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Advancements in Genetic Enhancement: CRISPR/Cas-Mediated Genome Editing in Leguminous Crops

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Legumes play a crucial role in human nutrition and sustainable agriculture due to their high protein content and health-promoting phytochemicals. To accelerate genetic gain in yield, stress resilience, and nutritional quality, extensive efforts are underway. Recent advancements in genomic resources have paved the way for the application of cutting-edge breeding technologies like genomic selection and genome editing in legume crops. This review focuses on the latest advancements in CRISPR/Cas9-based gene editing technology, specifically tailored for improving traits in legume crops. While successful gene-editing methods have been established for crops like soybean, cowpea, and chickpea, challenges remain, particularly in overcoming the recalcitrance of certain legumes to gene transfer and regeneration in vitro. Strategies to enhance transformation and regeneration rates through modifications in culture methods and DNA delivery are discussed.

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Despite the potential benefits of gene editing in legume breeding, regulatory barriers pose significant challenges. A comparison of regulatory environments in different regions sheds light on the importance of favourable regulations and public acceptance for realizing CRISPR's potential in enhancing global food security. Additionally, leguminous crops are vital for their nutritional properties, nitrogen-fixing abilities, and contribution to sustainable agriculture. Traditional breeding methods focused on increasing yield, while recent advancements in genome editing techniques, notably CRISPR-Cas technology, have revolutionized the improvement of agronomic traits in legumes. This chapter provides insights into the application of genome editing tools for enhancing traits such as stress tolerance, yield, and seed content in grain legumes. Furthermore, it discusses the challenges and prospects associated with enhancing grain legumes using molecular breeding techniques. Genome editing has been successfully employed in diverse legumes, including model species like *Medicago* and widely cultivated crops such as soybean and chickpea, offering exciting opportunities for enhancing agronomic characteristics.

Keywords: Stress resilience; nutritional quality; CRISPR/Cas; in vitro; food security; Medicago.

1. INTRODUCTION

Legumes, belonging to the Fabaceae (*Leguminosae)* family, represent a significant portion of the Earth's plant species, constituting approximately 5% of the known 400,000 plant species [1]. The term "legume" stems from the Latin word "Legūmen," which translates to "beans inside pods." Notably, legumes are among the earliest domesticated crops in human history, with four of them—Pea (*Pisum sativum*), chickpea (*Cicer arietinum*), lentils (*Lens culinaris*), and bitter vetch (*Vicia ervilia*)—being part of the "Big Eight" founder crops cultivated as early as 10,000 BC [2-3]. The cultivation of legumes holds paramount importance in meeting global demands for grain food and forage production. The edible seed fruits of leguminous plants, commonly referred to as pulses, are a staple in many diets worldwide. Among the vast array of legume species, common bean, faba bean, pea, chickpea, cowpea, pigeon pea, lentils, peanut, grass pea, and horse gram are extensively cultivated [4]. In 2018, common bean emerged as the most cultivated grain legume globally, followed by chickpea and cowpea, with the total production of grain legumes exceeding 92 million tons [5-7].

India stands as the largest producer of grain legumes globally, contributing a quarter of the total production [8]. However, significant production also occurs in countries such as China, Canada, Australia, the USA, Brazil, Argentina, and Russia. In Europe, soybeans, faba beans, and field peas are the primary legumes cultivated, with soybean production witnessing a substantial increase due to its high demand in livestock feed [9]. From a nutritional perspective, legumes are often hailed as the "poor man's meat" owing to their high protein content. These seeds are rich in storage proteins, making them one of the most significant plant-based sources of proteins, ranging from 16% to 50% of the total dry weight [10]. Additionally, legume seeds offer essential dietary fibers, vitamins, complex carbohydrates, sugars, minerals, and fatty acids, rendering them a vital component of a healthy diet [11]. However, some non-nutritional components in legumes, such as phytosterols, polyphenols, trypsin inhibitors, phytate, lectin, and saponins, are deemed as anti-nutrients, potentially hindering nutrient absorption and bioavailability [12].

