing, quality traits, biofortification*

INTRODUCTION

A distinctive crop with a high economic worth and a wonderful supply of both oil and protein is the soybean. To meet market demand by increasing yield and improving quality due to the growing demand for soybeans worldwide, it is vitally necessary to clarify the functions of genes and speed up functional gene research and breeding techniques. However, mature soybean seeds contain lipoxygenases (LOXs) that can catalyze the oxidation of unsaturated fatty acids like linoleic and linolenic acids to produce conjugated unsaturated fatty acid hydroperoxides. These compounds are then transformed into volatile compounds that give beany an unpleasant taste (*Two Soybean Seed Lipoxygenase Nulls Accumulate Reduced Levels of Lipoxygenase Transcripts | SpringerLink*, n.d.). Human intake of soybeans is limited by the flavor of items made from soybean seed(*Flavor Problems of Vegetable Food Proteins - (*Rackis, 1979*) - Journal of the American Oil Chemists' Society - Wiley Online Library*, n.d.). The cost of soybean production and processing has increased due to the use of treatments like heat, microwave processing, and organic solvent extraction in the food sector to remove the beany flavor from soybean products (oil, soymilk, tofu, etc.). Genetic engineering techniques including hybridization, mutagenesis, and transgenesis are largely responsible for the steady rise in soybean yield over the past century. To fulfill the soaring worldwide demand for soybean products, soybeans must be improved; yet, a lack of genetic resources and complex societal difficulties with the use of transgenic technology prevents this (Xu et al., 2020). Since the majority of cultivars are discovered to have been chosen from the same initial set of progenitors, soybean has relatively little genetic variation (Singh, 2017). Even though human selection has reduced the genetic diversity of soybeans since domestication, it is noteworthy that current cultivars have kept 72% of the diversity observed in Asian landraces while losing 79% of their uncommon alleles (frequency 0.10). Simulations showed that the few Asian transplants and not the later artificial selection imposed by selective breeding were primarily responsible for the variety lost via the genetic bottlenecks of introduction and plant breeding. The major bottleneck was domestication, which resulted in the loss of 81% of the unusual alleles and evidence of significant allele frequency shifts in 60% of the genes. The minimal sequence diversity found in the wild species was halved (Hyten et al., 2006). Soybean has a genome that is extensively duplicated, with over 75% of the genes present in more than one copy (Schmutz et al., 2010). Opportunities for soybean breeding have recently arisen as a result of the quick development of genome editing technologies such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/CRISPR associated protein (CRISPR/Cas). These methods have several potential applications in studying gene function and enhancing vital agronomic characteristics in various crops (Bao et al., 2020). CRISPR (Clustered regular interspaced short palindromic repeat) and Cas9 (CRISPR-associated) have offered an efficient tool for targeted genome editing and gene function studies, as well as a fresh perspective on reverse genetics. The CRISPR/Cas9 system enables the deletion and replacement of huge fragments of DNA as well as the knockout and knockin of genes at various loci. Originally shown the CRISPR/Cas9 system's potential for crop genome editing by generating site-directed mutations in certain genes in Arabidopsis and rice (Gratz et al., 2013; Shan et al., 2013). The first targeted editing for nine soybean endogenous genes was performed by (Jacobs et al., 2015) using CRISPR/Cas9 technology to delete the *GFP* gene. The CRISPR system consists of the nuclear-localized CRISPR-associated (Cas) 9 protein and the guide RNA (gRNA). The most often utilized nuclease domain in Cas9, a big protein with two, comes from *Streptococcus pyogenes*. The Cas9 protein interacts with the 3′ end of

the gRNA, a synthetic 100 nucleotide (nt) RNA molecule that contains the targeting site for the first 20 nt or so (Jinek et al., 2012). When Cas9 and the gRNA work together, they may recognize DNA sequences that are complementary to the gRNA and cause a DNA double-strand break (DSB). Non-homologous end joining (NHEJ), an ineffective repair method for DNA double-strand breaks (DSBs), can lead to the insertion and/or deletion of sequences at the breakage site, which often results in frame-shift mutations (Mladenov & Iliakis, 2011). Such targeted DSBs may be employed in plants to knock out genes (Curtin et al., 2011), alter gene expression by destroying promoter regions (Li et al., 2012), or introduce transgenes by homologous recombination at a particular site (Zhang et al., 2013). With the recent fast advancements in CRISPR/Cas9-mediated genome editing technologies, several plant species, including soybeans, have benefited. A single CRISPR target or several targets in a single destination vector may be designed and copied on a variety of platforms. This review offers a revised functioning technique for using CRISPR/Cas9 technology to concurrently target one or more genes in soybeans. The CRISPR/Cas9 system is frequently employed in agricultural biotechnology and plant genetic studies because Cas9 are crucial part of a quick and effective genome editing process. We provide two systems for cloning single CRISPR targets and multiplexing targets, as well as approaches for delivering reagents. In soybean hairy roots, composite plants, and tissue culture-based regenerated entire plants, the techniques address important limiting processes that may restrict CRISPR editing. As of now, it has been possible to create transgenic soybean plants with up to three target genes mutated using this procedure (Liu et al., 2019b). Variation produced using such CRISPR/Cas-enabled advanced breeding procedures may be difficult to identify from naturally occurring variation, making it likely to be suitable for commercialization. Genome editing might play a significant part in future attempts to enhance crops thanks to CRISPR/Cas's accuracy, reach, and versatility (Chilcoat et al., 2017). Early CRISPR/Cas9 research on plants mostly consisted of functional investigations in model systems and proof of concept experiments. In addition to single gene editing, CRISPR/Cas9 has several other applications that have been developed as a result of these investigations and those in other branches of biology. These tools include site-specific transgene insertion, regulating gene expression, and multiplexing for triggering numerous cleavage events. Plant scientists are starting to use CRISPR/Cas9 for agricultural trait modification now that much of the conceptual work is practically finished (Schaeffer & Nakata, 2015). When the CRISPR/Cas9 system is delivered through a transfer DNA (T-DNA) vector, the DNA fragment encoding the guide RNA targeting the gene of interest and the endonuclease-coding region are typically cloned in the vector (Le et al., 2020). Despite being a common delivery method, vectors frequently have unintended consequences, such as off-target cleavage and random insertion of foreign DNA into the genome (Amirkhanov & Stepanov, 2019). This conversion of nucleotides occurs without the use of a donor template, fully active CRISPR-Cas9 nuclease, or DNA repair processes that follow double-stranded breaks. Based on some data, base editing appears to produce fewer undesired insertions and deletions than the original CRISPR-Cas9 method, according to proponents who claim base editing is a simpler approach to delivering editing machinery to cells (Marx, 2018). As a result, CRISPR/Cas9 is a useful tool for targeted genome editing in soybeans. It also provides a theoretical and technological foundation for future study on the soybean genome while increasing breeding efficiency and speeding up the breeding process.

