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CRISPR/Cas Genome Editing in Legume Crops: Challenges and Opportunities: A Review

Murugavelu Girija Sangari[1,2], S.R. Harish Chandar[1], Subramanian Mahalakshmi[3],
P. Latha[4], Swaminathan Amutha[2], Chinnaswamy Appunu[1]

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ABSTRACT

Legumes are an important source of protein and provide a health-rich diet for human beings. It contains essential amino acids. It mainly plays a significant role in soil enrichment. Due to their role in agriculture and human nutrition, scientists have made efforts to develop new traits. The genetic enhancement of legumes was achieved using traditional breeding over the years however, the progress is very slow. Recent developments in genome editing technologies, specifically CRISPR-Cas technology, have improved key agricultural traits in legumes and offer a wealth of opportunities for studying traits like improved seed nutrient content, enhancing productivity and resilience to biotic and abiotic stresses recently introduced in legumes. So far, the genome editing technology has been effectively used in various legume crops, mainly soybean, peanut, cowpea and chickpea. Still, the transformation and regeneration of other legumes have remained a significant hurdle to the implementation of gene editing. This review mainly highlights the use of different gene editing technologies in legumes, progress and updates of CRISPR/Cas9 tools in legumes and challenges of legume crops face during production.

Key words: CRISPR/Cas9, gRNA, Legumes, TALENs, ZFNs.

By 2050, the global populace is projected to surpass 9.5 billion, so to ensure food security, the agricultural output must be boosted by 60-100%. Cereals and legumes are the two main crops cultivated worldwide to feed billions of people. Legumes belong to the Fabaceae family of angiosperms and comprise about 19,500 species in 751 genera. It consists of small herbs and large trees. Legumes are essential for sustainable agriculture as they perform an integral function in the natural ecosystem. Legumes supports with high-quality organic matter into the soil due to their symbiotic nitrogen-fixing ability and increase soil fertility (Baloglu *et al.*, 2022). Leguminous crops are considered as the most significant due to their various uses, including animal pasture, aquaculture feed and human food. Legumes are popular for their protein content and contain health-promoting chemicals, including lignans, folate, saponins and antioxidants (Mousavi Derazmahalleh *et al.*, 2019). Legumes can effectively solve the issues related to nutrient deficiency. Nevertheless, global legume production is not up to the mark and various biotic and abiotic stresses heavily influence the yields (Choudhury and Rajam, 2021).

Advanced molecular techniques have supplanted conventional plant breeding methods, which are time-consuming and expensive, to tackle the global food shortage. It takes years to establish specific crop traits, while genetic engineering, which involves introducing foreign genetic material, creates social biosafety issues. Hence, due to the rise of advanced molecular techniques such as genome editing, the focus of targeted crop improvement has shifted towards a new path (Rasheed *et al.*, 2022). The availability of complete genome sequence of organisms substantially contributes to the advancement of next-generation genome editing research. New-generation genome-editing experiments are more common in legumes, namely *Lotus japonicus*, *Glycine max* and *Medicago truncatula,* than in other family members (Baloglu *et al.*, 2022).

Gene editing is an emerging process to generate precise DNA modifications at specific genomic locations. The precise gene edits can be achieved via gene editing technologies such as Transcription activator like effector nucleases (TALENs), Zinc finger nucleases (ZFNs) and CRISPR/Cas systems. While other gene editing technologies still have their role, the emergence of CRISPR/Cas systems has significantly improved the accuracy and

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[1]ICAR-Sugarcane Breeding Institute, Coimbatore-641 007, Tamil Nadu, India.
[2]Avinashiligam Institute for Home Science and Higher Education for Women, Coimbatore-641 043, Tamil Nadu, India.
[3]Department of Bioinformatics, Bharathiar University, Coimbatore-641 046, Tamil Nadu, India.
[4]Regional Agricultural Research Station, Acharya N.G. Ranga Agricultural University, Tirupathi-517 502, Andhra Pradesh, India.

