"Review on Modern Breeding and Biotechnological Techniques for Enhancing Breeding Potential of Legume Crops"

Abstract

Legume crops are critical to global food security and sustainable agriculture, providing essential plant-based proteins and amino acids, while also contributing to soil fertility through symbiotic nitrogen fixation. Despite their nutritional and ecological significance, legume crops production faces numerous challenges, including low yields, susceptibility to biotic and abiotic stresses, and the impacts of climate change on water and land resources. Addressing these issues requires innovative solutions that combine traditional breeding with cutting-edge biotechnological approaches. The recent advancements in legume crops improvement through modern breeding and genome-editing technologies, such as CRISPR/Cas9, TALEN, and ZFN, which enable precise modifications to enhances the suitability and genetic potential of agronomic traits. CRISPR/Cas9, in particular, has emerged as a powerful tool in legume breeding, facilitating targeted mutations, gene knockouts, and gene expression regulation. The review discusses its application across various legume species, including soybeans, cowpeas, chickpeas, and peanuts, to improve traits for example, CRISPR/Cas9 has been used to increase oleic acid content in peanuts and improve photoperiodic flowering in soybeans. However, the complexity of legume transformation and regeneration has historically hindered the broader use of genome-editing technologies. Advances in transformation protocols, such as Agrobacterium-mediated methods and biolistic technologies, as well as improvements in tissue culture and phenotyping techniques, are helping to overcome these challenges. Despite significant progress, challenges in legume transformation and regeneration remain, but recent improvements in tissue culture protocols and high-throughput phenotyping have enhanced the efficiency of these genome-editing techniques. It also explores the potential of integrating genome-editing technologies with traditional breeding programs to accelerate genetic gains and develop bio fortified, climate-resilient legume varieties. By leveraging the vast genetic diversity in legumes and employing advanced genomics tools, researchers can create crops that are not only high-yielding but also nutritionally superior and environmentally sustainable.

Keywords: legumes, biotechnological, Genome editing, CRISPR/Cas9, Agrobacterium-mediated transformation.

Introduction

The legume family ranked as the third largest among flowering plants, encompassing over 19,500 recognized species distributed across 751 genera (Lewis, 2005). This family included numerous food crops that were vital providers of plant-based proteins and essential amino acids. Leguminous crops were crucial for sustainable agriculture, as they enhanced soil health through symbiotic nitrogen fixation and contributed high-quality organic matter to the soil. Despite their health benefits and ecological importance, legume production faced challenges due to low yields. The reasons of low yield in legume are very from region to region e.g. low productivity is due to weed infestation, poor irrigation management in Chickpea, Urdbean and Mungbean in Bundelkhand region in Uttar Pradesh and Madhya Pradesh while highly productive land is one of the major cause for poor yield in Punjab, Haryana and Western Uttar Pradesh. (Kumar 2020 and Kumar *et al*., 2023).

Addressing these yield issues was complex but could be improved by implementing innovative strategies like genomics-assisted selection (including marker-assisted or genomic selection) and precision breeding (Bhowmik *et al*., 2021). Ensuring food security with a focus on high nutrition and yield was anticipated to be a major challenge for researchers and the farming community by 2050.

Grain legumes were rich in proteins, vitamins, minerals, iron, calcium, zinc, magnesium, and essential omega-3 fatty acids. Soybeans had the highest protein content, ranging from 33% to 45%, followed by common beans (21%–39%), wing beans (30%–37%), cowpeas (21%–35%), groundnuts (24%–34%), and peas (21%–33%). Other protein-rich legumes included moth beans and urd beans (21%–31%), lentils (20%–31%), grass peas (23%–30%), chickpeas (15%–30%), horse grams (19%–29%), and rice beans (18%–27%) (Pandey *et al*., 2016). Increasing legume production to desired levels was hindered by biotic and abiotic limitations. Furthermore, the reduction in available land and water resources due to climate change was expected to intensify these adverse conditions in the coming years, placing protein-rich crops like legumes at risk. Eliminating anti-nutritional factors, such as tannins (Mikic *et al*., 2009), was a key focus in legume crop improvement programs.

Efforts were aimed at accelerating genetic gains related to yield, stress tolerance, and nutritional quality. Over the past fifty years, most legume genetic improvements had been achieved through pedigree and performance-based selection. To boost genetic gains more rapidly, innovative genomics techniques and high-throughput phenomics were widely applied, leading to improved legume varieties with valuable agronomic traits (Varshney *et al*., 2018). With growing access to information on genes and haplotypes associated with agronomically important traits, genome editing now enabled the modification of multiple

SNPs without altering the original characteristics of popular cultivars. Genetic barriers, such as differences in ploidy levels, often prevented natural genetic exchange between legume species. As a result, the vast genetic diversity found in their wild relatives remained largely untapped (Varshney *et al*., 2018).

The availability of complete genome sequences for various organisms played a crucial role in advancing next-generation genome editing research. Among legumes, there was a greater number of recent genome editing studies focused on Lotus japonicus (Sato *et al*., 2008), Glycine max (Schmutz *et al*., 2010), and Medicago truncatula (Young *et al*., 2011), all of which had been fully sequenced. This underscored the significance of having complete genome data for facilitating such studies. This article discussed the mechanisms behind new-generation genome-editing technologies, including TALEN (transcription activator-like effector nucleases), ZFN (zinc finger nucleases), and the CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9 nuclease), along with detailed examples of their applications in legume species. Additionally, it provided an in-depth examination of the regulatory framework and the future prospects of genome-editing technologies in these crops. This study served as a comprehensive resource on genome-editing research in plants within the legume family.