Genome editing in soybean via CRISPR/Cas9

The CRISPR/Cas system has been divided into five kinds and two classes as a result of the Cas protein's classification. Type II of the CRISPR Cas9 system, which serves as an adaptable tool for genome editing, has been taken from a bacterial species, *Streptococcus pyogenes*. It has been altered for efficient use in genome editing (Hsu et al., 2014). The type II of the CRISPR/Cas9 system is comparatively uncomplicated in structure. It has been studied thoroughly and understood well (Liu et al., 2020). This system comprises three main components including the target site which is just upstream to the sequence of protospacer adjacent motif, sgRNA, and Cas9 protein (Doudna & Charpentier, 2014).

Genome editing takes place in three steps: recognition, cleavage, and repair respectively. sgRNAs are oligonucleotides that are 20-22 nucleotides long and are simple to design. Hence, by altering the 20-22 nucleotide guide sequence, any DNA sequence having 5'(20-22)-NGG can be targeted by Cas9 nuclease (Jain, 2015). The guide RNA (gRNA) has a complementary sequence at the 5' end. This sgRNA will guide the Cas9 nuclease to recognize the target sequence in the gene of interest. If sgRNA is not present, the Cas9 enzyme will be unable to cut the target sequence. At the location of three base pairs that are upstream of the PAM motif, the Cas9 enzyme will make double-stranded breaks (Ceasar et al., 2016). Plant cells repair these double-stranded breaks in two ways. One way to repair the cleavage is by non-homologous end joining (NHEJ), which eventually causes minor sequence changes and results in gene knockout and eventually loss of protein function (Liu et al., 2019a). The other way to repair the cleavage is by activation of HDR. This causes the DNA repair template to be inserted into the desired location of the genome (Sun et al., 2016).