Corresponding Author: Chinnaswamy Appunu, ICAR-Sugarcane Breeding Institute, Coimbatore-641 007, Tamil Nadu, India.
Email: cappunu@gmail.com

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effectiveness of editing (Karavolias *et al.*, 2021). In legumes, the CRISPR/Cas9 technology was first demonstrated in Soybean (*Glycine max*) (Wang *et al.*, 2017). This review, primarily focus on the application of gene editing technology in legumes and the challenges of legume production.

Modifying the genome of legumes using genome editing technologies

In recent years, genome editing technologies have reformed nearly every domain of biological research fields, expanding the scope of agricultural studies. These technologies enable the creation of novel plant varieties by introducing precise mutations that modify or deactivate target gene functions. With the progression of these technologies, there has been a rise in the amount of legume genomes that have been sequenced. Zinc finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and CRISPR stand out as highly advanced tools for precise genome editing (Wang *et al.*, 2017). At present, CRISPR genome-editing technique is being employed to address challenges in agriculture, aiming to meet the growing demand for food in the future.

Zinc finger nucleases (ZFNs) is an initially established genome editing tool that revolutionizes genome manipulation research. Its functionality is quite similar to TALEN. ZFN has two components: the DNA binding domain (housing custom-engineered Cys2-His2 (C2H2) zinc finger protein) to spot specific DNA sequences and the DNA cleavage domain (containing the Fok1 restriction enzyme) to cut the target DNA site (Urnov *et al.*, 2005). These two domains are linked to make the ZFN functional. This DNA binding domain consists of 2 to 3 zinc finger domains, each recognising one amino acid, *i.e.*, 3 DNA bases. ZFN is used to either knockin or knockout the gene of interest (Daniel *et al.*, 2023). Two FokI endonuclease domains must dimerize to become active; dimerization of FokI nuclease generates a functional nuclease complex that results in double-strand breaks (DSBs). The resulting double-stranded breaks can be repaired either by homologous recombination (HR) or nonhomologous end joining (NHEJ). ZFN is a complex process that might lead to off-target effects and unexpected mutations. Despite the availability of kits, producing ZFNs is still more time-consuming and less adaptable than TALENs or the CRISPR/Cas system due to target sequence suitability constraints (Puchta and Fauser, 2014). In legume plants, targeted mutagenesis was performed targeting the soybean genes DICER-LIKE (DCL) genes - DCL4a and DCL4b which underwent successful knockout by utilising both hairy root and stable *Agrobacterium*-mediated transformation methods to deliver ZFNs (Curtin *et al.*, 2011).

Transcription activator-like effector nucleases (TALENs) is also a genome editing tool similar to ZFNs. Like ZFNs, TALEN is also formed by combining the DNA binding domain of TALE protein repeats with the cleavage domain of FokI nuclease. The DNA binding domain comprises multiple repeats; however, unlike ZFNs, each DNA binding domain recognizes just one nucleotide (Christian *et al.*, 2010). Their adherence to the target sites triggers the dimerization of the FokI nuclease domains, forming a functional complex that induces double-strand breaks (DSBs) at the designated site (Cermak *et al.*, 2011). Currently, there are limited studies on the use of TALENs in soybeans and no advancements have been documented with other legumes. In Soybean, TALENs were employed to knock out two genes, FAD2-1A and FAD2-1B, influencing soybean oil quality. Challenges such as the complexity of creating DNA-binding modules, ineffective genome targeting and frequent off-target consequences have considerably limited the application of TALENs in legume research (Haun *et al.*, 2014).

“Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)” is an advanced genome editing method