Gene editing revolutionizes genetic manipulation by employing engineered nucleases and cellular DNA repair mechanisms to make precise alterations to an organism's genome. The journey of gene-editing methodologies commenced nearly thirty years ago with the discovery that specific double-stranded breaks could be induced in chromosomes using a meganuclease called I-SceI [13]. Despite its potential, the application of meganucleases was restricted due to the limited frequency of target sites in most genes. Subsequent advancements emerged with the introduction of programmable zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), which marked significant progress in the field [14]. However, the true breakthrough occurred with the adaptation of RNA-guided Cas9 nuclease from the prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system for genome editing in eukaryotic cells [15].

CRISPR/Cas9-based gene editing stands out as the simplest, most versatile, and precise method

of genetic manipulation in plants. It relies on two essential components: the Cas9 endonuclease and a guide RNA (gRNA), comprising CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA). Target site recognition by Cas9 necessitates the presence of a specific protospacer-adjacent motif (PAM) flanking the target site [16]. While the canonical PAM associated with widely used Cas9 variants like *Streptococcus pyogenes* (SpCas9) is 5'-NGG-3', the strict PAM requirement limits target site flexibility [17]. To overcome this limitation, recent efforts have focused on utilizing Cas9 orthologs with broader PAM compatibility and longer gRNAs. Despite its versatility, concerns regarding off-target binding and cleavage persist, prompting the development of Cas9 nickase variants to reduce off-target activity. Furthermore, technological advancements have expanded the CRISPR/Cas9 toolbox to include Cas9 variants for gene modulation and base editing. The latest addition, prime editing, enables a wide range of editing applications without double-stranded DNA breaks or donor DNA templates, signifying a significant leap forward in genome editing capabilities [18,19].

2. CRISPR-MEDIATED GENOME IMPROVEMENT IN LEGUMES

The advent of genetic engineering, heralded by the discovery and application of restriction endonucleases, opened new avenues in biotechnology. Genetic engineering involves the introduction of foreign genetic material into a host organism using restriction enzymes and ligases to confer desired traits. Notably, the incorporation of CRY1 family genes from *Bacillus Thuringiensis* into crops for insect pest resistance exemplifies the impactful success of genetic engineering. Subsequent breakthroughs unfolded with the identification of homing endonucleases (HEs) or meganuclease I-SceI encoded by mobile genetic elements (MGEs), which generate double-stranded breaks in DNA at specific sites [20]. Repair of these breaks by natural DNA repair pathways, such as Homology Directed Repair (HDR) or Non-Homologous Ends Joining (NHEJ), often leads to genome editing through insertions or deletions (Indels) of base pairs in a locus-specific manner [21].

However, the limited recognition sites of meganucleases posed challenges, driving the development of synthetic genomic scissors known as Zinc Finger Nucleases (ZFNs) in 2002. ZFNs, hybrid molecules comprising DNA-binding domains (zinc fingers) and a nuclease domain of Fok1 endonuclease, enabled genome editing by recognizing specific sites in the genome [22]. Despite advancements, concerns persisted regarding compromised specificity in ZFNs due to context-dependent specificity. Transcription Activator-Like Effector Nucleases (TALENs) emerged as an alternative, combining DNA-recognizing domains of transcription activator-like effectors (TALEs) with the nuclease domain of the restriction enzyme Fok1 for site-specific mutagenesis [23].

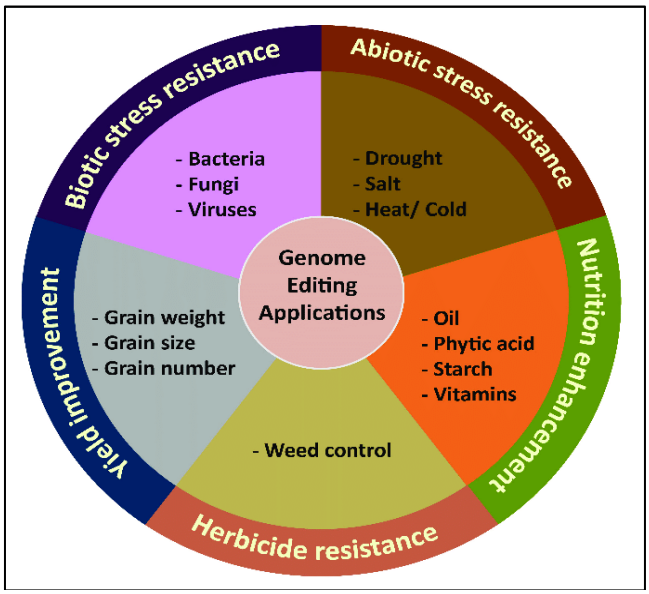

Fig. 1. Application of genome editing in crops improvement [26]