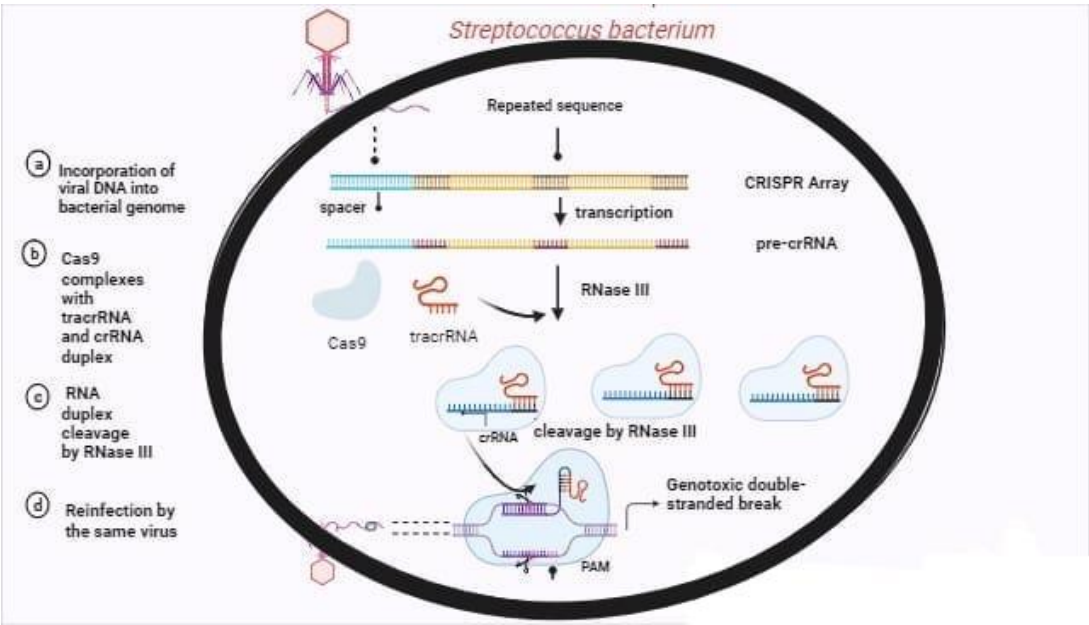

Figure 1. The genome editing mechanism of CRISPR/Cas9 system

Several works have been done in the last years to improve the traits of soybean crop (Table 1). Gene Paralogous family members that share the same sequence are targeted with single gRNA. On the other hand, multiplexing can be performed by utilizing two or more gRNAs each of which target the different DNA sequence. Five gRNAs that target nine genes were introduced by the particle bombardment method into embryonic cells using a combination of these two methods. As a result of this, 200 plants were regenerated (Li et al., 2010). Besides this, *ALS* gene editing was performed in soybeans. *ALS* genes (*ALS1,2,3,4*) were located at chromosome 4. On a single allele of the ALS1 gene, the codon was changed from CCC to AGC. Due to this, proline was altered to serine at position 178. The edited P178S *ALS1* allele conferred resistance against sulfonylurea herbicides in soybean crops (Sebastian et al., 1989). In *Phytophthora sojae*, the pathogen virulence gene (*Avr4/6*) was disrupted using CRISPR (Ma et al., 2021). The function of the virulence gene in pathogen identification by plants that carry the soybean *R* gene loci, *Rps4* and *Rps6*, was emphasized by the homologous gene replacement of *Avr4/6* by a marker gene (*NPT II*) mediated by the CRISPR/Cas9 technology. Besides this, in the following T2 generation, flowering time gene *GmFT2* of the soybean was successfully knocked out using CRISPR., and the homozygous *GmFT2a* mutants showed delayed flowering in both conditions i.e. short and long day conditions (Cai et al., 2018). While performing soybean hairy root

experiments, scientists used the CRISPR/Cas9 system to identify different targets (Jacobs et al., 2015).

Table 1. Applications of CRISPR/Cas9 based genome editing in soybean

Application	Target gene	Mode of action/ method	Improved trait	References
Increase crop yield (Regulating architecture and flowering period)	*SPL9a, SPL9b*	Knockout	Influence features of plant architecture	Bao et al., 2019
	SPL9c, SPL9d	Knockout	Influence features of plant architecture	Bao et al., 2019
	GmLHY1a, b	Knockout	Influence features of plant architecture	Cheng et al., 2019
	GmLHY2a, 2b	Knockout	Influence features of plant architecture	Cheng et al., 2019
	GmFT2a, GmFT4	Base editing	Affect time of flowering	Cai et al., 2020
	GmAP1	Knockout	Affect time of flowering	Cai et al., 2020
	Gmprr37	Knockout	Affect time of flowering	Wang et al., 2020
	GmE1	Knockout	Affect time of flowering	Han et al., 2019
	GmFT2a	Knockout	Affect time of flowering	Cai et al., 2018
Enhance crop quality & nutrition (Storage protein/seed oil/sugar content/allergenic properties/bean flavor free Soybean)	*Glyma.03g163500*	Knockout	Regulate storage of protein	Li et al., 2019
	Glyma.20g148400	Knockout	Regulate storage of protein	Li et al., 2020
	Glyma.19g164900	Knockout	Regulate storage of protein	Li et al., 2021
	FAD2-2	Knockout	Improve content of fatty acid	Al Amin et al., 2019
	GmFAD2– 1A	Knockout	Improve content of fatty acid	Do et al., 2019b
	GmFAD2– 1B	Knockout	Improve content of fatty acid	Do et al., 2019b
	GmFAD2-1A	Knockout	Increase content of fatty acid	Wu et al., 2020
	GmFAD2-2A	Knockout	Increase content of fatty acid	Wu et al., 2020
	Glyma.19G147300	Knockout	Increase content of fatty acid	Wu et al., 2020
	GmFATB1 (GmFATB1a and GmFATB1b)	Knockout	Develop fatty acid content	Ma et al., 2021
	GmGOLS1A	Knockout	Develop fatty acid content	Ma et al., 2021
	GmGOLS1B	Knockout	Develop fatty acid content	Le et al., 2020
	GmSWEET15a	Knockout	Develop fatty acid content	Wang et al., 2019
	Gly m Bd 30 K	Knockout	Regulate allergenic	Sugano et al., 2020

As we know carotenoids play an important role in the nutritional benefits of the soybean crop. It includes reducing the risk of cardiovascular diseases and cancer (Elvira-Torales et al., 2019). Targeting the carotenoid-related *GmPDS11* and *GmPDS18* genes via CRISPR/Cas9 was another advancement in soybean crops (Du et al., 2016). Seeds of soybean that can also be consumed by human beings contain a significant class of water-soluble carbohydrates known as raffinose family oligosaccharides (RFOs). These include the mono-, di- and tri-galactosidase of sucrose, raffinose, stachyose, and verbascose Hou et al., 2009. RFOs cannot be digested by people and other monogastric animals because they lack an enzyme required to cleave the α-1,6 glycosidic linkage in RFOs (Coon et al., 1990). Galactinol synthase (GOLS) generates galactinol which is the primary checkpoint for biosynthesis of RFOs. A study was conducted in which the CRISPR/Cas9 system was used to cause mutations into the two GOLS-encoding homeologs, GmGOLS1A and GmGOLS1B for the purpose of investigating the functions of these genes in soybean seed RFO metabolism. RFOs level significantly reduced in both single and double mutant knockout seeds. The soybean nutritional quality was improved without influencing key agronomic traits like plant morphology and seed germination etc. (Le et al., 2020). In the year 2013, CRISPR/Cas9 technology was first time utilized for the modification of genes in plants. After its successful utilization, many researchers have focused on using this system to improve the crop quality, yield and inducing stress resistance. Micro-organisms that are

pathogenic in nature cause biotic stress due to which disease resistance crops' development becomes challenging. These stresses are responsible for the loss of 15% of the world's food production and a reduction of more than 42% in the crop yield (Oerke, 2006). For this, the CRISPR/Cas9 system has been utilized to improve the resistance against disease in crops. A total of 41 crop species, including 15 industrial crops, 8 ornamental crops, 6 oil crops, and 1 fiber and feed crop have had genome editing using the CRISPR/Cas9 system reported thus far (Ricroch et al., 2017). Additionally, a search of the literature showed a great increase in the number of publications using the CRISPR/Cas9 system for crop improvement during the past five years, from 5 to 125.