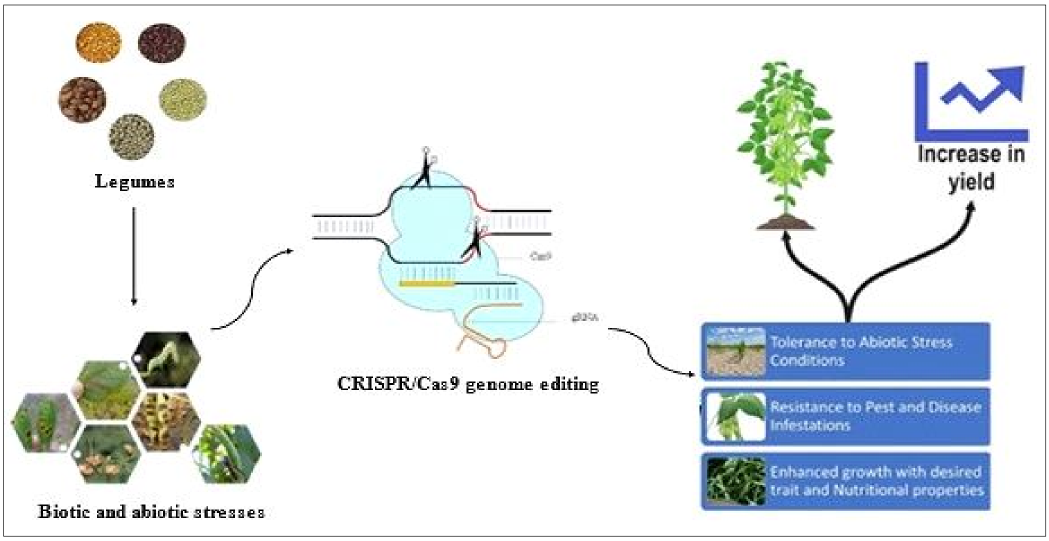

Fig 1: Genome editing for enhancement of yield and its attributing traits.

to alter the crop genome to improve its traits suitable for changing environmental conditions (Fig 1). It is an adaptive immune system of prokaryotes that helps to recognize and destroy the invading phages and pathogens (Bao *et al.*, 2020). It has been grouped into two classes depending on the organization of Cas effector module - Class I and Class II. Class I consists of type I, type III and type IV subunits, whereas Class II comprises type II, type V and type VI subunits. Among these various types, the CRISPR-Cas9 belonging to type II is an extensively used genome editing tool owing to its simplicity. CRISPR-Cas process mainly uses 2 key molecules, a Cas9 an endonuclease and a guide RNA (gRNA) to guide the Cas9 enzyme to cleave the target DNA for precise editing. Cas9 includes 2 nuclease domains, HNH and RuvC, to cleave the complementary and non-complementary target DNA strands. The Cas9 requires the presence of a protospacer-adjacent motif (PAM) sequence that helps the Cas9 recognize the target site. PAM sequence varies according to the Cas protein; in case of Cas 9 it is 5′-NGG-3′. The Cas9 protein is obtained from *Streptocococcus pyogenes*. The guide RNA is made up of a crRNA (CRISPR RNA) that attaches to the target sequences and tracrRNA (transactivating RNA) that promotes target detection and cleavage (Deltcheva *et al.*, 2011). The Cas9 makes a double-stranded break (DSB) at that specific location which can be mended by the non-homologous end joining (NHEJ) pathway or homology-directed repair (HDR). Plants mostly repair double-strand breaks (DSBs) using the non-homologous end joining (NHEJ) mechanism (Jinek *et al.*, 2012). This route is error-prone, which typically result in base insertion or deletion. As a result, genomic changes happen at the repair site (Ahmar *et al.*, 2020).

CRISPR-mediated gene editing has successfully modified a variety of legumes (Table 1). It has increased crop output and resilience to both biotic and abiotic challenges. Extensive research has been undertaken on the domestication of legume species and the CRISPR/Cas9 mechanism is currently being used to improve different features. Recent breakthroughs demonstrate great success in enhancing the nutritional value of legumes using CRISPR/Cas9 technology (Das and Acharjee, 2023).