The pinnacle of genome editing technology arrived with the development of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - CRISPR associated system (CAS) system [24]. Inspired by bacterial and archaeal defense mechanisms against bacteriophages, the CRISPR-Cas system leverages virus-specific sequences (spacers) to guide CAS proteins in cleaving viral DNA, preventing infection. Artificially designed guide RNA (gRNA) and CAS protein constructs enable precise genome editing, marking the dawn of third-generation genome editing [18,25]. This technology, including CRISPR-Cas9, has been harnessed for various applications in legumes, albeit hindered by challenges such as tissue regeneration and off-target effects, prompting ongoing research and regulatory discussions to overcome these barriers.

CRISPR/Cas9 plays a vital role in crop enhancement, addressing challenges such as disease, drought, and salinity. It also improves grain yield, biomass, and nutrient efficiency, enhancing crop quality and morpho-physiological traits (Fig. 1) [26]. However, limitations include off-target effects. Future prospects involve refining techniques for precise genome editing, promising sustainable agriculture and food security.

3. GENOME EDITING IN LEGUME CROPS FOR ENHANCED VARIETIES

Genome editing through CRISPR-Cas technology has opened new avenues for improving legume crops, offering precise manipulation of targeted genes to enhance desirable traits. CRISPR-Cas operates with the Cas9 endonuclease and a guide RNA (gRNA), which includes a crisper RNA (crRNA) binding the target sequence and a transactivating RNA (tracer RNA) that facilitates recognition and cleavage [27]. This process allows for a wide range of applications, from improving crop yield to enhancing resistance to pests and diseases. However, there are challenges in applying CRISPR-Cas to legume crops, primarily due to the complexities of transformation and callus regeneration. *Agrobacterium*-mediated transformation with seed tissues has shown success in some legumes, especially in soybean and model species like *Medicago truncatula* and *Lotus japonicas* [28]. While the focus of many studies has been on nutrient delivery to the soil and plant through CRISPR-edited legumes, further research is required to establish comprehensive protocols for successful transformation and regeneration.

Despite these challenges, CRISPR-Cas-based genome editing has yielded positive results. In soybean, genome editing has led to improved isoflavone content and resistance to Soybean mosaic virus (SMV), as well as better seed-oil composition with an 80% increase in oleic acid content [29,30]. In chickpea, CRISPR-Cas was optimized with 42% mutation efficiency, indicated by albino phenotypes, while in pea, mutation efficiencies ranged from 16% to 45% [31]. Peanut and alfalfa have also been targets for genome editing, focusing on improving oleic acid content and understanding genes associated with plant growth and biomass development, respectively Genome Editing in Legume Crops for Enhanced Varieties tabulated in (Table 1). The advancements in genome editing hold significant promise for enhancing legume crops, contributing to more resilient, high-yielding, and sustainable agricultural practices.

4. APPLICATION OF CRISPR/Cas9 IN GRAIN LEGUMES

The successful application of CRISPR/Cas9 technology in various legumes, including soybean, chickpea, lentil, and *Medicago truncatula*, underscores its potential to revolutionize legume crop improvement [46]. With leguminous plants playing a crucial role in global agriculture, CRISPR/Cas9 has shown remarkable promise in enhancing crop productivity, quality, and resilience to environmental stressors [47,48]. Furthermore, CRISPR/Cas9 is now being utilized in the domestication process of legume species, offering opportunities to introduce and enhance diverse characteristics that are of commercial significance. As a result, the forthcoming process of crop domestication is expected to witness significant acceleration, fueled by the capabilities of CRISPR/Cas9 technology to expedite trait development and crop improvement. Recent advancements in CRISPR/Cas9 have also led to major breakthroughs in enhancing the nutritional value of legumes, further highlighting its potential to address global food security challenges by improving the nutritional content of staple crops [49].