Quality Improvement in soybean due to CRISPR/Cas9

Protein and oil content are important qualitative traits that discover the nutritional quality and economic value of soybeans (Patil et al., 2018). The major target of soybean breeding programs is to increase soybean nutritional quality (Lu et al., 2011; Patil et al., 2018). Seed quality traits also include sugar content like sucrose, stachyose, and raffinose (Cicek et al., 2006). Unluckily, crushed soybean seeds become a reason for the off-flavors and unpleasant grassy taste, especially in the development of soybean-based beverages which ultimately decreases the soybean-derived product quality (Mellor et al., 2010). The important qualitative traits of soybean are its oil and protein content. The nutritional quality and economic value of soybeans are mostly determined by protein and oil contents (Patil et al., 2018). An increase in the nutritional content of the crop is one of the major goals of breeding projects for soybeans (Lu et al., 2011; Patil et al., 2018). Seed quality attributes also take into account sugar content, which includes sucrose, raffinose, and stachyose (Cicek et al., 2006). Unluckily, mashed or broken seeds of soybeans produce off-flavors and unpleasing grassy taste, particularly in beverages made from soybeans, which eventually lowers the quality of products made from soybeans(Mellor et al., 2010). Soy meal, a food made from soybeans that is valued as a source of protein, has allergenic qualities as well (Taliercio & Loveless, 2020). Soybean quality enhancement strategies face significant difficulty in managing unfavorable traits such as off-flavor and allergic qualities of soy protein (Kar et al., 2022). CRISPR/Cas9 has introduced new strategies that remove off-flavors and produce hypoallergenic food. The most popular and adaptable mechanism in functional genomics and breeding of crops is now the CRISPR/Cas9 system. Future usage of gene editing technology is anticipated to increase, and it will probably play a vital role in improving crop quality. To alter flowering time, plant architecture, and seed oil profile, CRISPR/Cas9 precisely modifies *GmFT2a*, *GmSPL9*, and *FAD2-2* genes respectively. This accomplishment suggests that it is feasible to use CRISPR/Cas9 mechanization to enhance the agronomic properties of soybeans (Siddique, 2022). Storage protein's quantity and quality in seeds of soybean have a significant impact on the quality of soy-based food items. Soybean seeds contain significant quantities of storage proteins that have a small number of sustained gene families (Nielsen et al., 1989; Schuler et al., 1982). CRISPR/Cas9 technology has enhanced characteristics connected to seeds, including seed oil profile, and soybean seed products having an unpleasing beany taste (Wang et al., 2020). CRISPR/Cas9 also enhances resistance to soybean mosaic virus and isoflavone content (Zhang et al., 2020; Li et al., 2019). Furthermore, gRNA testing for the production of mutants in genes of seed storage protein, which will be beneficial for breeding soybeans of the food variety, is another important development. The inevitable role of CRISPR/Cas9 in the quality of soybeans is further discussed below.

Improvement in yield

Yield is the result of plant development due to a variety of factors, including genes and environmental factors which are the main components that interact, and the outcome is referred to as yield (Li et al., 2020). Soybean yield is a complicated quantitative feature, significantly influenced by the environment. One of the most important characteristics of soybean is yield, which is influenced by indirect factors like plant height, growth period,

