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CRISPR/Cas9 Genome Editing in Legumes: Opportunities for Functional Genomics and Breeding

Dandan Huang
Hainan Institute of Biotechnology, Haikou, 570206, Hainan, China
Corresponding email: dandan.huang@cuixi.org

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Abstract Legumes play a crucial role in global agriculture and food security, yet they face significant challenges in breeding for improved traits. This study explores the potential of CRISPR/Cas9 genome editing as a transformative tool in legume functional genomics and breeding. It begins by outlining the importance of legumes and the limitations of traditional breeding methods. The study then delves into the mechanisms and advantages of CRISPR/Cas9, highlighting its application in functional genomics, such as gene knockout and activation studies. A case study on drought tolerance in soybeans demonstrates the practical application of CRISPR/Cas9 in identifying and enhancing key traits. Furthermore, the study discusses the broad applications of this technology in improving biotic and abiotic stress resistance, enhancing quality traits, and accelerating the breeding process, including a detailed case study on disease resistance in chickpeas. The study also addresses the challenges and ethical considerations associated with CRISPR/Cas9, such as off-target effects and regulatory issues. Looking forward, the study explores future innovations and the integration of CRISPR/Cas9 into legume breeding programs, emphasizing its potential for sustainable agriculture and global food security. This study underscores the vast opportunities that CRISPR/Cas9 presents for advancing legume breeding and anticipates its growing impact on agricultural practices.

Keywords CRISPR/Cas9; Legume breeding; Functional genomics; Drought tolerance; Disease resistance

1 Introduction

Legume crops, including chickpea, pigeonpea, cowpea, and groundnut, are vital for global nutrition and food security. They are a major source of proteins and health-promoting phytochemicals, particularly in Asia and Sub-Saharan Africa, where they are often grown in marginal environments (Varshney, 2016; Bhowmik et al., 2021). These crops also play a crucial role in sustainable agricultural production through their ability to fix atmospheric nitrogen, thereby improving soil quality and reducing the need for synthetic fertilizers (Pandey et al., 2016; Corte et al., 2019). The nutritional benefits and environmental sustainability of legumes underscore their importance in addressing global food security challenges.

Despite their significance, legume crops face several breeding challenges. Low productivity due to biotic and abiotic stresses is a major issue, particularly in marginal environments (Varshney, 2016). Traditional breeding methods have had limited success in overcoming these challenges due to the complex genetic architecture of traits such as drought tolerance, disease resistance, and nutritional quality (Pandey et al., 2016; Bhowmik et al., 2021). Additionally, the recalcitrance of some legumes to in vitro gene transfer and regeneration poses a significant hurdle to genetic improvement efforts. These challenges necessitate the adoption of advanced breeding technologies to accelerate genetic gains in legume crops (Abdelrahman et al., 2018; Chen et al., 2019).

CRISPR/Cas9 is a revolutionary genome-editing technology derived from the bacterial adaptive immune system. It involves the use of an endonuclease, Cas9, guided by a single-guide RNA (sgRNA) to introduce double-strand breaks at specific genomic locations. These breaks are then repaired by the cell's natural repair mechanisms, leading to targeted genetic modifications (Corte et al., 2019). This precise editing capability allows for the disruption, modification, or regulation of target genes, making CRISPR/Cas9 a powerful tool for functional genomics and crop improvement (Chen et al., 2019). CRISPR/Cas9 offers several advantages over traditional

breeding methods. It enables precise and targeted modifications, reducing the time and effort required to develop improved crop varieties. Unlike conventional breeding, which relies on random mutagenesis or genetic recombination, CRISPR/Cas9 allows for the direct manipulation of specific genes associated with desirable traits, such as stress resilience and nutritional quality. Additionally, the technology has been successfully applied to various legume crops, including soybean, cowpea, and chickpea, demonstrating its potential to overcome the limitations of traditional breeding. However, regulatory and public acceptance challenges remain, which need to be addressed to fully realize the benefits of CRISPR/Cas9 in legume breeding.

The study is to explore the potential of CRISPR/Cas9 genome editing in legume crops for functional genomics and breeding. By leveraging recent advances in genomic resources and gene-editing technologies, this study aims to address the current challenges in legume breeding and enhance the genetic gain related to yield, stress resilience, and nutritional quality. The study will also examine the regulatory landscape and public acceptance of CRISPR/Cas9 technology to ensure its successful implementation in legume crop improvement.