4.1 *Medicago truncatula* (Alfalfa)

Medicago truncatula, an invaluable model crop, showcases the potential of CRISPR/Cas9

despite challenges in editing its polyploid genome. Studies targeting genes like MsSGR have demonstrated the tool's ability to influence crucial traits such as color variation, impacting pollination success [50]. However, further refinement of CRISPR/Cas9 systems is necessary to enhance editing efficiency in complex genomes like alfalfa, paving the way for more precise genetic modifications and accelerated crop improvement.

Table 1. Genome editing in legume crops for enhanced varieties

Legume Plant	Desired Trait	Targeted Genes	Results	References
Soybean	Increased isoflavone content and resistance to Soybean mosaic virus (SMV)	GmF3H1, GmF3H2,& GmFNSII-1	stable inheritance; doubled isoflavone content in leaves; reduction (1/3) of SMV coat protein	[32]
Soybean	Understanding flowering time & adaptation to diverse environments	GmFT2a, & GmFT5a	Both genes collectively regulate flowering time; GmFT2a critical for short day conditions; GmFT5a essential for long day and adaptation in higher latitudes	[33]
Soybean	Improvement in seed-oil composition	GmFAD2-1A, & GmFAD21-B	80% increase in oleic acid; 1.3–1.7% reduction in linoleic acid	[34]
Soybean	Attempt to modify storage-protein composition of seeds	Nine soybean seed storage protein coding genes	three genes successfully mutated: Glyma.20 g148400, Glyma.03 g163500, Glyma.19 g164900	[35]
Soybean	Improvement in plant architecture	GmSPL9, GmSPL9a, GmSPL9b, & GmSPL9c	PL9a/b showed shorter plastochron length	[36]
Soybean	Improved taste	LOX1, LOX2, & LOX3	reduced lipoxygenase activity	[37]
Cowpea	Develop asexual plant lineage	Meiosis controlling gene VuSPO11-1	4.5–37% mutation efficiency	[38]
Chickpea	CRISPR-based genome editing and understanding drought tolerance	4CL, & RVE7	RNP complex-based editing of chickpea protoplast;	[39]
Chickpea	Optimization of genome editing through CRISPR-Cas	PsPDS	visible albino phenotypes	[40]
Pea	Optimization of genome editing through CRISPR-Cas	PsPDS	different vector constructs	[41]
Peanut	Enhanced oleic acid content	ahFAD2a, & ahFAD2b	higher oleic acid content	[42]
Alfalfa	Achieving genome editing through CRISPR	uidA, & NOD26	GUS gene successfully mutated	[43]
Alfalfa	Understanding genes for growth and biomass development	MsSPL8	early flowering, decreased internodal length, and plant height	[44-45]

4.2 *Glycine max* (Soybean)

Soybean, esteemed for its nutritional value, has emerged as a focal point for CRISPR/Cas9 applications. Efforts to improve gene-editing efficiency and target specific genes like GmLox have shown promise in enhancing desirable traits related to nutritional content and plant architecture [51]. Despite challenges in transformation methods, CRISPR/Cas9 continues to offer novel solutions for addressing key agricultural issues, positioning soybean as a prime candidate for genetic enhancement and sustainable crop production.

4.3 *Cicer arietinum* (Chickpea)

Chickpea, a globally significant crop, stands to benefit from CRISPR/Cas9 technology in addressing challenges such as drought tolerance. Studies targeting genes like 4CL and RVE7 have shown promising results in enhancing the resilience of chickpea plants to environmental stressors [52]. The utilization of CRISPR/Cas9 in chickpea research represents a pivotal step towards developing climate-resilient crop varieties capable of thriving in challenging growing conditions.

4.4 *Arachis hypogea* (Peanuts or Groundnut)

Peanuts, valued for their nutritional qualities, have witnessed significant improvements through CRISPR/Cas9-mediated gene editing. Targeting genes like ahFAD28 and Ara h 2 has led to enhancements in oil content and allergen reduction, respectively, offering healthier and more marketable peanut varieties [53]. These advancements underscore the versatility of CRISPR/Cas9 in addressing key challenges in peanut cultivation and enhancing crop quality for both farmers and consumers.