branches, and node number as well as direct factors like pod number, 100-seed weight, and grain weight (Li et al., 2020). Many yield-related factors for boosting soybean production have been studied (Kar et al., 2022). Soybean is a leguminous family crop whose economic value is very high; Soybean is enriched with protein and contains a lot of oil. Understanding gene function and moving forward with research on functional genes as well as breeding to boost quality and enhance yield are increasingly crucial now that there is a greater demand for soybeans globally. The issues brought on by the agricultural environment and the rising demand for soybean products cannot be satisfied with traditional soybean breeding practices. For creating amended varieties, especially those with upgraded quality, stress tolerance, or resistance and yield, it is crucial to employ swift, exact, and successful breeding techniques (Siddique, 2022). The *E1* gene, which regulates soybean blooming, was used to create targeted mutants. Two new mutation types were discovered: reduced *E1* proteins and termination codons of immature translation by mutations of frameshift, responsible for primal flowering during long days, and deletions of 40 bp and 11 bp in the *E1* coding region, respectively. Additionally, there were no off-target effects discovered after forecasting and examining the likely E1 targets' off-target regions. *GmFT2a/5a* was uninhibited by the shorter *E1* protein, and increasing *GmFT2a/5a* gene expression caused two additional mutants with much lower *E1* gene expression to manifest early blooming (Zhu et al., 2014). CRISPR/Cas9 was used to replace four essential genes, "*GmSPL9a*, *GmSPL9b*, *GmSPL9c* and *GmSPL9d*, from the *SPL9* family. "Following the induction of mutations in four genes, the architecture of soybean plants was examined (Bao et al., 2019). There is a low plastochoron value of mutant *spl9a* and *spl9b* obtained from T2 double homozygous, according to studies. The investigation revealed that the plastochoron length was shorter in the T2 double homozygous mutant *spl9a/spl9b*. The soybean plant's trifoliate leaves, nodes, and branches as a result (Sun et al., 2019). More branches, an increase in dry weight, and more nodes are features that correlate favorably with yield (Bao et al., 2019). A long plant will have a longer stem and inter-nodal distance, which will result in a higher yield. Height affects yield because a long plant has a longer stem and inter-nodal distance, which causes lodging. As a result, grain loss ultimately lowers yield (Li et al., 2020; Yang et al., 2021). The decrease of seed, ailing, less lusty seed, and seed loss were further effects associated with lodging that were noted (Hwang & Lee, 2019). Several procedures are used over time to reduce plant height in a variety of staple crops (Cheng et al., 2019). To understand the potential concerning plant height and inter-nodal distance, four main genes that are the reason for the Late Elongated Hypocotyl (LHY) family of genes were targeted in soybeans by CRISPR/Cas9 system. As we compare to wild-type plants, fourfold mutant soybean plants substantially increase crop production by reducing plant height and inter-nodal spacing. Another finding is that concerning wild-type soybeans, the experimental mutant soybean has less Gibberellic acid. The wild type has a lot more of it. Gibberellic acid contributes to plant height, elongation, and leaf expansion during the developmental stages (Naveed et al., 2022).

Regulation of flowering time

A significant factor in regulating grain production and crop productivity is flowering time (Kishchenko et al., 2020). The length of the day can control when a certain kind of plant blooms. The soybean refers to a short-day plant, and only a specific region of the world can be used for its planting (Kishchenko et al., 2020). Early blooming with a prolonged post-blooming phase in greater latitude areas results in greater soybean yield harvests, but early flowering with a short development stage and low yields in low-latitude zones (Kantolic et al., 2007; Vogel et al., 2021) Hence, Among the goals of breeders, one goal is to produce advanced plants with improved tolerance to climate change by regulation of the genes included in increasing or inhibiting soybean blooming. In the soybean plant, numerous genes are in charge of flowering (Kim et al., 2012). The *Floral Locus T* (*FT*) contains two of these homologous genes, *GmFT2a* and *GmFT5a*, which are included in photoperiod coordinated soybean blooming (Nan et al., 2014). Beneath both long-day and short-day photoperiodic coincidences, *GmFT2a* mutants of homozygous T2 produced

through CRISPR/Cas9 method slowed blooming time (Cai et al ., 2018).During various photoperiods, mutagenesis due to CRISPR/Cas9 was used to produce mutant *Gmft5a*, *Gmft2aft5a*, and *Gmft2a* (Cai et al., 2020). According to this study, during short-day conditions, *GmFT2a* has a stronger effect on controlling blooming time but during long-day conditions, *GmFT5a* has a considerable impact. During conditions of a short day, *ft2aft5a* double mutant soybean plant displayed slowed blooming by roughly 31 days having higher seeds and pods for each plant in contrast to soybean of wild-type (Cai et al., 2020) According to (Cai et al., 2018), homozygous mutants made in the soybean *GmFT2a* or *FLOWERING LOCUS T2a* by mutagenesis (CRISPR/Cas9-mediated) delayed blooming. Another study targeted the *GmFT4* and *GmFT2a* genes by inducing the substitution of a single base in soybean using a CRISPR/Cas9-mediated base editing method. They effectively replaced the target nucleotide with CRISPR/Cas9 for soybean enhancement, through which amino acid proline was substituted into alanine (Cai et al., 2020). In short-day conditions, the soybean plant with the desired mutation displayed delayed flowering (Imaizumi & Kay, 2006).

Increase in isoflavonoid content

A subclass of flavonoids is isoflavonoids, which can be separated from both non-leguminous and leguminous plants. They are generated through the phenylpropanoid pathway. This bioactive has a lot of biological advantages, including reduced risk of cancer, osteoporosis, cardiovascular disease, and other diseases, as well as use as an antioxidant (Bhagwat et al., 2022). Notably, soybean has an isoflavonoid level that is almost 100 times higher than that of other legumes. These substances serve a variety of purposes; for example, as frequent dietary components, they play important roles in maintaining human health by lowering the prevalence of certain malignancies and improving risk markers for cardiovascular disease (Budryn et al., 2018; Malloy et al., 2018) (Sathyapalan et al., 2018). Moreover, they contribute significantly to plant disease resistance. Isoflavonoids are produced through the phenylpropanoid pathway. This bioactive is used as an antioxidant and has a wide range of biological advantages, like decreased hazard of cancer, osteoporosis, cardiovascular disease, and other disorders. The enzymes that control the synthesis of isoflavonoid compounds include *flavone synthase* (*IFS*), *flavanone-3-hydroxylase* (*F3H*), and *flavone synthase II* (*FNS II*) (Liu et al., 2007). In the numerous locations in hairy roots of soybeans and plants that were targeted with CRISPR/Cas9, genes involved in isoflavone synthase synthesis are *GmF3H1*, *GmF3H2*, and genome of transgenic line T0 exhibited a triple mutation that was stably passed down into the following generation. The isoflavone concentration was significantly higher in these T0 triple-mutant leaves. When compared to soybean (wild type), the triple mutant's leaf of the T3 homozygous generation doubles the amount of isoflavones. Moreover, the leaf developed immunity to strain SC7 of soybean mosaic virus (SMV), opening up new opportunities for the mass production of these genome-edited plants with promising agronomic features (Zhang et al., 2020). One of the most dangerous soybean diseases is the soya bean mosaic virus (SMV), which severely lowers soya bean quality and the gene associated with SMV is *GmIFS1* (Hill & Whitham, 2014).