2 Functional Genomics in Legumes

2.1 Role of functional genomics in crop improvement

Functional genomics plays a crucial role in understanding the specific roles of genes in legume crops. By utilizing advanced genome-editing technologies such as CRISPR/Cas9, researchers can precisely target and modify genes to study their functions. This approach has been successfully applied in model legumes like *Medicago truncatula* and crop legumes such as soybean, enabling the identification of genes responsible for various traits (Bhowmik et al., 2021). The simplicity and efficiency of CRISPR/Cas9 make it a standout choice for targeted genome editing, facilitating the investigation of gene functions and the development of valuable traits in legumes (Meng et al., 2016).

Functional genomics also aids in the identification of key traits that are essential for crop improvement. Traits such as yield, stress resilience, and nutritional quality are of particular interest. Recent advancements in genomic resources for legumes have laid a solid foundation for the application of transformative breeding technologies, including CRISPR/Cas9, to enhance these traits (Bhowmik et al., 2021). For instance, the CRISPR/Cas9 system has been used to target genes involved in fatty acid composition in soybean, resulting in improved nutritional profiles (Do et al., 2019).

2.2 CRISPR/Cas9 as a tool for functional genomics

CRISPR/Cas9 has revolutionized gene knockout studies in legumes by enabling precise and efficient gene disruption. This technology has been employed to create knockouts in various legume species, including soybean and cowpea, to study gene functions and improve traits (Ji et al., 2019; Bhowmik et al., 2021). For example, targeted mutagenesis using CRISPR/Cas9 in *Medicago truncatula* has facilitated the investigation of gene functions related to forage quality and yield (Meng et al., 2016).

Beyond gene knockouts, CRISPR/Cas9 can also be used for gene activation and repression. This is achieved by fusing the Cas9 protein with transcriptional activators or repressors, allowing for the upregulation or downregulation of target genes. Such manipulations can help in understanding gene regulatory networks and their impact on important agronomic traits (Arora and Narula, 2017; Thomson et al., 2019). The ability to modulate gene expression using CRISPR/Cas9 provides a powerful tool for functional genomics studies in legumes.

2.3 Case study: functional genomics of drought tolerance in soybeans

Drought tolerance is a critical trait for soybean cultivation, especially in regions prone to water scarcity. Functional genomics approaches, including transcriptomics and CRISPR/Cas9, have been employed to identify genes responsive to drought stress. These studies have revealed several candidate genes that play significant roles in drought tolerance (Figure 1) (Arora and Narula, 2017; Cai et al., 2020). Understanding the functions of these genes is essential for developing drought-resistant soybean varieties.

CRISPR/Cas9-mediated gene editing has been utilized to enhance drought tolerance in soybeans by targeting and modifying drought-responsive genes. For instance, specific genes involved in water use efficiency and stress response pathways have been edited to improve the plant's ability to withstand drought conditions. These advancements demonstrate the potential of CRISPR/Cas9 in developing soybean varieties with enhanced drought tolerance, contributing to sustainable agricultural practices (Niazian et al., 2022).