Challenges in major legumes and minor legumes

Generally, legumes face numerous challenges during cultivation and production, including socio-economic, genetic, biotic and abiotic factors. These constraints reduce yield, ultimately limiting farmers from cultivating it in more areas (Govindaraj *et al.*, 2015). Legumes are classified into 2 types: major and minor, depending on their value and low-cost. Major legumes are commonly used and widely recognized. These legumes possess established domestication, cultivation and agronomic practices. The major legumes include *Glycine max* L., *Vigna unguiculata* L., *Arachis hypogaea* L., *Phaseolus vulgaris* L., *Pisum sativum* L. and *Cicer arietinum* L. However, minor legumes are not popular as they are less well-known and are regarded as underutilized. The minor legumes include *Psophocarpus tetragonolobus* L., *Cajanus cajan* L., *Lablab purpureus* L., *Phaseolus lunatus* L., *Canavalia* sp., *Vigna mungo* L., *Vigna subterranea* L., *Tylosema esculentum* L., *Kerstingiella geocarpa*, *African yam bean*, *Sphenostylis stenocarpa* Harms and *Vigna angularies* L. (Popoola *et al.*, 2019).

Arachis hypogaea (Peanut), an allotetraploid is one of the important promising legume crops. Originating in South America, it is now cultivated throughout the temperate and tropical regions of the world. It is rich in high oleic acid content and is globally recognized as an important oilseed crop because of its oil content, nutritional value and other industrial uses. It covers a vast expanse of 32.72 million hectares (M ha) with a 1648 kg/hectare productivity rate. The world's major peanut producers are China, India and Nigeria. China, producing 18.36 million tons (MT), is the world's leading peanut producer. Despite all, the peanut faces numerous challenges in yield and quality because of various biotic and abiotic stresses (You *et al.*, 2024). Common fungal pathogens include *Botrytis cinerea*, *Macrophomina phaseolina*, *Cylindrocladium crotalariae*, *Cetcospora arachidicola* and *Puccinia arachidis,* which causes rot, spot and rust diseases in peanut crops. Viral diseases that affect peanut production include Peanut Bud Necrosis Virus (PBNV), Tomato Spotted Wilt Virus (TSWV) and Peanut Stunt Virus (PSV) (Palekar *et al.*, 2023). A significant global challenge in peanut cultivation is aflatoxin (AFs) contamination, posing considerable concern due to the toxicological effects of these toxins. High doses of aflatoxin consumption may lead to mortality (Torres *et al.*, 2014). Hence CRISPR/Cas9 genome-editing tool can be used to develop varieties resistant to these diseases which will have an impact on the global peanut industry. Apart from this, climate change is also a main challenge that causes severe effects in groundnut yield. Increased CO_2 level, unreliable rainfall and temperature fluctuations inhibit the physiology, disease tolerance, yield and fertility. To overcome these threats, it is essential to develop climate-resistant varieties with stable genetic enhancements (Gangurde *et al.*, 2019; Ghosh *et al.*, 2022; Puppala *et al.*, 2023). Peanut breeding was challenging in ancient times due to its polyploid genome. However, recent advances in molecular techniques, leveraging the peanut genome, have illuminated successful strategies for its genetic improvement (Sharma *et al.*, 2023; Sun *et al.*, 2013).

Glycine max (Soybean) holds significant economic value as it contains abundant protein and oil sources for both animal and human feed (Li *et al.*, 2019). The challenges like inefficient farming systems, pests, diseases, environmental stresses like drought and lack of disease-resistant cultivars hinder soybean production. Diseases like bacterial leaf blight, downy mildew, stem and root blight, purple seed stain, brown spot, charcoal rot and soybean mosaic virus are the common familiar diseases that affect

Table 1: Traits targeted using gene-editing techniques in various legume crops.