Editing technologies

1. CRISPR/Cas9

Recently, CRISPR/Cas9 technology has gained significant popularity for genome editing across a range of organisms, including plants. This advancement has broadened agricultural research by enabling the development of new plant varieties that either eliminate undesirable traits or introduce beneficial characteristics. CRISPR is a fast-evolving method applicable for various genetic modifications, such as creating gene knockouts, making accurate edits, facilitating multiplex genome engineering, and regulating gene expression through activation and repression (Arora and Narula, 2017). The CRISPR/Cas9 system is composed of two main elements: the Cas9 protein and the guide RNA (gRNA). The Cas9 protein functions as an RNA-guided DNA endonuclease, forming a complex with the gRNA. The gRNA comprises 20 nucleotides complementary to the target DNA sequence and functions as a recruitment signal for Cas9. Unlike other genome-editing technologies, which rely on DNA–protein interactions, CRISPR/Cas9 utilizes RNA–DNA interactions to identify the target DNA sequence. In contrast, ZFNs and TALENs require two distinct DNA-binding domains for each target site due to their use of DNA–protein interactions, making the process more complicated. With CRISPR/Cas9, an 18–20 base pair sequence is all that's required,

since Cas9 and the guide RNA (gRNA) need to bind to a specific protospacer adjacent motif (PAM) located at the 3′ end of the target sequence. (see Figure 1). The most commonly used PAM sequence for Cas9 from *Streptococcus pyogenes* is 5′-NGG-3′. Cas9 induces DNA repair by creating double-stranded breaks (DSBs) in the target DNA. This repair can occur through non-homologous end joining (NHEJ) to produce gene knockouts or homology-directed repair (HDR) to facilitate gene modifications and insertions (Huang and Puchta, 2019) (Figure 1). A frameshift mutation takes place when Non-Homologous End Joining (NHEJ) randomly inserts or removes DNA strands within a coding region, leading to a gene knockout. This process does not require a homologous repair template, making NHEJ the most widely used and optimized DNA repair method in plants, including legumes. In contrast, the Homology-Directed Repair (HDR) technique allows for the precise insertion of specific sequences from a donor DNA template. However, HDR has limited application in plants due to its low editing efficiency (Atkins and Voytas, 2020). CRISPR technology offers various applications, such as gene expression analysis, and gain- or loss-of-function studies. CRISPR has also been applied in legume agriculture, and this genome-editing tool holds potential for creating sustainable, high-quality agricultural products, including legumes.

A.gRNA design and validation

The guide RNA (gRNA) is a critical element of the CRISPR/Cas9 system, determining the specificity and efficiency of gene editing. In plants, the presence of large gene families with high sequence similarity increases the risk of off-target binding by the gRNA, which can lead to unintended effects. Therefore, it is essential to design gRNAs that offer high on-target accuracy while minimizing off-target interactions. For species with low transformation rates and extended tissue culture processes, it is advantageous to select highly active and multiple gRNAs. Conversely, in species that are more easily transformable, approximately two gRNAs per gene are generally sufficient to achieve a mutant..

B. gRNA delivery

i) Transformation of legume crops

For successful transformation, including CRISPR/Cas9 gene editing, it is essential to deliver DNA/RNA components and regenerate a whole plant from transformed tissues. Legumes are notably challenging in this regard, as they tend to resist both DNA uptake and integration (Yadav *et al*., 2017) and are generally difficult to regenerate. Complicating matters further, the tissues that are amenable to transformation and those capable of regeneration do not always overlap in legumes. This highlights the need for a transformation

protocol that integrates the transformation vector from the start, rather than merely focusing on developing a regeneration protocol.

Large-seeded legumes primarily utilized Agrobacterium-mediated transformation, though biolistics technologies were also applied. Successful transformations generally involved explants from seed tissues, including whole or partially developing or mature embryos, as well as cotyledonary nodes, sometimes including deembryonated cotyledons. A Common treatments for explants involved wounding, often paired with acetosyringone to activate Agrobacterium virulence genes. Wounding methods included puncturing, cutting embryos in half, or making longitudinal slices. Additional techniques to enhance DNA uptake involved sonication and/or vacuum infiltration, along with other compounds such as lipoic acid.

Transformation protocols typically consisted of several phases. For organogenesis in species like pea and chickpea, these phases included a co-cultivation period, shoot initiation, shoot elongation, and rooting, each requiring distinct media formulations. Protocol development often utilized a reporter gene like gus (β-glucuronidase) to monitor progress (figure 1). Although using a reporter gene required sacrificing some tissue, it was often easier to detect than fluorescence in green, photosynthetic tissues. Regardless of the reporter method, molecular analysis techniques such as PCR or ddPCR were essential to confirm the successful regeneration of a transgenic plant.

Protocols utilizing embryogenesis typically included stages such as embryogenesis, proliferation of embryonic tissue for bombardment, maturation, desiccation, and germination. Media formulations, incubation conditions, and durations varied significantly among different species and protocols.

ii) CRISPR/Cas9 mutagenesis in doubled haploids

A functional microspore-based gRNA delivery system presents several advantages for rapidly achieving homozygosity and shortening the generation cycle. It allows for the quick isolation of a large number of genetically identical and physiologically uniform microspores, facilitating functional gene validation, genetic characterization, and the enhancement of valuable traits. Additionally, fluorescent reporter systems enable the high-throughput screening of multiple gRNAs simultaneously using microspores. One of the initial experiments involving microspore mutagenesis with the CRISPR/Cas9 system was conducted by Bhowmik *et al*. (2018), who utilized a Neon electroporation system to deliver gRNAs into

isolated wheat microspores. Furthermore, Bilichak *et al*. (2020) demonstrated that a complex of cell-penetrating peptides and zinc-finger nucleases (CPP–ZFN) could effectively introduce edits in haploid embryo-like structures regenerated from wheat microspores.