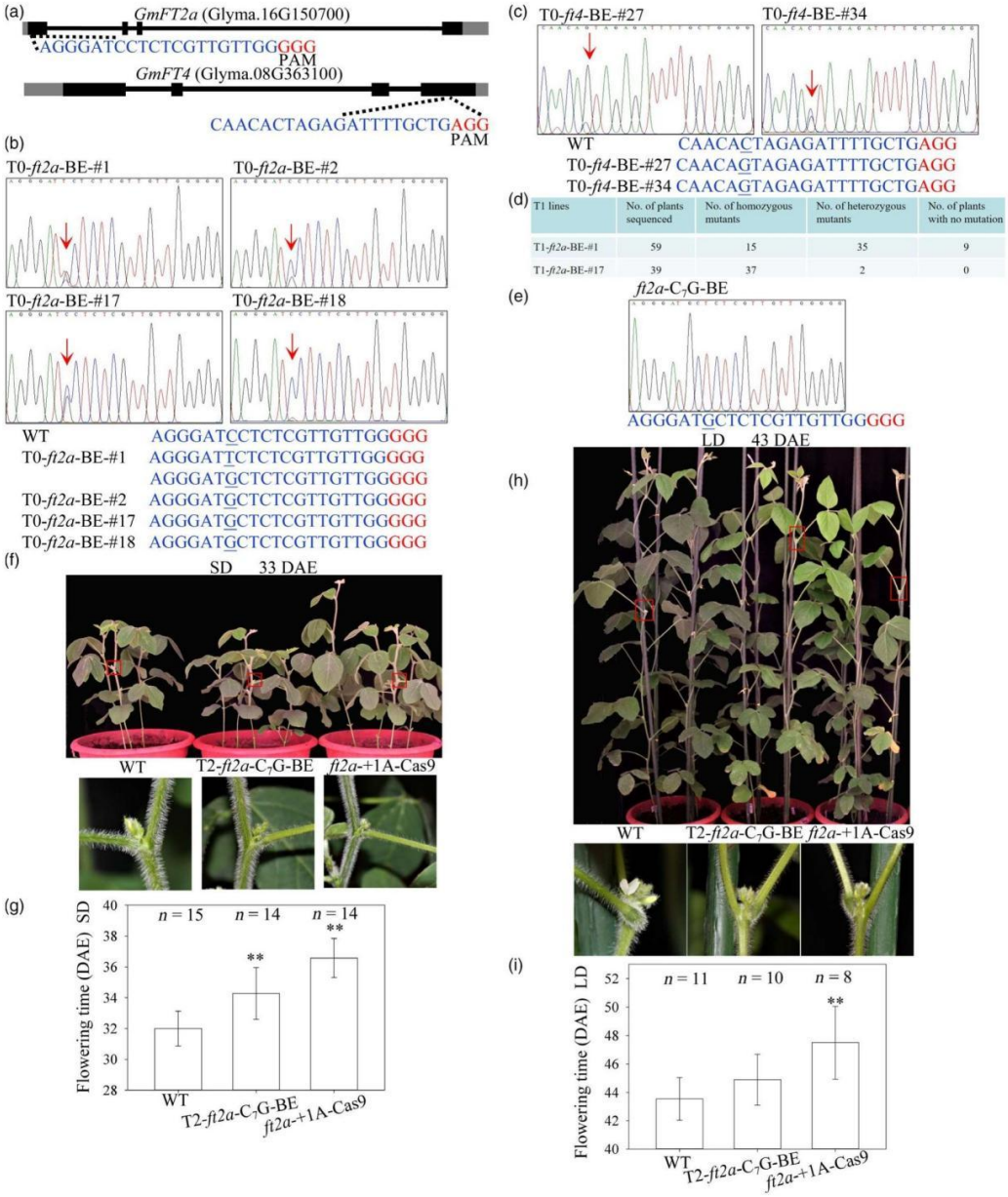

Figure 1 Base editing of *GmFT2a* and *GmFT4* in soybean (Adopted from Cai et al., 2020)

Image caption: (a) Gene structures of *GmFT2a* and *GmFT4* with target sites for base editing. Black stripe, exon. Black line, intron. Grey stripe, untranslated regions. Nucleotides in blue represent the target sequences. Nucleotides in red represent the PAM (protospacer adjacent motif). (b, c) Sequences and peaks of representative mutation types of base editing of *GmFT2a* and *GmFT4* in the T0 lines, respectively. The red arrowheads and underlines indicate the positions of these base editing mutations. (d) Base editing mutation types of *GmFT2a* in the T1 generation. (e) Sequence and peak of the homozygous *ft2a* mutant with C to G change. (f) and (g) Flowering time of WT, T2-*ft2a*-C7G-BE plants and *ft2a*-+1A-Cas9 plants under SD conditions. Red box, magnified view. *n*, exact numbers of individual plants identified. **, $P < 0.01$. DAE, days after emergence. The flowering time is shown as the mean values ± standard deviation. (h) and (i) Flowering time of WT, T2-*ft2a*-C$_7$G-BE plants and *ft2a*-+1A-Cas9 plants under LD conditions. Red box, magnified view. *n*, exact numbers of individual plants identified. **, $P < 0.01$. DAE, days after emergence. The flowering time is shown as the mean values ± standard deviation (Adopted from Cai et al., 2020)

3 Applications of CRISPR/Cas9 in Legume Breeding

3.1 Enhancing biotic stress resistance

CRISPR/Cas9 technology has been effectively utilized to enhance disease resistance in legumes by targeting and modifying genes associated with susceptibility to pathogens. For instance, the CRISPR/Cas9 system has been employed to develop resistance against various plant pathogens, including fungi, viruses, and bacteria, by knocking out or modifying specific genes that facilitate pathogen entry or proliferation (Bhowmik et al., 2021; Wang et al., 2022). This approach not only improves the resilience of legume crops but also reduces the reliance on chemical pesticides, promoting sustainable agricultural practices. In addition to disease resistance, CRISPR/Cas9 has been used to enhance pest resistance in legumes. By targeting genes that are involved in the plant's defense mechanisms against insect pests, researchers have been able to develop legume varieties that are less susceptible to pest attacks. This genetic modification can lead to a significant reduction in crop losses and improve overall yield (Haque et al., 2018).