Altering plant architecture

The architecture of plants has a substantial effect on the production of a grain of many crops inclusive of Glycine max or soybean, yet there is currently little understanding of how to optimize plant architecture to maximize yield potential. Due to the CRISPR/Cas9 method, genome editing recently transformed, it has been extensively used for a wide variety of crops to modify the genomes. Plant structure is essential for increasing output. Ideotype is a quality that is required for good yielding, and the soybean cultivar's growth habitat determines how it will behave. In this manner, editing modifies genes like *GmLHY* related to gibberellic acid (Bao et al., 2019).

Production of the hypoallergenic soybean crop

People who are sensitive to soybeans or have food allergies may be at risk due to the growing usage of Glycine max or soybean derivatives in the processing of foods. IgE irritability to insufficient stored proteins and some lower insufficient seed proteins has been observed in in vitro experiments on seed proteins of soybeans using the serum of persons who are irritable to soybeans. Subsequently, Gly m Bd 30 K, which is one of the rare proteins, commonly called P34 is a significant source of allergy from soybean also referred to as immunodominant (Naveed et al., 2022). An immunologically dominant protein that has already been recognized is Gly m Bs 30 K. Replicating low-P34 soybean germplasm was another attempt to create hypoallergenic soybeans for human consumption (Herman et al., 2003). For those who are allergic to soybeans, fifteen distinct proteins have been taken from their serum. Gly m Bd 60k, Gly m Bd 30k, and Gly m Bd 28k are vital allergy-causing proteins that have been recognized. The genes that were redesigned by CRISPR/Cas9 to form soybean non-allergenic are Gly m Bd 30k and Gly m Bd 28k. Immunodominant protein in mutant seeds of soybean identified (Kar et al., 2022).

Alteration of shape and size

To increase the production of their extremely nutritious seeds, several leguminous crops need loss of seed-coat impermeability during domestication. One gene, *GmHs1-1*, which produces the transmembrane protein calcineurin-like metallophosphoesterase regulates seed coat durability in wild type of soybean. *GmHs1-1*, which is related to the amount of calcium, is mostly present in the Malpighian layer of the seed shell (Sun et al., 2015).

Decreasing the unpleasant seed beany taste

Large amounts of protein are present in soybeans. Also, it has a unique taste similar to soy flavor, which hinders the soybean and its derivative's acceptability for human beings on a large scale. Soybean has three lipoxygenases whose work is carried out by three genes *Lox1*, *Lox2*, and *Lox3*. Due to lipoxygenases linoleic acids and linoleic acids are oxidized. As a result, they give soybean and its derivatives a grassy and beany taste. The *GmLox3*, *GmLox2*, and *GmLox1* genes were modified by the use of CRISPR/Cas9 technology to produce soybean lines that do not produce lipoxygenase. They could be utilized to lessen the flavor of beany in soybeans (Kar et al., 2022).

Biofortification

Currently, World's population is 7.8 billion which is growing rapidly and will reach 8.3 billion in 2030. With the growing population, another serious and continuously increasing problem is malnutrition. According to the report, about 800 million people are facing the malnutrition problem, among them 98% belong to developing countries (Sinha et al., 2019) Intake of insufficient energy and deficiencies of essential micronutrients which are required for different cell processes results in malnutrition. Around 340 M people are malnourished with one or more micronutrients such as iron, zinc, vitamin A, iodine, and others which are very necessary for a healthy lifestyle (UNICEF, 2021) According to the WHO, deficiencies of micronutrients and vitamins such as Zinc, Iron and vitamin A are most common within the peoples are contributing to many diseases. After these findings, there is an urgent need to overcome the problem of malnutrition or undernutrition. Consumer demand is also increasing for nutritionally rich foods. Researchers have been struggling to develop such strategies to reduce nutrient deficiencies. Almost every crop is rich in one or more nutrients, so by combining all these essential nutrients from different crops, the problem of malnutrition can be addressed (Siddique, 2022). Biofortification and its efficient technique by which the quality parameters of crops can be enhanced by

boosting the concentration of micronutrients, minerals, and vitamins present in them. Biofortification differs from other methods in a way that it aims to develop crops with high nutritional content naturally. This can be done by different techniques such as agronomic practices, conventional breeding, genetic engineering, and gene editing. Among all, gene editing methods are a more reliable option. CRISPR/Cas system is the most widely used technique in gene editing methods. CRISPR/Cas is a gene editing tool that allows researchers to make changes in the DNA sequences of living organisms. Among the leguminous crops, Soybean (*Glycine max* L.) has the highest oil and protein content, therefore, having great economic importance. Furthermore, all over the world soybean demand is growing very fast in the coming days as compared to the past, hence it is mandatory to increase the yield with high nutritional content. For this purpose, first of all, understanding and identification of genes contributing to the production of micronutrients is a need (Siddique, 2022). Due to its highly duplicated genome, understanding the gene's function through conventional genetic strategies is a major challenge. CRISPR/Cas9 system was first applied in 2015 in this crop (Cai et al., 2018). After some initial successful experiments, researchers are continuously trying to make more improvements in a soybean crop by using the CRISPR/Cas system. Many other crops are being utilized to enhance their nutritional value and fight against hidden hunger (Malnutrition). Scientists are making continuous efforts to develop such crops with high vitamin content, and increased iron, iodine, and zinc levels.