Microspores from legume species held potential for gRNA delivery and CRISPR/Cas9-mediated gene editing. However, the recalcitrant nature of these legumes significantly hindered the production of double haploid (DH) plants. Various research groups attempted to regenerate DH plants (Bobkov, 2010; Gupta, 1975; Lulsdorf *et al*., 2011; Ochatt *et al*., 2009; Ribalta *et al*., 2012), but the frequency of successfully regenerating complete haploid plants remained very low (Bobkov, 2014; Ochatt *et al*., 2009). To date, successful regeneration of DH plants had only been achieved in chickpea through anther culture and androgenesis.

Recognizing the importance of double haploid (DH) technology in pulse breeding, several laboratories, including the National Research Council of Canada, have recently initiated efforts to re-evaluate and enhance strategies for producing DH plants. These advancements aim to overcome previous challenges and improve the efficiency and success rates of DH plant production in legumes

Detection of genetic modifications

Identifying and monitoring modifications caused by genome editing were crucial at various stages, including the optimization of experimental conditions, characterization, selection, and tracking of edited lines. The methods available for detecting these modifications were diverse and continually evolving, with no single method meeting all genotyping requirements.

Key challenges in reliably detecting modifications stemmed from the biological characteristics of gene-editing proteins, which could induce a variety of possible mutations, as well as the biological traits of the target organism, which might involve multiple homologs and varying efficiencies in creating edited lines. Given the difficulties associated with regenerating legumes, it was often necessary to screen a large number of lines or heterogeneous tissues, emphasizing the need for methods that were both cost-effective and sensitive.

Although most economically important legumes were not complicated by recent polyploidization, they exhibited a higher propensity for gene duplications (Young *et al*., 2011). High-quality genome assemblies played a crucial role in the reliable interpretation of detection experiments and were especially important for assessing off-target effects.

Therefore, developing and utilizing detection methods that ensured high on-target accuracy while minimizing off-target interactions was essential, particularly in species with low transformation rates and lengthy tissue culture processes. In this section, we discuss some of the most widely used and promising methods for detecting edited sequences in legumes. For a more comprehensive comparison of established methodologies, we refer the reader to a recent review dedicated entirely to this topic.

Mismatch detection

One of the original and most popular methods for screening mutations involves detecting mismatches formed during the denaturation and annealing of heterogeneous DNA populations. Several endonucleases, such as cell, I that can cleave at mismatches have been employed for this purpose. While cell I may excel in single nucleotide polymorphism (SNP) detection compared to T_7EI, the latter is recommended for CRISPR/Cas9 experiments, as it tends to perform better on insertions and deletions (indels) and generally produces less background signal, which can be a limiting factor for this approach. Although mismatch detection is relatively easy to implement and requires only standard molecular biology equipment, it is limited by low sensitivity and high rates of both false positives and negatives.

Electrophoretic separation

Electrophoretic mobility was used to directly provide a simpler method for identifying mutants. Large insertions or deletions were easily monitored through PCR followed by agarose gel electrophoresis, while PCR coupled with nondenaturing polyacrylamide gel electrophoresis (PAGE) was able to detect indels as small as 1 bp. Although these methods are quick, they have limited sensitivity for detecting low-abundance edits, making them more suitable for systems with high editing efficiency or for screening later generations to identify homozygous lines.

When targeting a restriction enzyme recognition site is feasible, restriction enzyme-suppressed PCR (RE-PCR) offers a more sensitive alternative (Xie & Yang, 2013). This method involves conducting a restriction digest before PCR amplification, allowing only the mutated sites to be amplified.

Annealing at critical temperature PCR (ACT-PCR) is a specialized PCR strategy designed to detect homozygous mutant lines by exploiting the annealing characteristics of primers in relation to the presence of mutations. Key follwing points:

1. Principle- ACT-PCR relies on the concept that primers will have different annealing efficiencies based on the presence or absence of mutations in the template DNA. The strategy

involves finding the highest possible annealing temperature at which a primer can anneal to the wild-type template but fails to anneal when a mutation is present.

2. Detection of Mutations- By adjusting the annealing temperature, it is possible to ensure that the primer overlaps the target site, thereby creating conditions where it will successfully anneal to the wild-type sequence while failing on the edited (mutated) sequence. This results in no amplification of the target from the mutant template.

3. Internal Positive Control- To enhance the reliability of the assay, an additional competing flanking primer can be included, which serves as an internal positive control. This primer should amplify a region that is not affected by the mutation, ensuring that the PCR reaction is working properly.

4. Equipment Requirements- While the method has minimal equipment requirements, utilizing a gradient thermocycler is recommended. This allows for precise identification of the critical annealing temperature, enabling researchers to fine-tune the PCR conditions for optimal results.

5. Applications- ACT-PCR can be a powerful tool in genetic studies, particularly for identifying homozygous mutations in plant breeding, genetic engineering, and crop improvement programs.

This method represents a valuable approach in molecular biology, particularly for detecting specific genetic changes with high sensitivity and specificity.

Incorporating fluorescence-based detection methods can significantly enhance the sensitivity of PCR techniques. The application of fluorophore-labeled primers has been shown to produce labeled amplicons that can be detected using DNA capillary electrophoresis. This approach allows for the detection of insertions and deletions (indels) with a limit of detection (LOD) of approximately 0.1%, and it can differentiate mutation populations with a resolution of 1 to 2 base pairs. Additionally, this method capitalizes on the automation capabilities of capillary electrophoresis equipment. Although not yet demonstrated, it has the potential to simultaneously monitor multiple targets by using different fluorophores for each amplicon, similar to the high-throughput analysis of simple sequence repeats (SSRs). The method's combination of low cost, high sensitivity, and scalability makes it an appealing choice for screening in legumes, especially in settings where quick turnaround times are feasible due to available instrumentation.