3.2 Improving abiotic stress tolerance

Drought tolerance is a critical trait for legumes, especially in regions prone to water scarcity. CRISPR/Cas9 has been used to edit genes that regulate water use efficiency and root architecture, thereby enhancing the plant's ability to withstand drought conditions. For example, targeting regulatory genes that control stomatal closure and water retention has shown promising results in improving drought tolerance in legume crops (Abdelrahman et al., 2018; Haque et al., 2018; Zafar et al., 2020).

Legumes often face challenges from salinity and cold stress, which can severely impact their growth and productivity. CRISPR/Cas9 technology has been applied to modify genes associated with ion transport and osmotic balance to enhance salt tolerance. Similarly, genes involved in cold stress response pathways have been targeted to improve cold tolerance. These modifications help legumes maintain their physiological functions under adverse environmental conditions (Nazir et al., 2022; Wang et al., 2022).

3.3 Quality trait improvement

Improving the nutritional quality of legumes is another significant application of CRISPR/Cas9. By editing genes involved in the biosynthesis of essential nutrients, such as proteins, vitamins, and minerals, researchers have been able to enhance the nutritional profile of legume crops. This can lead to the development of legume varieties with higher protein content, improved amino acid profiles, and increased levels of health-promoting phytochemicals (Arora and Narula, 2017; Zhu et al., 2020; Bhowmik et al., 2021).

CRISPR/Cas9 has also been used to modify the seed composition of legumes to improve their processing and storage qualities. For instance, altering the fatty acid composition of legume seeds can enhance their oil quality, while modifying carbohydrate content can improve their digestibility and reduce anti-nutritional factors. These modifications can make legume seeds more suitable for various food and industrial applications (Tiwari et al., 2023).

3.4 Accelerating the breeding process

One of the most significant advantages of CRISPR/Cas9 technology is its ability to accelerate the breeding process. Traditional breeding methods are time-consuming and often require several generations to achieve the desired traits. In contrast, CRISPR/Cas9 allows for precise and rapid genetic modifications, enabling the development of new cultivars in a much shorter time frame. This is particularly beneficial for addressing urgent agricultural challenges and meeting the growing demand for food (Abdelrahman et al., 2018; Haque et al., 2018).

CRISPR/Cas9 facilitates precision breeding by allowing targeted modifications at specific genomic loci. This precision reduces the risk of off-target effects and ensures that only the desired traits are introduced or modified. Precision breeding approaches using CRISPR/Cas9 can lead to the development of legume varieties with enhanced traits such as yield, stress tolerance, and nutritional quality, while maintaining the overall genetic integrity of the crop (Arora and Narula, 2017; Chen et al., 2019; Zhu et al., 2020).

4 Case Study: CRISPR/Cas9-Driven Improvement of Disease Resistance in Chickpeas

4.1 Identification of major disease resistance genes

The identification of major disease resistance genes in chickpeas is a critical step towards enhancing disease resistance through genome editing. Recent advancements in CRISPR/Cas9 technology have enabled the precise targeting and modification of specific genes associated with disease resistance. For instance, the 4-coumarate ligase (*4CL*) and Reveille 7 (*RVE7*) genes have been identified as key players in drought tolerance and stress response in chickpeas. The *4CL* gene is involved in the phenylpropanoid metabolism pathway, which regulates lignin accumulation under stress conditions, while the *RVE7* gene is a MYB transcription factor that regulates circadian rhythm and stress responses (Badhan et al., 2021; Zhang et al., 2022).

4.2 CRISPR/Cas9-mediated targeted mutagenesis

CRISPR/Cas9-mediated targeted mutagenesis has been successfully employed to edit the *4CL* and *RVE7* genes in chickpea protoplasts. This approach utilizes ribonucleoproteins (RNPs) composed of the Cas9 enzyme and a synthetically designed single guide RNA (sgRNA) to achieve high-efficiency editing. The DNA-free CRISPR/Cas9 system has shown promising results, with high-efficiency editing achieved for the *RVE7* gene in vivo compared to the *4CL* gene. This targeted mutagenesis represents a novel approach for achieving precise gene modifications in chickpeas, paving the way for improved disease resistance and stress tolerance (Figure 2) (Badhan et al., 2021).