Biofortification to improve oil content

For the past many decades, dieticians suggested that to lower down the use of saturated fatty acids (SFA) (Figure 2) as they are the main cause of heart disease.

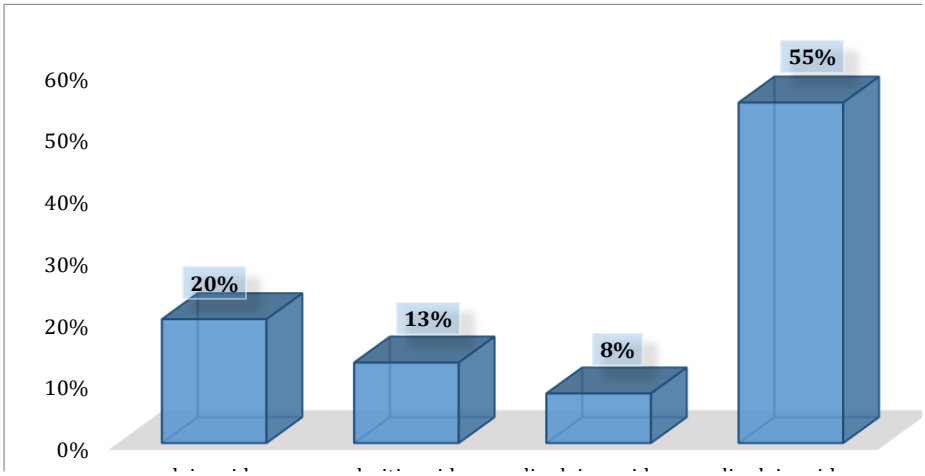

Figure 2. Graphical representation of concentrations of fatty acids

Soybean oil is most widely used because it contains 20% oleic acid. Soybean oil contains five fatty acids: monosaturated fatty acid-oleic acid (20%), palmitic acid (13%), linoleinc acid (8%), and polyunsaturated fatty acids like linoleic acid (55%). The extensive material of polyunsaturated fatty acids soybean use at the industrial level is highly hindered (Demorest et al., 2016). Monosaturated oleic acid is a beneficial fatty acid and is considered to improve cardiovascular health by lowering cholesterol levels. It also has antioxidant properties. On the other hand, polyunsaturated linoleic acid deteriorates the soybean oil's nutritional importance by causing unstable frying. That's why bio-fortification of soybean with higher oleic acid is needed (Wu et al., 2020). By considering the relation to the composition of fatty acid in soybean, genes FAD2 and FAD3 coding for fatty acid desaturase enzymes have been mutated by the CRISPR Cas system. In another experiment, researchers made mutations in two homologous genes GmFAD2 which enhanced the oleic acid content to 80% and lowered the linoleic acid content from 50% to

4.7% in 2019 (Do et al., 2019a). The first bio-fortified soybean crop with less than 20% linoleic acid and up to 80% oleic acid is now available in US markets (*First Commercial Sale of Calyxt High Oleic Soybean Oil on the U.S. Market | AP News*, n.d.).

Biofortification to improve sugar content

The Soybean crop has also gained importance due to its sugar content. The soybean seeds are rich in sugars and belong to the raffinose family of oligosaccharides (RFOs). The problem with their presence is that they cause difficulty in digestion in humans (Hua et al., 2019). To reduce the accumulation of RFOs soybean crop genes *GmGOLS1A* and *GmGOLS1B* were altered with the help of the CRISPR/Cas 9 system. Results were quite satisfactory as a significant reduction in the RFOs was observed with no morphological changes (Le et al., 2020).

Vitamin A biofortification

Carotenoids are isoprenoid pigments that are necessary for photosynthesis and play a vital role in photosynthetic organisms. Humans cannot produce carotenoids but they use them for metabolic processes as carotenoids are the precursor to vitamin A production. Biofortification of crops with higher carotenoid content is necessary to reduce vitamin A deficiency in the population (Maoka, 2020). Vitamin A deficiency is related to many serious health issues such as blindness in children, night blindness, and maternal mortality (Sommer, 2008). By using the CRISPR Cas system, carotenoid biofortification in tomatoes, rice, and bananas has been done to overcome vitamin A deficiency (Table 2). One example is the Golden Rice cultivar Kitaake in which a 5.2-kb carotenogenesis cassette containing *Crtl* and *PSY* genes has been developed by knocking-in. The resultant variety contains 7.9μg/g dry-weight β- carotene in the endosperm (Dong et al., 2020).