4.5 *Vigna radiata* (Mungbean)

Mung bean breeding initiatives are poised to benefit from CRISPR/Cas9 technology, with potential applications in disease resistance and climate resilience. Leveraging the extensive genomic resources available for mung bean, CRISPR/Cas9 offers a powerful tool for accelerating the development of resilient crop varieties capable of withstanding fluctuating environmental conditions and contributing to global food security [54].

4.6 *Vigna ungiculata* (Cowpea)

Cowpea, known for its resilience in warm and arid regions, presents unique challenges for genetic modification due to transformation constraints. However, recent advancements in CRISPR/Cas9-mediated gene editing offer promising solutions for overcoming these barriers. Enhanced transformation efficiencies and transient evaluation methods provide avenues for optimizing CRISPR/Cas9 applications in cowpea research, ultimately contributing to the development of improved crop varieties with enhanced productivity and resilience [19,55].

5. CRISPR/Cas9 MECHANISM AND FUNCTION

The CRISPR/Cas9 system orchestrates a multi-step process to confer immunity against foreign genetic elements in prokaryotic organisms. Subsequently, in the expression phase, these spacers are transcribed and processed into mature CRISPR RNAs (crRNAs) [56]. Finally, in the interference phase, the crRNAs guide Cas proteins to complementary DNA sequences, facilitating their cleavage and neutralizing the invading genetic material. The CRISPR/Cas9 system is categorized into three main groups (Types I, II, and III), with Cas9 being a key player in the Type II system. Unlike Types I and III, which employ multiple Cas proteins, Cas9 functions as a large, multifunctional protein responsible for both crRNA processing and DNA cleavage [57].

Among the CRISPR/Cas systems, Type II, involving Cas9, offers relative simplicity in its architecture and function. This simplicity renders it particularly amenable to being harnessed as a genome editing tool. The mechanism of genome editing utilizing CRISPR/Cas9 involves several steps: formation of a complex between a single-guide RNA (sgRNA) and Cas9, unwinding of the target DNA by the sgRNA, and subsequent cleavage of the DNA by Cas9 [49,6]. This process enables precise modification of the genome at targeted locations. Following genome editing, subsequent steps include analysis of the modified genome, cloning of sgRNA, transformation and selection of edited plants, regeneration of plant tissues, extraction of genomic DNA, and, finally, sequencing analysis to confirm the desired genetic alterations (Fig. 2).