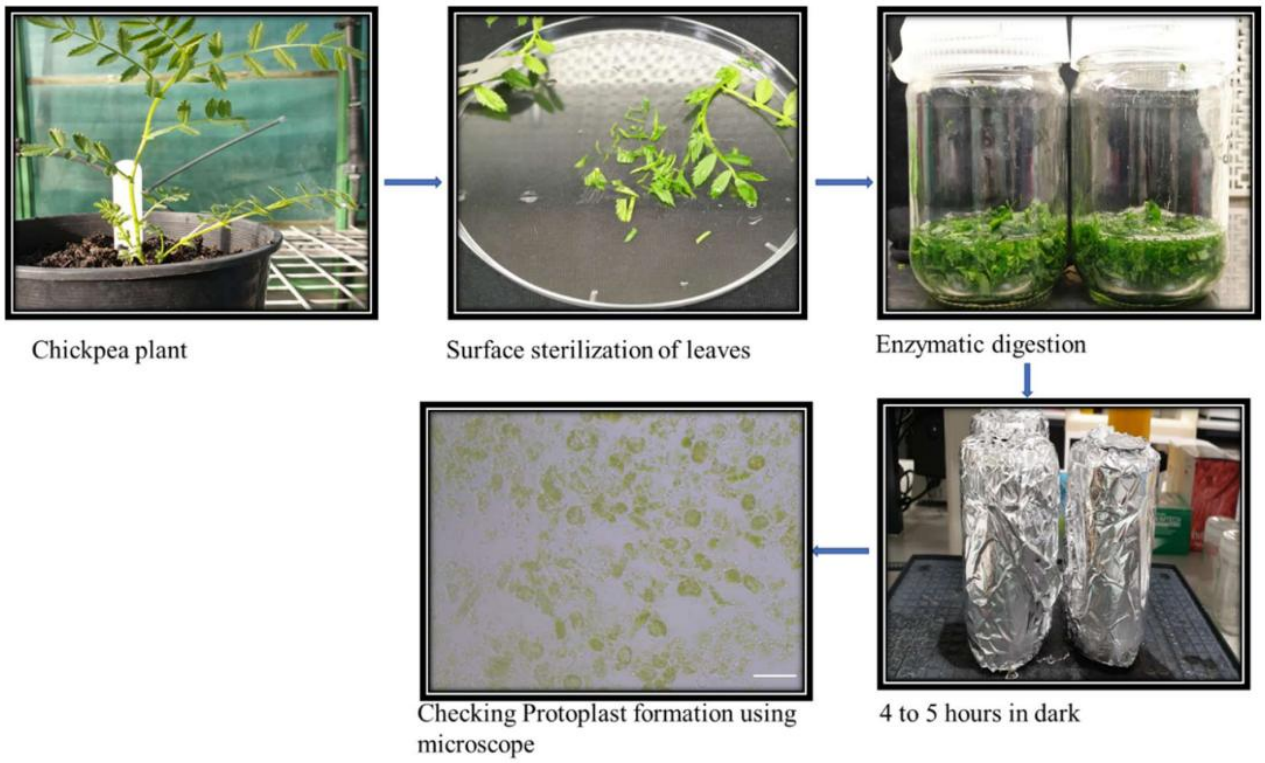

Figure 2 Flow chart of the steps performed during the protoplast isolation from leaf tissue of chickpea plants (Adopted from Badhan et al., 2021)

Image caption: The leaves from the 4 to 5 weeks old plants were collected and surface sterilized. The leaves were cut into small pieces. The enzyme solution was prepared and cut leaf sections were kept in the dark for 4 to 5 h digestion resulting in protoplast formation. After 2 h, samples for protoplast formation were collected to check the protoplast formation stage, and once the protoplast were isolated, they were used for the PEG mediated transfections (Adopted from Badhan et al., 2021)

4.3 Field trials and performance evaluation

Following successful gene editing, field trials are essential to evaluate the performance of the edited chickpea lines under natural conditions. Similar studies in other crops, such as soybean, have demonstrated that CRISPR/Cas9-mediated gene editing can enhance disease resistance without compromising agronomic traits. For

example, the knockout of the GmTAP1 gene in soybean resulted in enhanced resistance to *Phytophthora sojae* without affecting plant height, pod number, or yield (Liu et al., 2023). These findings suggest that CRISPR/Cas9-edited chickpea lines should be subjected to rigorous field trials to assess their disease resistance, growth, and yield performance under various environmental conditions.