Crops	Modified genes	Techniques	References
Arachis hypogaea L.	AhMULE9A	CRISPR/Cas9	(Li *et al.*, 2023)
	FAD2	CRISPR/Cas9	(Neelakandan *et al.*, 2022)
	AhNFR1 and AhNFR5	CRISPR/Cas9	(Shu *et al.*, 2020)
	Ara h1, Ara h2 and Ara h3	CRISPR/Cas9	(Kim and Jun, 2022)
	Ara h2	CRISPR/Cas9	(Biswas *et al.*, 2022)
Brassica napus	FAD2	CRISPR/Cas9	(Okuzaki *et al.*, 2018)
	BnWRKY11 and BnWRKY70	CRISPR/Cas9	(Sun *et al.*, 2018)
	BnaMAX1	CRISPR/Cas9	(Zheng *et al.*, 2020a)
	BnALC	CRISPR/Cas9	(Braatz *et al.*, 2017)
	JAG	CRISPR/Cas9	(Zaman *et al.*, 2019)
	BnaFAE1	CRISPR/Cas9	(Liu *et al.*, 2022)
Cicer arietinum	4CL and RVE7	CRISPR/Cas9	(Badhan *et al.*, 2021)
Glycine max	GmPRR3b	CRISPR/Cas9	(Li *et al.*, 2020)
	LNK2	CRISPR/Cas9	(Li *et al.*, 2021)
	GmLox1, *GmLox2* and *GmLox3*	CRISPR/Cas9	(Wang *et al.*, 2020)
	AGO7	CRISPR/Cas9	(Zheng *et al.*, 2020b)
	GmFT2a	CRISPR/Cas9	(Cai *et al.*, 2018b)
	qFT13-3	CRISPR/Cas9	(Li *et al.*, 2023)
	DCL4a and DCL4b	ZFNs	(Curtin *et al.*, 2011)
	FAD 2	TALENs	(Haun *et al.*, 2014)
	GmFEI2 and GmSHR	CRISPR/Cas9	(Cai *et al.*, 2015)
	SPL	CRISPR/Cas9	(Bao *et al.*, 2019)
	GmExpA and GmExpLB	CRISPR/dCas9	(Freitas Alves *et al.*, 2024)
	FAD2–2	CRISPR-Cas9	(Al Amin *et al.*, 2019)
	DD20 and DD43	CRISPR-Cas9	(Cai *et al.*, 2015)
	GFP	CRISPR-Cas9	(Jacobs *et al.*, 2015)
	GmPDS11 and GmPDS18	TALENs and CRISPR/Cas9	(Du *et al.*, 2016)
	GS1	CRISPR/Cas9	(Michno *et al.*, 2015)
	CHI20	CRISPR/Cas9	(Michno *et al.*, 2015)
	Conglycinins (7S) and glycinins (11S)	CRISPR/Cas9	(Li *et al.*, 2019)
	GmPHYA or *GmPHYB*	CRISPR/Cas9	(Zhao *et al.*, 2022)
	CENH3	CRISPR/Cas9	(Wang *et al.*, 2023)
	GmFEI2 and *GmSHR*	CRISPR/Cas9	(Cai *et al.*, 2015)
	GmFT2a and GmFT5a	CRISPR/Cas9	(Cai *et al.*, 2018b)
Lotus japonicus	*CYP716A51* and *LjCYP93E1*	CRISPR/Cas9	(Suzuki *et al.*, 2019)
	LjαCA2, LjαCA6 and LjβCA1	CRISPR/Cas9	(Wang *et al.*, 2021)
	LHK1	CRISPR/Cas9	(Cai *et al.*, 2018a)
	LjBAK1	CRISPR/Cas9	(Feng *et al.*, 2021)
	HAR1	CRISPR/Cas9	(Okuma *et al.*, 2020)
	NIN	CRISPR/Cas9	(Akamatsu *et al.*, 2021)
	SNF	CRISPR/Cas9	(Wang *et al.*, 2019)
	Lbs	CRISPR/Cas9	(Wang *et al.*, 2019)
Medicago truncatula	FMO1-LIKE, RFP1-like, ERDJ2, MEL1, PEN3-like, ACRE1, HLZ1-like, HLZ1-like, PHO2-like, PNO1-like and FBL1-like	CRISPR/Cas9	(Curtin *et al.*, 2017)
	MtHen1	CRISPR/Cas9	(Curtin *et al.*, 2011)
	MtPDS	CRISPR/Cas9	(Meng *et al.*, 2017)
	GUS	CRISPR/Cas9	(Michno *et al.*, 2015)
	NPD	CRISPR/Cas9	(Trujillo *et al.*, 2019)

Table 1: Continue...

Table 1: Continue...