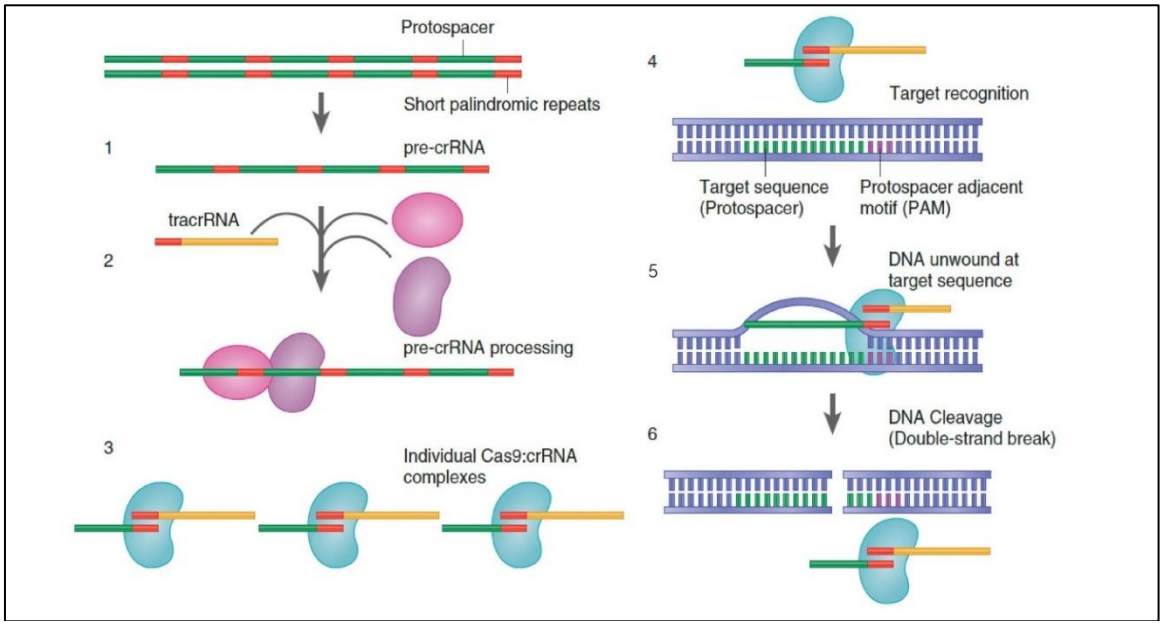

Fig. 2. Mechanism of genome editing using Cas9

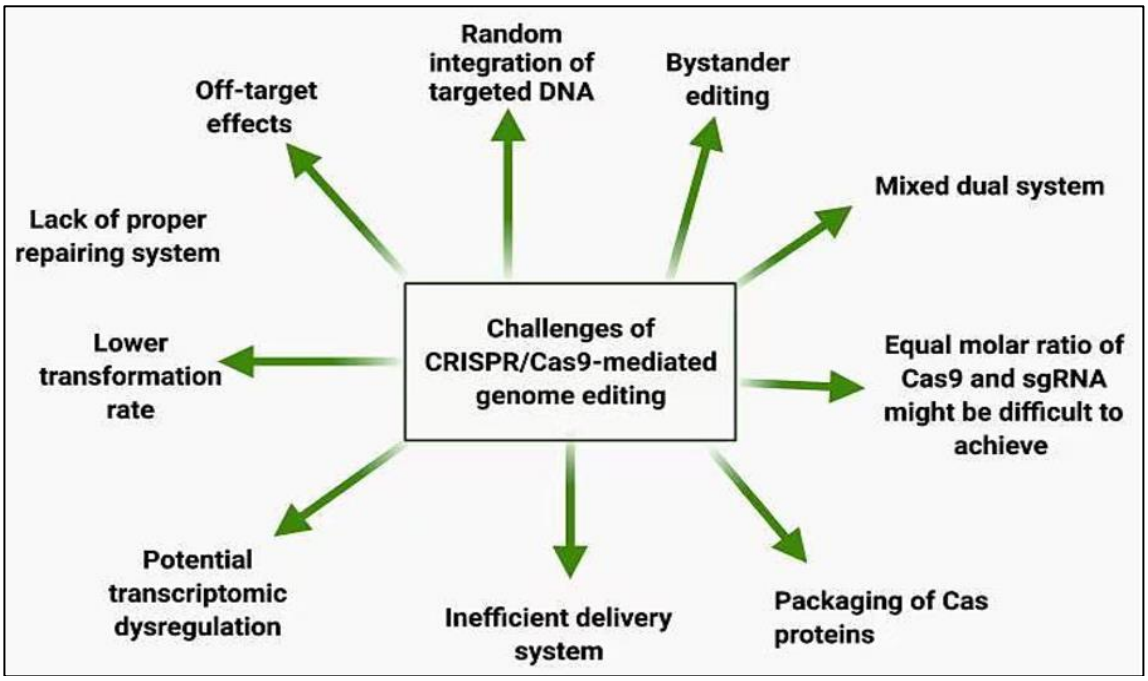

Fig. 3. Challenges of CRISPR/Cas9 gene editing [58]

6. CHALLENGES AND LIMITATIONS OF CRISPR/Cas9 APPLICATION

While CRISPR/Cas9 holds promise for various applications in plant breeding, it also faces several limitations. A significant challenge lies in the availability of a limited gene pool for important traits, highlighting the importance of accessing genomic sequence data and exploring valuable genetic resources for crop improvement. Additionally, inefficient transformation techniques and complex plant regeneration processes pose hurdles, along with biosafety concerns impacting its application in crop development. The issue of off-target effects remains a concern, but advancements in identification methods offer solutions to recognize and eliminate off-target mutants during

subsequent breeding cycles [23-25]. Future strategies aim to mitigate off-target effects by optimizing sgRNAs and selecting Cas9 variants with high fidelity. Commercialization of genome-edited crops faces regulatory challenges, as evidenced by recent rulings in the European Union, potentially delaying investment in CRISPR technology in certain regions.