Fluorophore binding-based detection

Various highly sensitive techniques for detecting edits were employed, including high-resolution melt analysis (Thomas *et al*., 2014), quantitative PCR (qPCR) (Peng *et al*.,

2018), and droplet digital PCR (ddPCR) (Miyaoka *et al*., 2018), which utilized fluorescent probes or dyes for binding. Although these methods were both sensitive and cost-effective, they required access to specific equipment.

Sanger sequencing

Sanger sequencing is an affordable and reliable technique for genotyping that can verify modifications. Traditionally, Sanger sequencing data analysis required clonal samples, but newer methods like TIDE (Tracking of Indels by Decomposition; Brinkman & van Steensel, 2019) and ICE (Inference of CRISPR Edits; Hsiau *et al*., 2018) now allow for the interpretation of Sanger sequencing data from mixed populations of DNA fragments. This capability, combined with its low cost and speed, has made Sanger sequencing popular in plant research. However, it has limitations, such as a moderate limit of detection (LOD) and low tolerance for off-target amplification. While the LOD can be as sensitive as 1–2% (Brinkman *et al*., 2014) depending on the length of indels, in practical applications, the LOD for these Sanger-based methods is typically around 10%.

Next-generation sequencing

Massively parallel sequencing technologies enabled the simultaneous examination of numerous sites across multiple samples. Next-generation sequencing (NGS) techniques were highly sensitive (with a limit of detection, LOD, of less than 1%, as noted by Kim *et al*., 2019) and could characterize the range of edits within a cell population. Sample preparation for NGS involved attaching sequencing adapters to the target sequences. For Illumina platforms, a common method for preparing amplicon libraries was to add a partial adapter sequence as a tail on the target-specific primers. A second PCR then completed the adapter sequence and added sample-specific barcodes. This method allowed the use of universal indexing primers, facilitating the pooling of multiple amplicons from a single sample after the initial PCR step. Generic indices became commercially available with up to 384 unique dual-index combinations, enabling greater scalability in multiplexing. Additionally, it was possible to multiplex the initial target-specific PCR step, further simplifying the preparation of numerous amplicons per sample. Various tools, like Oligogo (Hendling *et al*., 2018), supported multiplex PCR design, and methods such as rhAmp-seq from IDT could handle multiplexing for over 100 targets.

While there are currently no established methods for utilizing Oxford Nanopore Technologies for detecting edits, the smaller MinION or Flongle platforms are appealing for their quick turnaround capabilities. Despite the high error rates associated with this platform, both the technology and its application strategies are advancing swiftly. One approach to

manage these higher error rates involves adding unique molecular identifiers (UMIs) to amplicons (Karst *et al*., 2021). Although this adds an extra step to the process, it significantly enhances the reliability and sensitivity in detecting mutations.

Although per-experiment costs remain relatively high compared to other strategies mentioned here, the extensive data output and multiplexing capability of NGS methods offer the potential for reduced costs per sample. NGS of genomic libraries is also currently the only method that can provide complete genome coverage, allowing for a thorough examination of genomic alterations (Tang *et al*., 2018). Despite its powerful capabilities, NGS approaches often have longer turnaround times and require batching to fully capitalize on their advantages. NGS methods yield information for individual library fragments, which offers some tolerance for off-target amplification; however, off-target sequences and discrepancies between the experimental line and the reference genome are primary contributors to false positives. As a result, incorporating negative controls is essential for accurately interpreting variant calling data. We anticipate that NGS screening methods will continue to gain popularity as editing experiments advance towards higher multiplexity, and as the efficiency of NGS experiments continues to improve.

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APPLICATIONS IN LEGUMES

1.*M. truncatula* (Alfalfa)

Medicago truncatula is widely used as a model organism for legume crops because of its short life cycle, ease of genetic transformation, self-fertility, diploid genome, and relatively small genome size. These features make it a preferred option for conducting molecular and physiological research on legumes. In a study by Michno *et al*. (2015), mutations were induced in the soybean genes for glutamine synthase (GS1) and chalcone-flavanone isomerase (CHI20) in *Glycine max*, as well as in the β-glucuronidase (GUS) gene in *M. truncatula* using hairy root transformation.

Bottero *et al*. (2021) generated two transgenic alfalfa lines, designated as 3-1 and 5-1, using the CRISPR/Cas9 system with a pBI121 binary vector that included the GUS gene. This resulted in an average GUS inactivation of 55%. In the scientific literature, phytoene desaturase (PDS) genes were often chosen for study due to the ease of observing phenotypic changes, which made it simpler to evaluate the effectiveness of CRISPR/Cas9 gene editing. For example, Meng *et al*. (2017) established an effective CRISPR/Cas9 system to induce mutations in the MtPDS gene in M. truncatula, finding that 32 of 309 T0 transgenic plants displayed an albino phenotype. Sequencing 16 randomly selected albino plants confirmed that all had mutations at the targeted MtPDS gene site. Additionally, Wolabu *et al*. (2020) discovered that the UBQ10 promoter-driven Cas9 resulted in high mutation efficiency (95% in Arabidopsis and 70% in M. truncatula). Similarly, Zhang *et al*. (2020) targeted MtPDS genes with CRISPR/Cas9, and all homozygous/biallelic MtPDS mutant seedlings showed albino traits.

The CRISPR/Cas9 gene-editing tool was also applied in studies focusing on flowering and secondary metabolite production. Rodas *et al*. (2021) used CRISPR/Cas9 to edit the SUPERMAN (MtSUP) gene in *M. truncatula*, which disrupted MtSUP function, leading to defects in floral development and inflorescence structure in plants carrying the mtsup mutant allele. Similarly, Confalonieri *et al*. (2021) utilized CRISPR/Cas9 to knock out two cytochrome P450 genes, CYP93E2 and CYP72A61, which were involved in the production of soyasapogenol B in Medicago species. Their findings revealed that 51 putative CYP93E2 mutant plant lines, with an 84% editing efficiency, did not produce soyasapogenols in leaves,

stems, or roots. Instead, this alteration shifted the metabolic pathway towards producing valuable hemolytic sapogenins.