	CYP93E2 and *CYP72A61*	CRISPR/Cas9	(Confalonieri *et al.*, 2021)
	MtSUP	CRISPR/Cas9	(Rodas *et al.*, 2021)
Medicago sativa L.	*NOD26*	CRISPR/Cas9	(Bottero *et al.*, 2021)
Pisum sativum	*PsLOX2*	CRISPR/Cas9	(Bhowmik *et al.*, 2023)
Vigna unguiculata	SNF	CRISPR/Cas9	(Ji *et al.*, 2019)
	VuSPO11-1	CRISPR/Cas9	(Juranic *et al.*, 2020)
	PDS	CRISPR/Cas9	(Bridgeland *et al.*, 2023)

the soybean (Ayilara *et al.*, 2023). Soybean rust, caused by *Phakopsora pachyrhizi*, is a most significant foliar disease worldwide. So proper control measures can make the plant survive under these adverse conditions (Nair *et al.*, 2023). Implementing modern breeding technologies that are quick, accurate and effective is vital for obtaining varieties with good yields, quality and stress tolerance, thereby addressing agricultural challenges. Genome editing technology is a suitable and relevant method for specifically improving cultivating crops with these desirable features, making it an ideal tool for genetic improvement in soybeans. The precise modifications enabled by genome editing enhance soybean traits, ensuring better agricultural outcomes (Bao *et al.*, 2020).

Vigna unguiculata (Cowpea) is a widely cultivated legume crop possesses rich nutritional benefits and is also recognised as a black-eyed pea. It was first domesticated in Africa and about 200 million people consume it in Africa. It is consumed by many people in Africa, Asia and America as a daily diet. Due to its ability for symbiotic nitrogen fixation and valuable agronomic characteristics, including resilience to limited rainfall and minimal fertilizer needs, it is regarded as the most valuable legume crop in the world. These factors have led to the attention of cowpea gene-editing techniques (Ji *et al.*, 2019; Rasheed *et al.*, 2022). Until 1967, the United States was one of the world's top 10 producers of cowpea. With the expected growth in the minority population of the United States of about 47% in 2050, the consumption of cowpea is projected to increase substantially. Like other crops, cowpea productivity faces significant challenges from pests and diseases. Root-knot nematode (RKN) poses a significant threat to cowpea production in the U.S. by causing substantial damage to its root system and resulting in yield loss. Various commercial cowpea cultivars resistant to these specific RKN species have been created. Nonetheless, certain nematode populations are RK-virulent, rendering the narrow genetic resistance the RK genes provide ineffective against them (Osipitan *et al.*, 2021). Weeds are considered one of the most serious threats to cowpea cultivation, which causes up to 76 % yield loss. Leveraging advanced technologies such as CRISPR/Cas9, endeavours have been undertaken to create varieties exhibiting resistance to herbicide, insects and diseases, significant impediments to cowpea production (Osipitan, 2017).

Cicer arietinum (Chickpea) is an essential legume crop cultivated worldwide. Its genome sequence has been published in 2013 (Badhan *et al.*, 2021). It is very sensitive to saline conditions to just 25 mM NaCl in hydroponics, resulting in yield loss of about 8 to 10%. However, it remains unclear why chickpeas are particularly prone to salt sensitivity. During seed germination, some chickpeas are more resistant to 320 mM NaCl. The reason for this dissimilarity also remains unclear. So, genome editing techniques can be used to generate plants that are tolerant to saline conditions (Sobh *et al.*, 2023). The CRISPR-Cas9 system has the capacity to enhance yield potential even when plants are under biotic and abiotic stress, leading to the development of plants with the necessary traits.

Challenges and opportunities in legume for genetic improvement

Legumes pose challenges for genetic transformation due to tissue-specific transformability and regeneration issues. Challenges in confident detection include diverse mutations induced by gene-editing proteins and target organism complexities like homolog multiplicity. Legume regeneration limitations may require screening numerous lines or tissues, demanding cost-effective and sensitive methods. While economically important legume genomes are typically not recent polyploids, gene duplications are common in this clade. Reliable interpretation of detection experiments benefits from high-quality genome assemblies, especially for assessing off-target effects (Bhowmik *et al.*, 2021).