Moreover, the unnatural occurrence of CRISPR/Cas9 in plants necessitates time-consuming processes for protein delivery into plant cells, often requiring codon optimization for compatibility [45]. Inadequate transfer of CRISPR/dCas9 into plants due to tissue resistance and regeneration limitations underscores the need for novel delivery techniques, such as direct transfer into plant apical meristems to bypass tissue culture. Despite these challenges, ongoing research and advancements in CRISPR/Cas9 hold promise for revolutionizing crop breeding, contributing to sustainable agriculture to meet the demands of a growing global population [17,22]. Efforts to improve delivery systems, reduce off-target effects, and enhance editing efficiency are essential for realizing the full potential of CRISPR/Cas9 in crop improvement. The imbalanced molar ratio of sgRNA and CRISPR/Cas9 poses a significant limitation to the effectiveness of CRISPR/Cas9, as illustrated in (Fig. 3).

7. FUTURE PROSPECTS OF MODERN BREEDING METHODS

The quest for safe, cost-effective crops to meet global food demands presents challenges, but modern techniques offer promising solutions. Advanced breeding methods, particularly CRISPR/Cas9 gene editing, enable rapid gene manipulation and insertion, revolutionizing crop improvement. In the coming years, leveraging CRISPR/Cas9 to enhance yield, quality, and disease resistance will be a key research focus [2,13]. While its application has surged in various plant systems, further enhancements are needed for target effectiveness. CRISPR/Cas9-based genome manipulation is poised to play a crucial role in creating genetically optimized crops, essential for sustainable food production and addressing global hunger. The acceptance of modern breeding methods has prompted reevaluation of regulatory frameworks. As gene editing tools become ubiquitous, robust regulatory mechanisms must differentiate between genetically engineered (GE) and genetically modified (GM) crops, ensuring safety and compliance [12-15]. Integrating systems biology, next-generation sequencing, and functional genomic methods with CRISPR/Cas9 offers opportunities for intelligent crop development, boosting yield and enhancing traits.

Combining CRISPR/Cas9 with speed breeding programs enhances global food security efforts. The integration of CRISPR/Cas9 with next-generation sequencing facilitates comprehensive mutational screening, vital for optimizing gRNA design and minimizing off-target effects [43-45]. The CRISPR/Cas9 library's advantages, including high multiplexing and specificity, ensure efficient and precise gene targeting, driving future advancements in crop improvement. Recent advancements in CRISPR/Cas9 technology have expanded the possibilities for precise and efficient gene modifications, including additions or deletions of genetic material. This article provides a comprehensive review of the tools and resources essential for designing and delivering gRNAs, as well as detecting genetic modifications. CRISPR/Cas9 technology opens up new avenues for functional genomics and enhancing various traits in grain legume crops. However, the successful implementation of genome editing for legume improvement hinges on the development of efficient protocols for plant transformation and whole plant regeneration. Additionally, a supportive regulatory framework and public acceptance of gene-edited crops are crucial factors for the widespread adoption of this technology.

8. CONCLUSION

The incorporation of CRISPR/Cas9 technology into legume crop improvement signifies a paradigm shift in agricultural practices. This review has illuminated the transformative role of CRISPR/Cas9 in enabling precise and efficient gene editing, offering unprecedented opportunities for enhancing various traits in legume crops. By harnessing this technology, researchers have unlocked new avenues for functional genomics and the development of legume varieties with enhanced yield, quality, and resilience to biotic and abiotic stresses. Nevertheless, the realization of CRISPR/Cas9's full potential in legume improvement hinges upon overcoming several hurdles. Efficient protocols for legume transformation and the regeneration of whole plants are imperative prerequisites for

successful genome editing. Furthermore, navigating regulatory landscapes and garnering public acceptance of gene-edited legumes are essential for their widespread adoption. Looking ahead, future research endeavors will concentrate on refining CRISPR/Cas9 tools and methodologies to enhance target specificity and mitigate off-target effects specifically tailored for legume crops. Collaborative efforts between researchers, policymakers, and stakeholders will be instrumental in establishing robust regulatory frameworks conducive to the responsible deployment of gene-edited legumes. By harnessing the capabilities of CRISPR/Cas9 and embracing innovative strategies, we can pave the way for a resilient and sustainable legume farming system that ensures food security and addresses the challenges of the future.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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