4.4 Potential impacts on chickpea cultivation

The successful implementation of CRISPR/Cas9-driven genome editing in chickpeas has the potential to revolutionize chickpea cultivation. By enhancing disease resistance and stress tolerance, CRISPR/Cas9-edited chickpea lines can contribute to increased yield stability and reduced reliance on chemical pesticides. This, in turn, can lead to more sustainable and resilient agricultural practices. Moreover, the precise and predictable nature of CRISPR/Cas9 technology allows for the rapid development of improved chickpea varieties, addressing the urgent need for nutritious and high-yielding crops in the face of global food security challenges (Abdelrahman et al., 2018; Chen et al., 2019).

In summary, the application of CRISPR/Cas9 genome editing in chickpeas holds significant promise for improving disease resistance and overall crop performance. Continued research and field trials will be crucial to fully realize the potential of this technology in chickpea breeding and cultivation (Belhaj et al., 2015).

5 Challenges and Ethical Considerations

5.1 Off-target effects and genome integrity

One of the primary challenges in using the CRISPR/Cas9 system for genome editing in legumes is the occurrence of off-target effects, which can lead to unintended mutations. Various strategies have been developed to minimize these off-target effects. For instance, the use of truncated guide RNAs (gRNAs) has been shown to significantly reduce off-target mutagenesis without compromising on-target efficiency (Fu et al., 2014). Another effective approach involves the use of ligand-dependent ribozymes called aptazymes, which have been successful in reducing off-target mutations in human cells and hold promise for plant applications as well (Hajiahmadi et al., 2019). Additionally, the double-nicking strategy using Cas9 nickase mutants with paired guide RNAs has been demonstrated to minimize off-target cleavage (Ran et al., 2013). Tools like CRISPOR.org also assist researchers in selecting gRNAs with high specificity and low off-target potential, further aiding in the reduction of unintended mutations (Concordet and Haeussler, 2018).

The long-term impact of CRISPR/Cas9-induced mutations on genome stability is another area of concern. While the system is highly efficient in inducing targeted gene edits, the potential for off-target effects raises questions about the stability of the genome over multiple generations. Studies have shown that while CRISPR/Cas9 can produce stable and heritable mutations, the frequency and impact of off-target effects need to be carefully monitored to ensure long-term genome integrity (Figure 3) (Zhang et al., 2014). Continuous advancements in detection methods and the development of high-fidelity CRISPR/Cas9 variants are crucial for mitigating these risks (Zhang et al., 2015).

5.2 Regulatory and ethical issues

The regulatory landscape for genome-edited crops varies significantly across different regions, posing a challenge for the global adoption of CRISPR/Cas9 technology in legume breeding. In the European Union, genome-edited crops are subject to stringent regulations similar to those for genetically modified organisms (GMOs), which can hinder their development and commercialization (Bhowmik et al., 2021). In contrast, the United States has a more favorable regulatory framework that facilitates the use of genome editing for crop improvement. This disparity in regulations can impact international trade and the global food supply chain, making it essential to harmonize regulatory standards to fully realize the benefits of CRISPR/Cas9 technology (Manghwar et al., 2020).

The application of CRISPR/Cas9 in legumes also raises several ethical considerations. One major concern is the potential for unintended ecological consequences, such as the spread of edited genes to wild relatives or non-target species. This could disrupt local ecosystems and biodiversity. Additionally, there are ethical questions

related to the ownership and control of genome-edited crops, particularly in developing countries where access to advanced technologies may be limited. Public acceptance of genome-edited crops is another critical factor, as societal concerns about the safety and ethical implications of genetic modifications can influence regulatory decisions and market adoption (Mao et al., 2019). Addressing these ethical considerations through transparent communication and inclusive policy-making is essential for the responsible use of CRISPR/Cas9 technology in legume breeding.