***Japonicus* (Lotus)**

L. japonicus served as a model organism for legume crops and shared similarities with *M. truncatula*. However, unlike *M. truncatula*, L. japonicus formed determinate nodules, similar to soybean (*G. max*) and cowpea (*Vigna unguiculata*). Wang *et al*. (2016) demonstrated that gene mutations related to symbiotic nitrogen fixation (SNF) were achievable in L. japonicus using CRISPR/Cas9 through hairy root transformation. In 2018, Cai *et al*. edited the cytokinin receptor Lotus histidine kinase I-interacting protein (LjCZF1) to investigate cytokinin signaling involved in rhizobia-legume symbiosis. They found that knock-out mutants had significantly fewer infection threads and nodules, indicating that LjCZF1 positively regulated symbiotic nodulation. Subsequently, Wang *et al*. (2019) used CRISPR/Cas9 to explore the role of leghemoglobin (Lbs) in L. japonicus, discovering that the absence of Lbs led to early nodule senescence. In another study, a loss-of-function analysis of the CYP716A51 gene, involved in triterpenoid C-28 oxidation, showed that cyp716a51 mutant L. japonicus hairy roots were unable to produce C-28 oxidized triterpenoids.

***G. max* (Soybean)**

The growing demand for soybean products, due to their high economic value as a protein and oil source for both animal and human nutrition, has highlighted the need to accelerate genetic advancements using gene-editing technologies. Bao *et al*. (2020) emphasized that these advancements are essential to meet rising demands and address environmental challenges. Curtin *et al*. (2011) initially optimized a gene-editing approach in soybean by targeting the green fluorescent protein (GFP) coding region with a zinc finger nuclease (ZFN) array, developed using context-sensitive selection strategies. This approach resulted in deletions of up to 71 base pairs at the target site. With further optimization, they subsequently targeted two distinct RNA-dependent polymerases in soybean.

The study's most intriguing result was the design of two independent ZFN pairs, both of which successfully recognized their specific targets and caused two base pair differences in both genes. This demonstrated the precise targeting and effectiveness of the ZFN approach in gene editing. In another study by Curtin *et al*. (2015), researchers investigated the disruption of miRNA maturation and the regulation of miRNA gene expression. They developed two distinct ZFN pairs aimed at the Dicer-like 1a (DCL1a) and Dicer-like 1b

(DCL1b) genes in soybean. Although single mutants of the DCL genes did not yield significant results, the double mutants of the DCL genes exhibited notable morphological changes. Additionally, these double mutants showed impaired processing efficiency of miRNA precursor transcripts and deregulated expression of miRNA target genes. In 2018, Curtin *et al*. employed TALENs to target the G. max Dicer-like2 gene and used whole-genome sequencing to reveal multiple transgene insertion events, producing a range of combinatorial mutant plants. With CRISPR-Cas, they targeted two endogenous soybean genes (GmFEI2 and GmSHR), designing sgRNAs against a transgene (bar) and creating targeted DNA mutations in hairy roots using six sgRNAs. Li *et al*. (2015) successfully utilized CRISPR/Cas9 to induce mutations at two genomic sites, DD20 and DD43, on chromosome 4 with success rates of 59% and 76%, respectively. Sun *et al*. (2015) further developed two vectors with Arabidopsis U6-26 and soybean U6-10 promoters to target Glyma06g14180, Glyma08g02290, and Glyma12g37050 in protoplasts. Biallelic mutations were observed in Glyma06g14180 and Glyma08g02290 within transgenic hairy roots.

Additionally, a comparative analysis of CRISPR/Cas9 and TALENs for editing GmPDS11 and GmPDS18 genes revealed albino and dwarf phenotypes (PDS knock-outs) following cotyledon node transformations. TALENs demonstrated a slightly higher mutation efficiency than CRISPR/Cas9 with the AtU6-26 promoter, though it was lower when using the GmU6-16g-1 promoter in hairy roots. The study concluded that both technologies effectively achieved gene targeting in soybean. Curtin *et al*. (2018) also used CRISPR/Cas9 and TALENs in parallel on G. max and M. truncatula, creating a bi-allelic double mutant for the soybean GmDrb2a and GmDrb2b genes and a mutation in the M. truncatula Hua enhancer1 (MtHen1) gene. Soybean flowering time is crucial for accelerating breeding processes to enhance yield and quality. Consequently, various studies have focused on editing genes associated with flowering time. Cai Y. *et al*. (2018) developed an efficient dual-sgRNA/Cas9 system to induce deletions in the GmFT2a and GmFT5a genes. They achieved deletion frequencies of 15.6% and 15.8% in GmFT2a and GmFT5a, respectively, with deletions over 4.5 kb occurring in 12.1% of GmFT2a samples. These deletions were inheritable in T2 transgene-free homozygous ft2a mutants, which displayed a late-flowering phenotype. In a separate study, Han *et al*. (2019) edited the soybean maturity gene E1, responsible for flowering regulation, producing 11 bp and 40 bp deletions that resulted in premature stop codons and truncated E1 proteins. Additionally, CRISPR/Cas9 was used to generate knock-out and overexpression mutations in the GmPRR37 gene, which encodes the flowering time regulator qFT12-2, demonstrating its role in controlling photoperiodic

flowering in soybean. Another study examined GmFT2a and GmFT5a genes using CRISPR/Cas9 and showed that these genes jointly regulate flowering time by analyzing ft2a, ft5a, and ft2a/ft5a mutants under short-day and long-day conditions.