Table 2. Vitamin A bio fortified crops through CRISPR/Cas9 system (Kumar et al., 2022)**.**

Vitamin A bio fortified Crops	CRISPR-Cas systems	Targeted Genes	References
Golden Rice	CRISPR-Cas9	Phytoene desaturase *Crtl* & phytoene synthase *(PSY)* genes were knocked-in	Dong et al. (2020)
Tomato	CRISPR-Cas9	Staygreen *(SGR)* gene a negative regulator of carotenoid systhesis was knock-down	Li et al. (2018)
Golden Banana	CRISPR-Cas9	Lycopene epsilon-cyclase (*LCY*) was knock-down by create INDELS	Kaur et al. (2020)

Iron biofortification

Micronutrient deficiencies are posing the same threat as deficiencies in vitamins. Among them, iron is an important micronutrient, present in every cell of the human body. Iron is crucial to the oxygen transportation from the lungs to tissues as it is a key component of the hemoglobin protein. Apart from that iron is also involved in the many enzymatic reactions which are important for cell functions (Jimenez et al., 2015). Currently, iron deficiency is the most common cause of malnutrition which leads to anemia, especially in women and children. According to the WHO reports, about 2 billion people are suffering from iron deficiency or anemia having symptoms of tiredness and poor metabolism. In developed countries, 30-40% of school-going children and pregnant women are suffering from iron deficiency. The percentage is even higher in developing countries (Lucca et al., 2006). Iron deficiency in the population can be fulfilled by crop biofortification. According to one research, by using CRISPR/Cas9 scientists disrupt the *inositol pentakisphosphate 2-kinase 1 (IPK1)* gene in wheat to biofortified the crop (Ibrahim et al., 2022). To increase the iron content in grain crops, the knockdown of vacuolar iron transporter (*VIT*) is also a potential use of the CRISPR Cas system. In the current study, changes in *VIT2* of rice crops enhanced the Fe content in the grain, endosperms, and embryo (Sun et al., 2021).

Zinc biofortification

Like many other minerals and vitamins, zinc is also crucial for the human body. Zinc plays a necessary role in many cell functions such as cell growth, immune system development, and cell division. Zinc is not needed in large amounts as other vitamins are required but the human body can't store it for long duration therefore, it should be taken in a regular diet to overcome the deficiency (Frassinetti et al., 2006). Zinc deficiency is related to many biological disorders such as alopecia, dermatitis, weight loss, emotional disturbance, poor immunity, and delay in wound healing. About 1.1 billion people are suffering from zinc deficiency across the world (Kumssa et al., 2015). To the best of our knowledge, zinc biofortification is done only on wheat crops by using CRISPR Cas9. In this experiment inositol pentakisphosphate 2-kinase 1 (*TaIPK1*) gene was disrupted resulting in a reduction of phytic acid which leads to the improvement of zinc accumulation in grains of wheat (Ibrahim et al., 2022). Multiple crops are obligatory to be biofortified by using the CRISPR Cas system to improve the zinc level in the diet and also to make the crops available across the world (Kumar et al., 2022).

Table 3. Biofortification of crops by using CRISPR/Cas9 system (Ricroch et al., 2017).

Crops	Biofortification
Camelina sativa	Enhancement of seed oil composition Seed oil biosynthesis
Oryza sativa	High amylose bio-fortified rice
Solanum tuberosum	Amylopectin potato starch (starch quality)
Hordeum vulgare	N-glycan modification in cereal grains

This technology is being used for the enhancement of the oil content in the *Camelina sativa* as well as used for starch properties modifications (Table 3).

CONCLUSION

Legumes are a key source of energy for people all over the world and are essential to a balanced diet in order to meet the body's protein needs. Another poor man's meat that has a greatly enhanced amount of protein is soybean (*Glycine max* L.). The daily rise in global population makes it extremely difficult to boost yield and nutritional benefits. The soybean is a unique crop with a significant economic value and a fantastic source of both protein and oil. Due to the increasing demand for soybeans globally, it is imperative to explain the activities of genes and accelerate functional gene research and breeding approaches in order to fulfil market demand by boosting yield and improving quality. The flavour of products manufactured from soybean seed limits the amount of soybeans that humans may consume. Over 75% of the genes in the soybean genome, which has undergone considerable duplication, are present in more than one copy. The CRISPR/Cas9 system permits the knockout and knockin of genes at multiple loci, as well as the deletion and replacement of large DNA pieces. By creating site-directed mutations in a few genes in Arabidopsis and rice, researchers first demonstrated the CRISPR/Cas9 system's potential for crop genome editing. Dieticians have long advised reducing consumption of saturated fatty acids (SFA), as they are the primary contributor to heart disease. Because soybean oil has 20% oleic acid content, it is the most often utilized oil. Five fatty acids are found in soybean oil: oleic acid, a monounsaturated fatty acid, palmitic acid, a polyunsaturated fatty acid, and linoleic acid, a polyunsaturated fatty acid, which makes up 55% of the oil. The utilization of soybeans, which contain a large amount of polyunsaturated fatty acids, is severely constrained.

AUTHOR CONTRIBUTIONS

All writers contributed to the completion of this work. The study was created and the protocol was written by author Ali Haider[1]. All the tables in whole manuscript were designed by Umar Azam. The critical study of CRISPR/Cas9, ZFNs, and TALENs was also carried by Umar Azam and Ali Haider. Authors Amina Zia, Aniqah Akhter, Rabia Iqbal covered the portion of Genome editing in soybean via CRISPR Cas9. Author Amina Zia desgin the genome editing mechanism of CRISPR/Cas9 system. Author Rabia Naz, Muhammad Atif, Muhammad Fahad Iqbal the literature searches and contributed a lot in Biofortification section of manuscript. The final part of manuscript that is related to the Zinc and Iron Biofortification covered by Saima Majeed and Ayesha Ashraf. References and citations were managed by Ali Haider. All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors have declared that no conflict of interest exists.

ETHICS APPROVAL

Not applicable.

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