CRISPR/Cas9 technology presents a promising avenue for revolutionizing plant breeding by streamlining processes and reducing labour-intensive tasks compared to traditional methods. However, despite its potential, several challenges hinder its widespread application in plant genome editing. Major challenges in improvement of some of the legumes are given in Table 2. One such challenge involves the editing of plant organelle genomes, where precise modifications are often complex due to their unique genetic features. Additionally, ensuring transgene-free editing remains a priority for regulatory and consumer acceptance, posing technical hurdles in achieving heritable genome modifications without foreign DNA integration. Another frontier is virus-induced genome editing, where leveraging viral vectors for delivery holds promise but requires meticulous control to avoid unintended consequences. Furthermore, editing recalcitrant elite crop inbred lines, which are crucial for agricultural productivity, presents significant obstacles due to their genetic complexity and limited regeneration capacity. Addressing

Table 2: Major challenges of few legume crops.

Crop	Challenges
Groundnut	Lines resistance to PBND and PSND
	Lines resistance to drought and heat stress
	Lines with high partitioning efficiency
Black gram,	Lines resistance to Yellow Mosaic Virus
Green gram	Lines resistance to drought and heat stress
	Lines with high partitioning efficiency
Cowpea	Lines resistance to drought and heat stress
Red gram	Lines resistance to sterility Mosaic Virus
	Early duration varieties

these challenges demands continued innovation in CRISPR/Cas9 technology and strategic collaborations across disciplines to unlock its full potential in shaping the future of plant genetic improvement (Son and Park, 2022).

Agrobacterium-mediated transformation is predominantly used, with biolistic methods employed in plant regeneration (Yadav *et al.*, 2017). CRISPR/Cas9 offers promise in overcoming transformation hurdles. Soybean stands out with successful CRISPR/Cas9 applications, yielding 41 cultivars (Bhowmik *et al.*, 2021). Large-seeded legumes face rooting challenges *in vitro*. Stable regeneration protocols are crucial for commercial production, yet many legumes lack successful protocols due to poor rooting (Deng *et al.*, 2022). Genetically Modified Organisms resistance hinders transformation acceptance, especially in smaller markets (Negi *et al.*, 2021). Techniques like sonication-assisted Agrobacterium transformation enhance transformation efficiency. Optimizing explants, host-plant affinity and culture media additives can improve transformation rates. Molecular science advancements will uncover solutions for legume transformation challenges (Rasheed *et al.*, 2022).

Legume research benefits from a wealth of genetic resources but grapples with the challenges of managing their diversity and complexity. A workshop identified key needs: developing strategies for storing and integrating genetic resources, standardizing data formats and metadata, recognizing the critical role of curators, implementing standardized software practices, creating tools for global plant genetic information management and centralizing database information while supporting training and outreach efforts (Bauchet *et al.*, 2019).

CONCLUSION

Legumes are significant in the human diet as they contain several nutritional values. Legume production is affected by numerous factors, namely biotic and abiotic stresses like drought, salinity, temperature and heavy metals stress. However, the need for agricultural improvement, driven by rapid climate change and population increase, highlights the importance of thinking beyond the box. Though transgenic technology is used to create plants that are tolerant to several stresses, due to numerous regulatory concerns, commercial success is very low across the world. The emergence of genome editing tools such as ZFNs, TALENS and CRISPR/Cas allows for the rapid production of site-specific alterations in the genetic makeup of plants. For efficient genome editing, thorough knowledge of legumes genomic sequences is essential. The discovery of genes that regulate various traits in legumes, such as seed size, quality, disease resistance and ability to withstand abiotic stresses, enables the development of new and unique varieties in the future. We conclude that further research is needed on the application of CRISPR/Cas9 in legume crops to unravel successful genetic transformation. Numerous legume crops remain unexplored for effective gene editing and studies should address the challenges and difficulties associated with legume transformation. The future is likely to witness an increased use of CRISPR/Cas9, providing additional insights into the genetic enhancement of legume crops to ensure global food security.

Conflict of interest

All authors declared that there is no conflict of interest.

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