The CRISPR/Cas9 strategy has also been used to target three GmLox genes (GmLox1, GmLox2, and GmLox3), which encode lipoxygenases (LOX1, LOX2, and LOX3) that contribute to a beany flavor limiting soybean's appeal for human consumption. In this study, 60 T0 positive transgenic plants were generated with combinations of sgRNAs, resulting in various mutations, including two triple mutants and one double mutant, all of which lost the corresponding lipoxygenase activities. Similarly, Li *et al*. (2019) applied CRISPR/Cas9 to edit the conglycinin (7S) and glycinin (11S) storage protein genes, achieving editing efficiencies of 5.8%, 3.8%, and 43.7% for Glyma.20g148400, Glyma.03g163500, and Glyma.19g164900, respectively. Furthermore, CRISPR/Cas9 has been used to modify plant architecture in soybean by targeting squamosa promoter binding protein-like genes (GmSPL9a, GmSPL9b, GmSPL9c, and GmSPL9). T2 double homozygous spl9a/spl9b mutants displayed a shorter plastochron length and, in T4 mutant plants, increased main stem node numbers and branch numbers were observed.

Improving the oleic acid content in soybeans has also become a significant breeding objective. Gene-editing technologies have played a crucial role in this area. While TALENs have been less frequently used in legumes, successful applications have targeted the Fatty Acid Desaturase 2 (FAD2) genes, which convert oleic acid to linoleic acid, to increase oleic acid levels. Haun *et al*. (2014) targeted the FAD2-1A and FAD2-1B genes, using four TALEN pairs; two pairs (FAD2_T01 and FAD2_T04) were expressed, with FAD2_T04 achieving a 7.2% mutation rate. As a result, they observed a decrease in linoleic acid (down to 4%) and an increase in oleic acid content (up to 80%). Demorest *et al*. (2016) conducted a similar study, targeting the FAD2-1A, FAD2-1B, and FAD3A genes, continuing the effort to enhance oleic acid content through targeted mutagenesis.

TALENs were designed to target the FAD3A gene in lines already mutated at FAD2-1A and FAD2-1B, achieving mutation rates of 11.2%, 16.0%, and 4.9%, respectively. These mutations led to an increase in oleic acid content of over 80% and a reduction in linoleic acid to 2%. Do *et al*. (2019) also targeted the GmFAD2-1A and GmFAD2-1B genes, generating T0 transgenic plants that displayed an 80% increase in oleic acid content and a 1.3%–1.7% decrease in linoleic acid in T1 seeds homozygous for both genes. Similarly, Al Amin *et al*. (2019) used CRISPR-Cas9 to mutate the FAD2-2 gene, resulting in significant changes in the oleic acid/linoleic acid ratios due to frequent deletions and insertions.

In 2020, Wu and colleagues applied CRISPR/Cas9 to GmFAD2-1A and GmFAD2-2A genes, producing single and double knock-out mutants. They found editing efficiencies of 95% for GmFAD2-1A, 55.56% for GmFAD2-2A, and 66.67% for double mutants. These mutants exhibited an increase in oleic acid content up to 73.5% and a decrease in linoleic acid down to 12.23% in the T2 generation, with similar trends continuing into the T3 generation. Di *et al*. (2019) enhanced CRISPR/Cas9 efficiency by utilizing highly active U6 promoters to target Glyma03g36470, Glyma14g04180, and Glyma06g136900, which resulted in insertion, deletion, and substitution mutations. In the following year, they constructed 70 CRISPR-Cas9 vectors to target 102 candidate genes, creating 407 T0 mutant lines with a mutagenesis frequency of 59.2%, of which 35.6% had multiplex mutations. These mutants showed increased nodule numbers in gmric1/gmric2 double mutants, while gmrdn1-1/1-2/1-3 triple mutants exhibited reduced nodulation.

***V. unguiculata* (Cowpea)**

Cowpea (Vigna unguiculata (L.) Walp.) is recognized for its high nutritional value and health benefits. It possesses efficient symbiotic nitrogen fixation (SNF) capabilities, tolerance to low rainfall, and minimal fertilization needs, making it one of the most significant legumes globally. Consequently, there has been growing interest in employing gene-editing techniques in cowpea.

In 2019, Ji and colleagues demonstrated CRISPR/Cas9-mediated genome editing in cowpea by targeting SNF genes, leading to non-inheritable mutations in hairy roots. They found that nodule formation was entirely blocked in mutants with disruptions in both alleles. Following this, Juranic *et al*. (2020) identified three cowpea meiosis-related genes: SPO11-1 (which encodes the SPO11 protein responsible for initiating meiotic double-stranded breaks), REC8 (which encodes a meiotic recombination protein), and OSD1 (which encodes Ophiostoma scytalone dehydratase, promoting meiotic progression). They used CRISPR/Cas9 to induce asexual seed formation, resulting in biallelic mutations in exons 1 and 3 of the SPO11-1 gene, causing complete male and female sterility in T0 plants. More recently, Che *et al*. (2021) knocked out the cowpea meiosis gene VuSPO11-1 using CRISPR/Cas9 and confirmed mutations at the target site.

This work underscored the potential of CRISPR/Cas9 technology to advance genetic research in cowpea, addressing both fundamental biological questions and practical agricultural challenges.

***C. arietinum* (Chickpea)**

Chickpea (*Cicer arietinum*) is an important commercial crop globally, and gene-editing tools offer potential solutions to challenges in its production. In a study by Badhan *et al*. (2021), the researchers targeted drought tolerance-related genes, specifically 4-coumarate ligase (4CL) and Reveille 7 (RVE7), using CRISPR/Cas9 editing in chickpea protoplasts. The knockout of the RVE7 gene resulted in highly efficient editing in vivo. These findings demonstrated that DNA-free CRISPR/Cas9 gene editing can be effectively applied to drought tolerance genes in chickpea protoplasts. To date, this study represents the first and only application of CRISPR/Cas9 gene editing in chickpea.