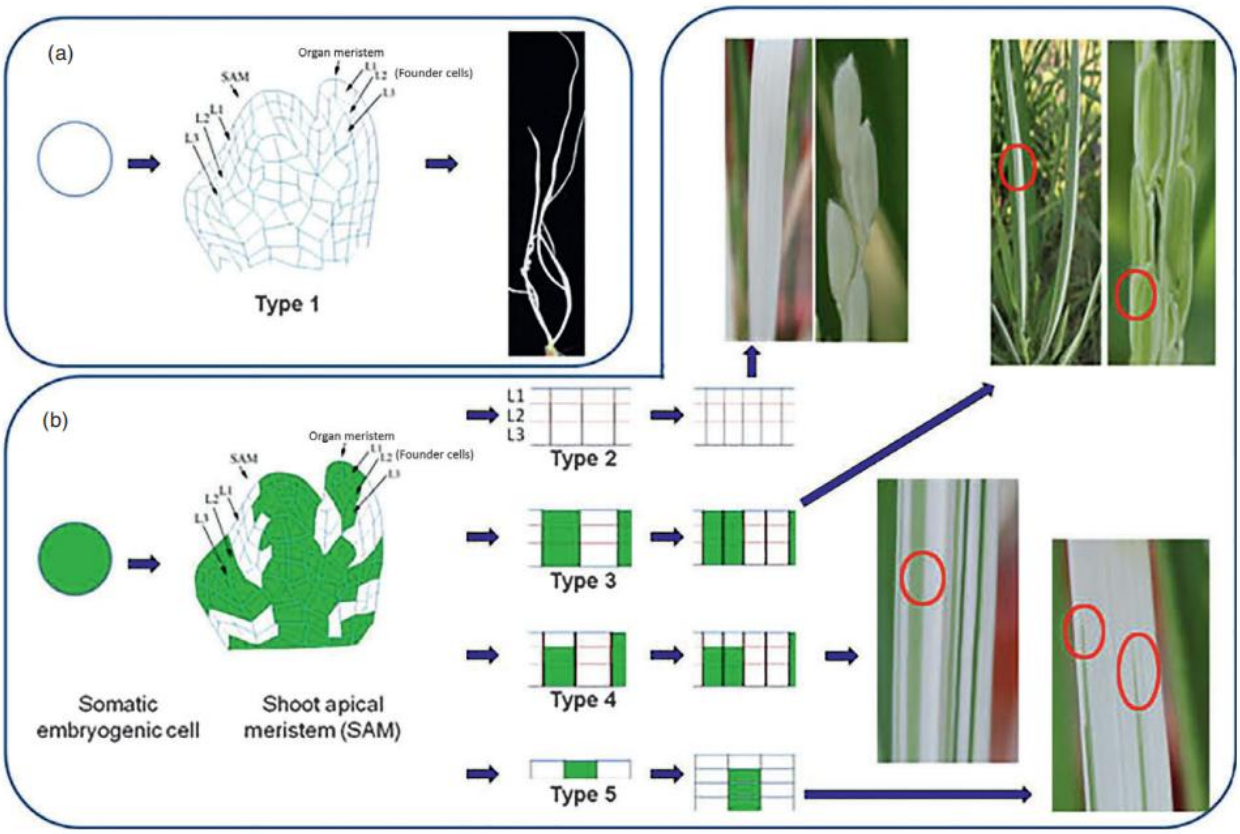

Figure 3 Diagramatic models of mutations in the cells transformed with the CRISPR/Cas9 system (Adopted from Zhang et al., 2014)
Image caption: The pictures shown were from T0 plants of the *OsPDS* target. Typical patterns of different mosaic types are marked by red circles. (a) When both copies of the *OsPDS* gene are simultaneously mutated, that is, in homozygous or bi-allelic plants, all the cells are albino (Type 1). (b) A cell with one or two copies of wild-type *OsPDS* gene is green. So, when two copies of the *OsPDS* are mutated after the division of the first embryogenic cell, the resulting cells have different genotypes and the regenerated plants exhibit different chimeric phenotypes (Types 2–5) depending on when the *OsPDS* gene is fully mutated (Adopted from Zhang et al., 2014)

6 Future Perspectives

6.1 Innovations in CRISPR/Cas9 technology

Base editing and prime editing represent significant advancements in CRISPR/Cas9 technology, allowing for precise nucleotide substitutions without inducing double-strand breaks. These methods have been successfully applied in various crops, including legumes, to introduce specific genetic changes that can enhance desirable traits such as disease resistance and nutritional quality (Chen et al., 2019; Cai et al., 2020). For instance, base editing has been utilized to modify specific genes in soybean, resulting in improved oil composition (Do et al., 2019). These innovations hold promise for creating legume varieties with enhanced traits while minimizing off-target effects and genetic instability.

Multiplexed genome editing enables the simultaneous targeting of multiple genes, which is particularly beneficial for complex traits controlled by multiple genetic loci. This approach has been demonstrated in legumes, where dual gRNA systems have been used to edit homeologous genes efficiently, leading to significant phenotypic

changes (Meng et al., 2016; Do et al., 2019). The ability to edit multiple genes concurrently can accelerate the development of legume varieties with improved yield, stress tolerance, and nutritional profiles (Arora and Narula, 2017; Bhowmik et al., 2021).