***A. hypogaea* (Peanut)**

Peanut (*Arachis hypogaea*) is a significant legume crop known for its high oleic acid content. A spontaneous mutant line with high oleate levels, designated F435, has been identified through a peanut germplasm screening project, which contains 80% oleic acid (Norden *et al*., 1987). In this line, two mutations were reported: a "G" to "A" substitution at 448 bp after the start codon (G448A) in the ahFAD2A gene and an "A" insertion between bp 441 and 442 (441_442insA) in the ahFAD2B gene. The study also identified a new mutation, G451T, in the ahFAD2B gene. These findings indicate that the mutations induced in ahFAD2B using CRISPR/Cas9 may be beneficial for developing high oleate peanut lines. Additionally, TALENs were employed to target the ahFAD2 genes to enhance oleic acid content (Wen *et al*., 2018). Two TALEN pairs were constructed; one pair inoculated 216 regenerated roots, while the other inoculated 105. The observed mutation frequencies were 8.33% and 12.38%, respectively, with most mutations consisting of small deletions ranging from 1 to 10 base pairs. In the resulting mutant lines, the oleic acid content of the seeds reached 80.45%, indicating a two-fold increase compared to wild-type plants, while linoleic acid content decreased to 3%, with no change in the total fatty acid content. Furthermore, Shu *et al*. (2020) edited nod factor receptors (NFRs) with CRISPR/Cas9 to investigate their roles in peanut nodulation. Mutants with edited AhNFR5 genes displayed a Nod- phenotype, while mutants with two selected AhNFR1 genes were still able to form nodules after inoculation.

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Non-edited Legume Species

Lentil (*Lens culinaris*) is a diploid, self-pollinating plant belong from the Fabaceae family, known for its high protein, mineral, fiber, and carbohydrate content. In developing countries and among populations that cannot afford costly animal proteins, lentils can play a significant role in combating malnutrition and micronutrient deficiencies (Kumar *et al*., 2015). Additionally, lentils enhance soil quality by fixing nitrogen. The availability of draft genome sequences allows for the targeted identification of candidate genes associated with nutritional value, responses to abiotic and biotic stresses, and herbicide resistance. Genomic tools and technologies have the potential to advance lentil breeding efforts. Some gene transformation studies have been conducted on lentils. A notable example involves the successful transfer of the dehydration-responsive element binding gene (DREB1A), which helps plants respond to abiotic stresses, into lentils via Agrobacterium, producing transgenic plants that exhibit drought and salinity resistance (Khatib *et al*., 2011. In vitro regeneration post-transformation was crucial, with findings suggesting that using decapitated embryos was more effective for shoot generation than other tissues (Sarker *et al*., 2003). These initiatives provided valuable resources for future genome-editing research. Several candidate genes related to abiotic and biotic stress factors, as well as agronomic traits, were identified in lentils (Kumar *et al*.). However, to date, there had been no genome-editing research on these candidate genes in lentils using ZFNs, TALENs, or CRISPR/Cas9 technologies. Genome-editing technologies could offer a cost-effective and straightforward method for elucidating the functions of these candidate genes, leading to the development of cultivars with desirable

characteristics, such as stress tolerance and improved agronomic traits (Bhowmik *et al*., 2021).

Pea (*Pisum sativum*) was a significant legume crop worldwide, second only to the common bean (Phaseolus vulgaris), and was rich in dietary proteins, mineral nutrients, complex carbohydrates, and fiber (Bastianelli *et al*., 1998). Its ability to fix nitrogen symbiotically enhanced soil fertility, making it a valuable addition to agricultural systems (Mabrouk *et al*., 2018). Additionally, peas were historically important as a model organism used by Mendel to establish the principles of inheritance (Ellis *et al*., 2011). The pea genome was approximately 4.45Gb in size, with a reference genome published in 2019 that offered insights into the evolution of legume genomes (Kreplak *et al*., 2019). Genomic approaches were crucial for identifying genes associated with important traits and developing tools for crop improvement. Despite significant advances in pea cultivation, efforts to enhance crop yield and quality had to continue to meet the needs of the growing global population.

Peas were susceptible to various threats, including parasitic weeds, viruses, bacteria, fungi, and abiotic stressors such as drought, salinity, heat, and cold, all of which could lead to significant yield and growth losses. A successful stable transformation study was conducted, transferring the cry1Ac gene (which encodes a protoxin) from Bacillus thuringiensis (Negawo *et al*., 2013) and the alpha-amylase inhibitor gene from Phaseolus vulgaris to enhance insect tolerance using Agrobacterium-mediated transformation. Another attempt involved introducing antifungal genes against Fusarium spp. into peas; however, this resulted in unstable expression over three years of field trials. Recent advancements improved Agrobacterium-mediated transformation efficiency and regeneration frequency through extended infection times and the addition of zeatin to the selection medium. The availability of genome sequence information, successful transformation methods, and regeneration techniques were essential for utilizing genome-editing tools in vegetable crops (Cardi *et al*., 2017). To date, there had been no studies employing new genome-editing technologies in peas, likely due to insufficient regeneration capabilities (Pandey *et al*., 2021). The advancement of new genome-editing methods could create opportunities in breeding for higher yields and the development of nutritionally enhanced plants.

Faba beans possessed several advantages over other legumes in cold climates, making them suitable for sustainable farming practices (Temesgen *et al*., 2015). Like other legumes, they contributed significantly to soil fertility. Faba bean breeding was essential to meet the growing demand for food and feed, as they were a rich source of protein, fiber, and other nutrients (Khazaei *et al*., 2021). However, publicly available genome sequence data for faba

beans were lacking, likely due to the challenges associated with assembling their large genome (Cooper *et al*., 2017). Transformation and regeneration of faba beans remained difficult, although there had been attempts at Agrobacterium-mediated transformation with limited success. The first successful transformation was reported by Böttinger *et al*. (2001), who used in vitro development of internode stem segments invaded by Agrobacterium, and later by Hanafy *et al*. (2005), who infiltrated cut embryo axes with Agrobacterium to obtain stable transgenic lines. Additionally, abiotic stress-tolerant lines were generated by Hanafy *et al*. (2013) by transforming the potato PR10a gene into faba beans using the same method. However, various biotic and abiotic stress factors, including heat, insects, viruses, and parasitic weeds, continued to reduce faba bean yields.

The availability of whole-genome data for commonly cultivated non-edited legume species such as lentil, pea, common bean, and mung bean—which were rich in nutrients for human diets—presented opportunities for developing genome-editing strategies. Identifying and demonstrating the functions of genes associated with abiotic and biotic stress tolerance, yield, and quality could facilitate the application of new genome-editing tools to enhance these traits in key legume crops essential for human nutrition. Additionally, legumes played a crucial role in maintaining soil fertility; thus, targeting symbiotic nitrogen-fixing pathways could be beneficial in genome-editing initiatives aimed at improving soil health. Despite some promising efforts, low transformation efficiency and challenges in regeneration remained significant obstacles, which may explain the limited application of new genome-editing techniques. To fully realize the potential for substantial improvements in these legumes, it was critical to develop innovative methods that enhanced transformation and regeneration efficiency, thereby supporting the nutritional needs of a large portion of the global population.

Conclusion and future perspective

The assessment of genetic resources in legume crops has revealed significant variation in protein content and micronutrient levels. This review highlights ongoing efforts to screen germplasm, map quantitative trait loci (QTLs) associated with seed protein and micronutrient content, and identify future focus areas for enhancing the nutritional quality of key pulses. The concentration of micronutrients in seeds is a complex trait influenced by multiple genes and QTLs, with its levels significantly impacted by various soil and environmental factors.

Currently, an integrated breeding strategy that combines QTL mapping, genome-wide association studies, and differential gene expression profiling is considered the most effective

approach for the genetic analysis of complex traits such as nutritional quality. There is considerable potential to improve the nutritional quality of pulse crops and develop biofortified varieties. The variability in iron (Fe) and zinc (Zn) content, along with other micronutrients in food legumes, can be utilized to enhance their levels and bioavailability.

Additionally, reducing anti-nutritional factors like phytates, trypsin inhibitors, and chymotrypsin inhibitors can further increase the bioavailability and consumption of legumes. A key objective in legume improvement programs is to identify genotypes with low anti-nutrient content, whether from existing germplasm or through methods like induced mutagenesis and genome editing, to achieve nutritional security. Furthermore, crop wild relatives of various legumes, which naturally possess lower levels of these anti-nutrients, can be integrated into pre-breeding programs (Singh and Jauhar, 2005).

TABLE 1. Gene-editing technology in different legume crops.

Legume	Technique	Target	Legume	Technique
Medicago truncatula	CRISPR/Cas 9	*MtSUP* (regulates the floral organ number)	MtSUP was found to be orthologous of *AtSUP*	Rodas *et al*. 2021
	CRISPR/Cas 9	CYP93E2 and CYP72A61 (soyasapogenol B biosynthesis)	51 CYP93E2 mutant plant lines	Confalonieri *et al*.
	CRISPR/Cas 9	GmGS1, GmCHI20, MtGUS	Mutated genes	Michno *et al*.
	CRISPR/Cas 9	MtHen1 (Hua enhancer1 gene)	Efficient mutation	Curtin *et al*. (2018)
Lotus japonicus	CRISPR/Cas 9	Lbs genes (nodule senescence)	Early nodule senescence	Wang *et al*. (2019)
	CRISPR/Cas 9	SNF (symbiotic nitrogen fixation) genes	CRISPR/Cas9 system can effectively induce	Wang *et al*. (2016)

			mutations in SNF related genes	
	CRISPR/Cas 9	LjCZF1 and LjCZF2 (root nodule symbiosis)	Decrease in nodule formation	Cai, K., (2018). *et al*.
Glycine max (Soybean)	CRISPR/Cas 9	GmPRR37 (photoperiodic flowering)	Changes in flowering time	Wang, L., *et al*. (2020)
	CRISPR/Cas 9	GmLox1, GmLox2, GmLox3 (encoding lipoxygenases)	Loss of lipoxygenase activity	Wang, J., *et al*.
	CRISPR/Cas 9	GmAGO7a and GmAGO7b (controlling leaf pattern)	Inherited mutation until T2 lines	Zheng *et al*. (2020)
	TALEN	*FAD2-1A/B* (seed content improvement)	Increased oleic acid content, reduced linolenic acid content	Haun *et al*. (2014)
Cowpea (Vigna *unguiculata*)	CRISPR/Cas 9	VuSPO11-1 (cowpea meiosis gene)	Mutations	Che *et al*. (2021)
	CRISPR/Cas 9	SNF (symbiotic nitrogen fixation) genes	Blocked nodule formation	Ji *et al*. (2019)
Chickpea (*Cicer arietinum*)	CRISPR/Cas 9	*4CL* (*4-coumarate ligase*) *RVE7*	High efficiency in editing	Badhan *et al*.

		(*Reveille 7*) (drought tolerance)		
Peanut (*Arachis hypogaea*)	CRISPR/Cas 9	*AhNFR1* and *AhNFR5* (nodulation)	Successfully edited genes	Shu *et al*. (2020)
	CRISPR/Cas 9	*AhFAD2* (seed content improvement)	G448A, 441_442insA, G451T mutations	Yuan *et al*. (2019)
	CRISPR/Cas 9	AhFAD2 (seed content improvement)	G448A, 441_442insA, G451T mutations	Yuan *et al*. (2019)
	TALEN	AhFAD2 (seed content improvement)	Increase in the oleic acid content	Wen *et al*. (2018)

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