6.2 Integration of CRISPR/Cas9 in legume breeding programs

Collaborative research initiatives are essential for the successful integration of CRISPR/Cas9 technology into legume breeding programs. These collaborations can facilitate the sharing of resources, knowledge, and expertise, thereby overcoming technical challenges and accelerating the development of improved legume varieties (Bhowmik et al., 2021; Baloğlu et al., 2022). Joint efforts between academic institutions, government agencies, and private sector companies can also help address regulatory and public acceptance issues, ensuring that the benefits of CRISPR/Cas9 technology are realized globally (Chen et al., 2019).

The adoption of CRISPR/Cas9 technology by the agricultural industry is crucial for translating research findings into practical applications. Industry adoption can drive large-scale production and commercialization of CRISPR-edited legume varieties, making them accessible to farmers and consumers (Arora and Narula, 2017; Chen et al., 2019). Successful examples include the development of high oleic acid soybeans, which have been achieved through CRISPR/Cas9-mediated gene editing and are now being produced commercially (Do et al., 2019). Industry involvement can also support the development of robust regulatory frameworks and promote public acceptance of genome-edited crops.

6.3 Potential for sustainable agriculture

CRISPR/Cas9 technology offers significant potential for developing climate-resilient legume crops. By precisely editing genes associated with stress tolerance, researchers can create legume varieties that are better adapted to changing environmental conditions, such as drought, heat, and salinity (Arora and Narula, 2017). For example, targeted mutagenesis in model legumes like *Medicago truncatula* has demonstrated the feasibility of enhancing stress resilience through CRISPR/Cas9 (Meng et al., 2016). These advancements can contribute to sustainable agricultural practices by reducing the need for chemical inputs and improving crop resilience to climate change.

The application of CRISPR/Cas9 technology in legume breeding has the potential to significantly contribute to global food security. Legumes are a vital source of protein and essential nutrients, and improving their yield, nutritional quality, and stress tolerance can help meet the growing demand for food (Bhowmik et al., 2021; Baloğlu et al., 2022). By accelerating the development of high-yielding, nutrient-rich, and resilient legume varieties, CRISPR/Cas9 technology can play a crucial role in addressing food security challenges and ensuring a stable food supply for the global population (Chen et al., 2019).

7 Concluding Remarks

The application of CRISPR/Cas9 genome editing in legumes has shown significant promise in advancing functional genomics and breeding. Key findings from recent studies highlight the successful establishment of gene-editing methods in various legume species, including soybean, cowpea, chickpea, and model legumes such as *Medicago truncatula* and *Lotus japonicus*. The CRISPR/Cas9 system has been particularly effective in enabling precise and targeted modifications, which are crucial for improving traits such as yield, stress resilience, and nutritional quality. Despite these advancements, challenges remain, particularly in the transformation and regeneration of certain legume species, which are recalcitrant to in vitro gene transfer. Modifications in in vitro culture methods and the development of efficient delivery systems have been proposed to overcome these hurdles.

CRISPR/Cas9 technology is poised to play a transformative role in the future of legume breeding. Its ability to introduce site-specific double-stranded DNA breaks allows for rapid and precise genome modifications, which can significantly accelerate the breeding process. The technology's versatility extends to various genetic manipulations, including gene knockouts, precise modifications, and the activation or repression of target genes. This opens up new opportunities for developing legume varieties with enhanced disease resistance, improved nutritional profiles, and greater resilience to environmental stresses. Furthermore, the development of DNA-free

delivery methods and high-throughput mutant libraries are expected to enhance the efficiency and applicability of CRISPR/Cas9 in legume breeding.

While the potential of CRISPR/Cas9 in legume breeding is immense, several challenges need to be addressed to fully realize its benefits. Regulatory hurdles and public acceptance remain significant barriers, particularly in regions with stringent regulations on genetically modified organisms. Additionally, the efficiency of transformation and regeneration in certain legume species needs to be improved to make the technology more broadly applicable. Despite these challenges, the ongoing advancements in CRISPR/Cas9 technology, including the development of more efficient delivery systems and the refinement of gene-editing techniques, offer promising solutions. The continued research and collaboration among scientists, regulatory bodies, and the public will be crucial in harnessing the full potential of CRISPR/Cas9 for the sustainable improvement of legume crops.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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